Effect of Plasticizers (Water and Glycerol) on the Diffusion of a Small Molecule in Iota-Carrageenan Biopolymer Films for Edible Coating Application

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Received February 27, 2006; Revised Manuscript Received April 6, 2006

Translational diffusion of a fluorescein probe has been measured in iota-carrageenan edible films containing different amounts of glycerol (0, 15, 30, and 45%), using fluorescence recovery after photobleaching (FRAP) experiments. The effects of this plasticizer as well as the plasticizing effect of water on the diffusion of fluorescein have been studied in this edible coating mainly composed of natural biopolymer. Diffusion coefficients of about 10^{-13} m^2 s^{-1} have been measured in these films for water activity (aw) lower than 0.7. Above this water content threshold, fluorescein translational diffusion coefficient increases up to 10^{-12} m^2 s^{-1}. Another interesting information obtained from FRAP experiments on this system is the ratio of the diffusing molecules which are immobilized in the carrageenan matrix at aw lower than 0.98. Moreover, films containing more than 30% glycerol (w/w carrageenan) present a huge increase of the diffusion coefficient of fluorescein at high water activity (about 2 orders of magnitude), this effect being less pronounced at low water activity. The increase of diffusion seems to be only related to the water content, and glycerol only acts through the enhancement of water adsorption. Therefore, in biopolymer films containing polyol plasticizers, the gain in mobility could be devoted to the effect of the ubiquitous plasticizing molecule, water, whose adsorption is increased by the plasticizer.

1. Introduction

Carrageenans are water-soluble polymers with a linear chain of partially sulfated galactans, which present high potentiality as film-forming material. These sulfated polysaccharides are extracted from the cell walls of various red seaweeds (*Rhodophyceae*). The number and position of sulfate groups on the disaccharide repeating unit determine the classification in three major types: κ, ι, and λ. The κ, ι, and λ-carrageenans have sulfate contents of 20%, 33% and 41% (w/w) respectively, resulting from one, two and three sulfate ester groups per dimeric unit. Iota carrageenan is mainly composed of alternated α(1,3)-D-galactose-4-sulfated and β(1,4)-3,6-anhydro-D-galactose-2-sulfate groups. A schematic of the disaccharide repeating unit is shown in Figure 1. In aqueous solutions, iota-carrageenans produce thermoreversible gels on cooling below the critical temperature, where the conformation changes from random coil single chains to the formation of double-helices of carrageenan chains, and consequently to a gel.[1] This mechanism of gelation is promoted by the presence of cations such as potassium, calcium and sodium. Except starch, carrageenan is, with pectin, the main natural gelling polysaccharide extracted from plants or seaweeds and used as a high-value functional ingredient in foods, cosmetics, and pharmaceuticals.[2] Iota-carrageenan is a hydrocolloid widely used in the dairy industry as it presents significant reactivity with milk proteins and is a functional ingredient in milk-based food products for stabilization (ice cream), thickening, and gelation (preparation of milk gels). Moreover, it is a naturally renewable and commercially resource available at a reasonable cost. The use of carrageenan as edible films and coatings already covers various fields of the food industry such as application on fresh and frozen meat, poultry and fish to prevent superficial dehydration,[3] ham or sausage-casings,[4] granulation-coated powders, dry solids foods, oily foods,[5] etc., but also manufacturing soft capsules[6,7] and

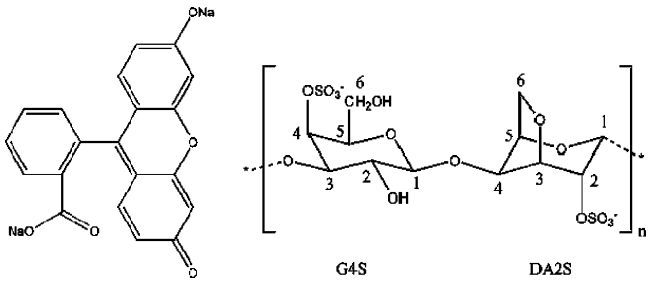

Figure 1. Fluorescein molecule (left drawing) and iota-carrageenan (right drawing) dimeric unit (two sulfate ester groups per unit). G4S = 3-linked β-D-galactopyranose 4-sulfate. DA2S = 4-linked 3,6-anhydro-α-D-galatopyranose 2-sulfate. Iota-carrageenans are linear polymers of about 25 000 galactose derivatives. Fluorescein ($C_{20}H_{10}O_5Na_2$, molecular weight = 376 g mol^{-1}, anionic weight = 330 g mol^{-1}) is a fluorophore presenting a excitation maximum at 494 nm and a peak emission at 520 nm.

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10.1021/bm060179r CCC: $33.50
Published on Web 05/06/2006

especially nongelatin capsules.[8] Indeed, this protective barrier can also be used in food domain in order to prevent the transfer of moisture, gases, flavors, or lipids and thus to maintain or improve food quality and to increase food product shelf life.[9,10] Another promising emerging technology that has been applied to various biopolymers, including, carrageenan-based coating, is their use as antimicrobial agent carriers in active packaging systems.[11,12] For most of these applications, film-forming substances such as polysaccharides are usually formulated in combination with a plasticizer molecule in order to improve their mechanical and structural properties. Effects of plasticizers on mechanical properties of edible films have been widely documented in scientific literature related to films.[9,10] However, little is known on their effect on the diffusion of other species in these matrixes.

The main purpose of our experiments has been to investigate the diffusion coefficient of a small molecule (fluorescein) in various edible films of iota-carrageenan at various aw and amounts of glycerol. To perform such experiments we have used a technique called fluorescence recovery after photobleaching (FRAP) which allows the measurement of translational diffusion coefficient of fluorescent particles. This technique is based on the photobleaching properties of specific fluorescent molecules under intense light illumination. Experimentally, a partial photobleaching of the fluorescent molecules within a small spot in the sample is achieved by a brief exposure to an intense laser beam. Once this irreversible destruction of fluorescence has occurred, the subsequent recovery of the fluorescence in the bleached area is monitored over time. Fluorescence recovery is due to the lateral diffusion of unbleached molecules into the bleached region, whereas at the same time, there is diffusion of bleached ones out of the bleached region. The FRAP technique was originally performed by using a spot as the bleach pattern, the accuracy has been improved by using a sinusoidal fringe pattern created by the interference of two laser beams,[13] thus introducing a well-defined diffusion length. This noninvasive technique has been applied to mobility studies in a wide variety of systems such as biological membranes,[14] cellular compartments, proteins or polyelectrolyte solutions and gels. However, most of studied systems remain liquid or in a high hydrated state. FRAP technique thus appears to be a valuable tool for studying diffusion of small molecules in polymer films and may contribute to obtain information about their molecular organization and their potential functionality as barrier. Moreover, in pharmaceutical field, there is a great potential for the characterization of the drug delivery in macromolecular systems, by studying the matrix effects on the diffusion rate of the active molecules[15,16] through the film envelopes.

The present work deals with structural and functional properties of iota-carrageenan-based edible films by studying the diffusion of a small model fluorescent molecule (fluorescein) in this matrix using FRAP measurements. Specific attention has been focused on the plasticization effects of water and glycerol and the ways they affect the diffusion of a small molecule in the carrageenan matrix.

2. Experimental Section

2.1. Material. Iota-carrageenan (Figure 1) was supplied by Degussa Texturant Systems (DTS, Baupte, France) and constituted the film matrix. Anhydrous glycerol was purchased from Fluka (98% purity, Fluka Chemical, Germany) and used as a plasticizer in order to improve mechanical properties of carrageenan films. The diffusing molecule, fluorescein (disodium salt), was purchased from Kuhlmann, France.

Table 1. Saturated Salt Solutions Used to Control Water Activity (Adapted from Bell and Labuza[19])

salt	chemical formula	water activity of the saturated salt solution (at 20 °C)
lithium chloride	LiCl	0.113
potassium acetate	CH_3COOK	0.231
magnesium chloride	$MgCl_2$	0.331
potassium carbonate	K_2CO_3	0.432
magnesium nitrate	$Mg(NO_3)_2$	0.544
sodium bromide	NaBr	0.591
strontium chloride	$SnCl_2$	0.725
potassium chloride	KCl	0.851
barium chloride	BaCl	0.90
potassium dichromate	$K_2Cr_2O_7$	0.98

This planar, heterocyclic molecule (Figure 1) has been chosen in reference to its molecular weight that closely matches the one of a sucrose molecule,[17] therefore, of small molecular size compared to the carrageenan matrix. This is consistent with the Stokes−Einstein equation for diffusivity with the assumption that the molecule is a sphere with a hydrodynamic volume proportional to its molecular weight. Another reason for the choice of fluorescein as a tracer is its ability to easily undergo photobleaching under laser irradiation.

The various saturated salt solutions used to fix water activity, listed in Table 1, were supplied by WVR International (Prolabo, Fontenay sous bois, France).

2.2. Carrageenan-Based Film Preparation. Iota-carrageenan-based films were made following the experimental procedure described in a previous paper.[18] A carrageenan film-forming solution was prepared by dispersing 6 g of Carrageenan powder in 200 mL of distilled water at 90 °C for 15 min under 700 rpm magnetic stirring. Glycerol was added at four different concentrations of 0, 15, 30, or 45% (w/w of the carrageenan dry matter), in the carrageenan solution. Fluorescein was introduced in this film forming solution at a concentration of 3 μM. The hot solutions were deaerated by sonication to remove dissolved air bubbles and poured into a thin-layer chromatography spreader to be spread at a 1000 μm thickness onto smooth poly(methyl methacrylate) (PMMA or Plexiglas) plates. These conditions were found to be very convenient for dried films to be peeled intact from the casting surface. To obtain a film, the water was removed by drying in a ventilated chamber (KBF 240 Binder, ODIL, France) for 8 h with temperature and relative humidity fixed at 30 ± 1 °C and 40 ± 2% RH, respectively. Film thickness after drying was about 35 μm.

2.3. Moisture Equilibration of Films. The determination of moisture sorption isotherm of the four different films (containing four different amount of glycerol) has been conducted using the gravimetric method,[19] by storing dry samples into controlled humidity chambers at constant temperature of 20 °C to obtain the equilibrium moisture content. The control of relative humidity of the film samples has been achieved by using saturated salt solutions (listed in Table 1) with different water activities ranging from 0.113 to 0.98. The same procedure was applied to prepare the films used in the FRAP measurements. During FRAP experiments, the film samples were placed between two glass plates to avoid moisture transfer with surrounding atmosphere and measurements were done at 21 ± 1 °C.

2.4. Fluorescence Recovery After Photobleaching (FRAP). *2.4.1. Experimental Set Up.* Principles of the FRAP technique used for this study have been discussed in detail by Davoust et al.[13] The experimental set up for FRAP measurements of fluorescein diffusion in films is shown in Figure 2. For our experiments, the laser beam was generated by an argon ion laser (Coherent Innova 90), emitting visible light at a wavelength of 488 nm (which is within the absorption band of the fluorescein). The irreversible bleaching of the fluorescein is caused by oxidation of the excited molecule. The main laser beam is split into two beams of equal intensity converging on the sample at an angle 2θ,

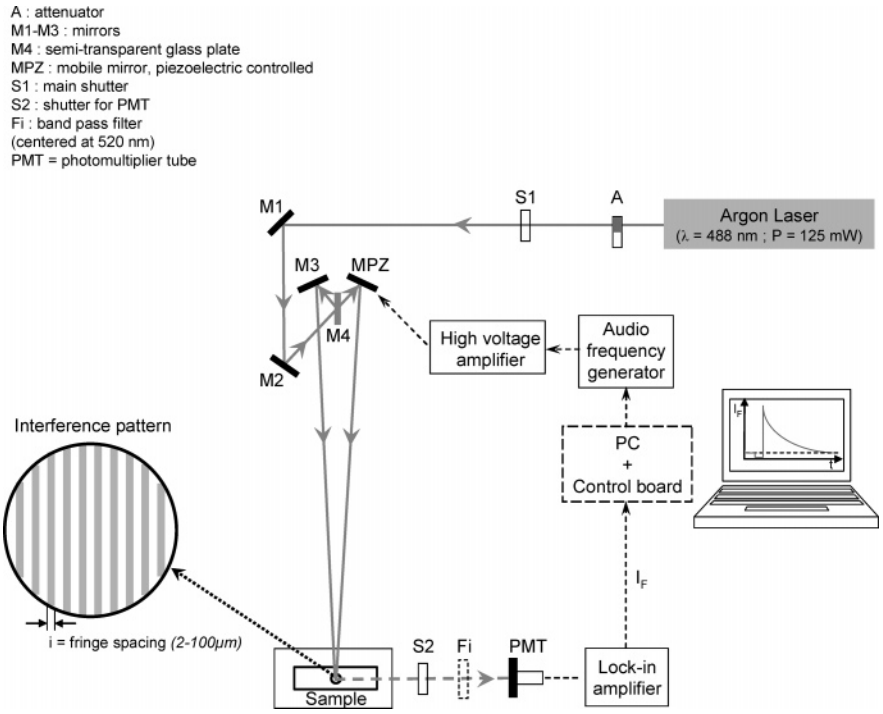

Figure 2. Experimental FRAP set up used to measure fluorescein translational diffusion coefficients in iota-carrageenan films. The interference pattern is obtained by splitting a laser beam and recombining the two resulting beams onto the sample (M1, M2, M3, and MPZ). The crossed beams give rise to a sinusoidal fringe pattern with spacing *i.* A short and intense pulse of an argon-ion laser irreversibly destroys the fluorescence of the fluorophores present in the exposed fringe pattern. A much attenuated beam is then used (A) for the monitoring of the fluorescence signal resulting from the recovery of fluorophores that diffuse from dark to illuminated regions. The fluorescent intensity emitted by the sample is recorded by a photomultiplier tube (protected by a shutter S2 during the bleaching pulse) through a band-pass filter centered at 520 nm. During monitoring, the fringes are spatially modulated by a piezoelectric driven mirror (MPZ) and modulation detection is used during the measurement. Processing and data collection is computer controlled.

thus creating in the crossing area a linear interference fringe pattern with a spacing of $i = \lambda/(2\sin(\theta))$. Inset of Figure 2 illustrates the bleaching pattern composed of alternating bright and dark stripes obtained onto the sample in the crossing region of the two Gaussian laser beams. The beams were focused to a spot size of about 1 mm diameter within the sample. The fringe spacing i, which controls the diffusion distance, can be adjusted from 1 to 50 μm by varying the intersection angle, θ, between the two interfering beams. The fringe spacing we used ranged between 5 and 25 μm. For photobleaching, the laser beam power was set to 100 mW during a typical bleaching time of 500 ms (which has to be much shorter than the characteristic diffusion time constant). We found no influence of the bleaching time (in the range of 100 ms to 1s) on the measured diffusion coefficients. After bleaching, the same light source, highly attenuated ($\sim 10^{-3}$ of the bleaching intensity), is used to monitor the recovery of fluorescence due to the diffusion of both fluorescent and bleached molecules within the bleached area. The beam intensity is reduced to avoid subsequent photobleaching. The fluorescence of the sample is collected by a condenser and after passing through an optical band-pass filter centered at 520 nm (about the center of the emission band of fluorescein) it is detected by a photomultiplier tube, protected from overexposure during the photobleaching pulse by an electronic shutter. To increase the signal-to-noise ratio of the experiment the fringe pattern position is modulated at a frequency f_0 and an amplitude $i/2$ giving a resulting signal modulated at a frequency $2f_0$ which is fed into a lock-in amplifier. The optical modulation is obtained by applying a sinewave signal to a piezoelectrically driven mirror which reflects one of the two incident beams. The outputs of the photomultiplier and of the lock-in amplifier are fed into an analog-to-digital converter. The whole FRAP apparatus and data collection system is controlled by a personal computer. Data from 4 to 16 individual FRAP measurements were averaged for each diffusion coefficient determination.

2.4.2. Diffusion Coefficient Calculation. The rate at which the recovery of homogeneous fluorescence process takes place is related to the translational mass diffusion coefficient of the diffusing molecules. The diffusion coefficients were deduced from the fluorescence recovery curves. The concentration of fluorescent and bleached molecules in the sample can be described by the mass diffusion equation according to Fick's second law as

$$\frac{\delta C}{\delta t} = D\frac{\delta^2 C}{\delta x^2} \tag{1}$$

where C is the concentration of fluorescent molecules at time t and location x.

A solution of this equation for the system used where x is a sinusoidal time function can be written

$$C = C_0 \exp^{-Dq^2 t} \tag{2}$$

or

$$C = C_0 \exp^{-t/\tau} \tag{3}$$

with

$$\tau = \frac{1}{Dq^2} \tag{4}$$

where C_0 = initial concentration of fluorescent molecules immediately after photobleaching; D = translational diffusion coefficient ($m^2\ s^{-1}$); t = time (s), the origin of time being chosen at the end of photobleaching pulse; τ = relaxation time of diffusion (s), with

$$q = \frac{2\pi}{i} \tag{5}$$

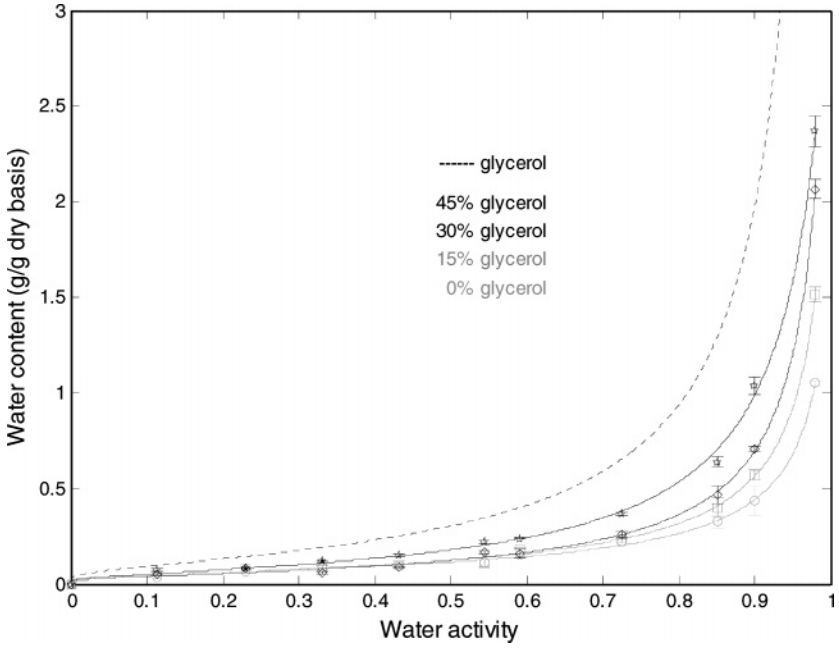

Figure 3. Sorption isotherms of the iota-carrageenan film containing 0% (○), 15% (□), 30% (◊), and 45% (☆) of glycerol (w/w of the carrageenan dry matter), fitted with Ferro−Fontan model.[20] A sorption isotherm of pure glycerol is added in the dashed line, extracted from Uniquac model.[21]

where q = wave vector (m^{-1}); i = fringe spacing of the bleaching light pattern (m).

The fringe spacing is directly related to the angle 2θ between the two interfering beams and to the excitation wavelength λ

$$i = \frac{\lambda}{2 \sin \theta} \tag{6}$$

In FRAP experiments, the fluorescence intensity is proportional to the concentration of fluorescent molecules for the fluorescein concentration used. In the simplest cases, the diffusion coefficient is estimated by fitting the recovery curve to an exponential function defined as

$$I(t) = A \exp^{-t/\tau} + B \tag{7}$$

where I is the level of intensity of the fluorescence light (in arbitrary units), A is the amplitude of fluorescence after photobleaching, and B is the baseline. The characteristic time constant, τ, of the system is inversely proportional to the diffusion coefficient.

If all of the fluorescent molecules are mobile, the baseline B has the value of the fluorescence intensity before the bleaching pulse, B_0. If at long time B is larger than B_0, this will suggest that within this time window there is in the sample an immobile fraction of fluorescent molecules. In the case of multiple diffusion kinetics, the fit should be done with more than one exponential; however, we never fit more than two exponentials, because going beyond that number requires a great accuracy of the data that we do not have. The values of the unknown parameters were determined by minimizing the sum of the square of the differences between the measured and predicted values, using a Levenberg−Marquardt algorithm.

3. Results and Discussion

3.1. Moisture Sorption Isotherms of Glycerol Plasticized Carrageenan Films. The water vapor sorption kinetics at 20 °C for films containing 0, 15, 30, and 45% glycerol (w/w carrageenan) was determined (three replicates) using the gravimetric method.[19] From equilibrium moisture content corresponding to each tested relative humidity (see Table 1), the experimental points of the sorption isotherm were obtained for aw ranging from 0 to 0.98. Then, the Ferro Fontan equation was used to model the sorption isotherm data for water activities lying between 0 and 0.98.[20]

Because of their hydrophilic nature, hydrocolloid film properties are highly dependent on environmental conditions such as relative humidity and temperature.[10] An important quantity of water can be held within their cross-linked polymer network. As presented in Figure 3, the water content of carrageenan films is far more sensitive to relative humidity when glycerol is included. This hydrophilic plasticizer increases the water sorption of carrageenan film. The sorption behavior of carrageenan films with different amount of glycerol is not linear: it becomes more significant at water activity above 0.7, whereas no significant difference is noticeable for water sorption at lower water activities. Moreover, the sensitivity to water activity appears also to be slightly more marked for high plasticizer contents above 30%. High water transfer inside the matrix containing glycerol has already been reported on the same materials (0% and 30% glycerol films).[18] Only 45% glycerol film water content appears to be systematically higher than the other glycerol concentrations used. Moreover, it tends to separate from the other sorption isotherm and to get closer to the pure glycerol sorption isotherm, added from Uniquac model[21] in Figure 3, the dashed line. This could correspond to a glycerol phase separation in the hydrocolloid system, as it is classically observed for this level of plasticizer introduced in films forming materials.[22] Glycerol is indeed a highly hygroscopic molecule generally added into film-forming solutions to prevent film brittleness. Water molecules also act as plasticizers. Water incorporation in a system increases molecular mobility, decreases solution viscosity, and increases elasticity in the rubbery state.[23] Sorbed water, as well as glycerol, acts as plasticizers by lowering the glass transition temperature, T_g, of the polymer.[24] According to Debeaufort and Voilley,[25] the plasticizers can also act as lubricants of the polymer chains and, thus, allow fibers to slide one over the other, improving flexibility of the film. More than the classical plasticizing effect of water and glycerol, it is also interesting to know what their real impact is on the diffusion process of another molecule incorporated in the polymer network.

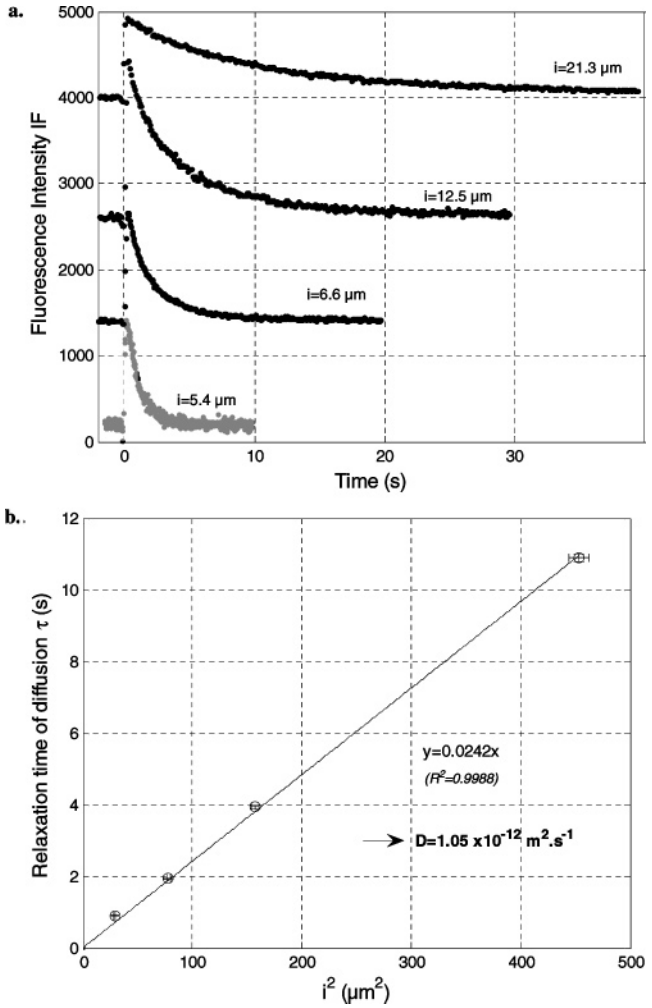

Figure 4. a. Fluorescence recovery intensity signal as a function of time, IF = $f(t)$, when the fringe spacing i is varying. These experiments have been performed on a 3% carrageenan film containing 3 μM fluorescein (without glycerol) equilibrated at 0.98 water activity. b. Characteristic constant time of diffusion (corresponding to the previous plot) as a function of the square of the fringe spacing.

3.2. FRAP Analysis. FRAP measurements have been performed on carrageenan films in order to estimate fluorescein diffusion when varying external relative humidity (therefore water content) and glycerol concentration. Typical fluorescence recovery curves using four different fringe spacings for the same system (aw = 0.98) are given in Figure 4a. Time 0 corresponds to the beginning of the fluorescence recovery process by diffusion following the photobleaching pulse. This process gradually takes place with a time constant τ related to the translational mass diffusion coefficient D (eq 4). If the motion is due to diffusion with a simple kinetic, the recovery signal can be fitted with a simple exponential, and the characteristic time constant of diffusion τ should be proportional to the square of the fringe spacing i^2 (eq 5). The fit of $\tau = f(i^2)$ conduces to a typical linear relationship, which is relevant to the diffusive phenomena. The relative uncertainty on i is of order 2%.[13] This linear dependence of τ on i^2 has been verified for our system on a 0% glycerol carrageenan film (equilibrated at aw = 0.98), demonstrating that diffusion of the fluorescein molecules is the only process responsible for the fluorescence recovery (Figure 4b). From the slope of this linear relationship (straight line with zero intercept; goodness of fit was evaluated by R^2), a fluorescein diffusion coefficient of 1.05 ($\pm$0.03) $\times$ 10^{-12} m^2 s^{-1} can then be easily calculated according to eqs 4 and 5.

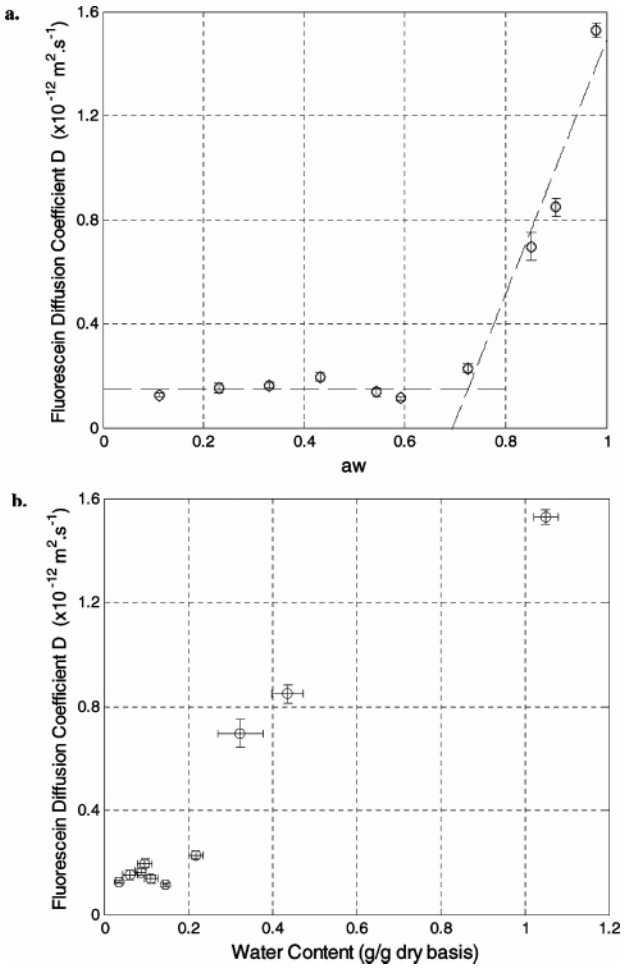

Figure 5. a. Fluorescein diffusion coefficient in a 3% carrageenan film (without glycerol) at various water activities. b. D as a function of film water content (corresponding to the above water activities).

3.2.1. Effect of Carrageenan Film Hydration Level on Fluorescein Diffusion. From fluorescence recovery data obtained for 0% glycerol carrageenan films for levels of hydration below aw = 0.98, it turns out that the fluorescence recovery curves are not simple exponentials but have to be fitted with at least two exponentials, one with a fast relaxation time ($D \sim 10^{-13}$ m^2 s^{-1}) and another with very long relaxation time ($D > 10^{-15}$ m^2 s^{-1}), the ratio of the two populations being roughly constant, $\sim$1. Furthermore, about half of the probe molecules seem to be frozen in the matrix.

We will first discuss the evolution of the fast relaxing species when the level of hydration of the films is increased, and the data are presented in Figure 5 a,b. The fluorescein diffusion coefficient has been estimated from a two-exponential fitting of the fluorescence recovery curves monitored over short-time experiments of 60 s. The diffusion remains approximately constant in the range of 0.1 and 0.6 water activity, with a diffusion coefficient of about 10^{-13} m^2 s^{-1}. Then for higher hydration state of the film, corresponding to an aw range between 0.7 and 0.98, D increases to 10^{-12} m^2 s^{-1}. This last value remains 2 orders of magnitude less than the fluorescein diffusion measured in pure water (D = 5.1 ($\pm$0.2) $\times$ 10^{-10} m^2 s^{-1} at 25 °C [26]), due to a high carrageenan polymer concentration even for high water activities. The diffusional processes involved in fluorescein transport in the polymer network are highly dependent on the water content of the films. It is indeed related to the higher water content of film for this aw range, as displayed on the sorption isotherm (Figure 3). Therefore, water acts on

the mobility of other hydrophilic molecules, such as fluorescein dissolved in the carrageenan polymer network, by decreasing "the apparent viscosity" of the system. From a certain threshold of water content (of about 0.2 g/g dry matter) in the system, the polymer network becomes more and more permeable to hydrophilic small molecules.

Another population of molecules can be identified from these recovery experiments. It corresponds to a much slower diffusing species with a diffusion coefficient ranging between 10^{-15} and 10^{-14} m^2 s^{-1}. The mobility of this population does not seem to be strongly affected by increasing the level of hydration of the films. However, our experiments do not enable us to discuss accurately this population. Nevertheless, in all cases, fast and slow species exhibit a highly enhanced diffusion for the highest level of hydration (aw = 0.98), compared to the lower hydrated states.

In addition, except for the 0.98 water activity, the previous experiments do not display a complete return of the fluorescence intensity to the intensity baseline, B_0, prior to the bleaching even for long time experiments. Long-time acquisition, over 4000 s for the lowest water activity, suggests that a third population of fluorophores is immobilized in the system, for all samples below 0.98 water activity. Incomplete recovery is indeed a direct indication of the existence of an immobile fraction of fluorescein within the three-dimensional polymer network, which is assumed to be immobile at the time scale of the experiment (the polymer diffusion should be more than 10^{-16} m^2 s^{-1} to interfere with fluorescein recovery in the time scale of the experiment, which is irrelevant for a polymer such as carrageenan, especially in a dry state). The relative proportion of (im)mobile fluorophore in the sample can then be estimated by amount of (non)-recovered fluorescence intensity. The estimated fraction of immobilized fluorescein molecules is found to be approximately 50% for aw < 0.6, 40% for aw = 0.7 and 0.8, and 30% for aw = 0.9, until total release from the polymer network for aw = 0.98. For this last level of hydration, the phenomenon seems thus to be more complex: the immobile fraction is free but its diffusion coefficient still remains lower than that of the initially mobile fraction. For aw < 0.98, this means that a finite amount (between 50% and 70%) of the population is composed by a fast diffusing population and a much slower one, and the rest of the population is retained by the carrageenan structure. Nevertheless, it is not possible by this technique to assess if the trapping of the fluorescent molecules is due to weak physicochemical interactions or is related to some physical retention due to steric hindrance (fluorescein molecules could be entrapped in the carrageenan double helices) or a combination of both phenomena. At least we can infer that the fluorescein is not strongly retained by the polymer, as the whole population becomes mobile at the highest level of hydration. Therefore, if interactions between fluorescein and the polymer matrix exist, they must be weak.

Another way to fit our experimental data could have been to use a Levy flight distribution to describe the fluorescein diffusion assuming that diffusion is anomalous (non-Brownian diffusion) due to the presence of more than a single diffusing species with a proper diffusion coefficient.[27] We could also have used nonlinear curve-fitting with a multiexponential distribution, the fit being obviously better as the number of exponentials increases. Periasamy and Verkman[28] used this method to analyze fluorescence recovery data, considering a continuous distribution of diffusion coefficients, which enabled them to characterize superdiffusive phenomena. Anyhow, though such treatments would increase the quality of the fit, we do not think that in our case we would have got a better comprehension of the physics of diffusion within the films.

To better understand how diffusional motion can occur in such a system, it is necessary to refer to the structure of the film. Iota-carrageenan molecules in aqueous solutions are known to undergo a coil-to-helix transition on cooling that led to the formation of a clear and elastic gel consequently to a double helix association.[29] This conformation change from a disordered state to an ordered state is strongly dependent on polymer concentration and the presence of ions.[30] The ι-carrageenan concentration used for film-forming solution is 3% in water solution, which is above the critical concentration required for gelation, as reported by Rees et al.[31] and by Hossain et al.[32] Moreover, cations play also a major role in the gelation mechanism through electrostatic interactions. Counterions shield the negative charges of the carrageenan sulfate groups and then promote formation of associations between double helices to form an infinite network.[33] The commercial sample of ι-carrageenan used as film-forming agent already contains the amount of ions in accordance with classically observed concentrations for promoting gelation (Na^+, 3.2%, K^+, 6.8%, from Degussa).[32] Thus, during the film formation, carrageenan polymers, under random coil state in hot solution, undergo on cooling a coil-to-helix transition followed by helices association (casting and drying are carried out at 30 °C, which is at a temperature below the helix melting point reported for this polymer[34]). This three-dimensional network formed by polysaccharide double helices is then dried to obtain a compact solid film. The three-dimensional structure of the iota-carrageenan double helix has been determined by Janaswamy et al.[35] using X-ray diffraction. The molecule forms a half-staggered, parallel, 3-fold, right-handed double helix, stabilized by interchain hydrogen bonds from hydroxyl groups in the G4S units (see Figure 1). This aggregated double-helical structure is moreover organized into a three helices trigonal unit, each pair of helices being 13.9 Å laterally spaced. The helix diameter is almost the same as the helix−helix separation, so that the helices do not interdigitate. The sulfate groups are strongly implied in helix−helix interactions. These interactions are also mediated by sodium ions and water molecules. Therefore, on the basis of this structure, an iota-carrageenan film can be seen at a microscopic scale as a joined pair of double helices whose molecular axes are set 13.9 Å apart. The diffusing substance inside this matrix, fluorescein, is a small molecule with a hydrodynamic radius of 5 Å.[26] Thus diffusion of fluorescein dissolved in water should easily occur in such a system. Besides the observed results of mobility enhancement with water are in accordance with the chemical nature of fluorescein (Figure 1). Indeed, fluorescein is a synthetic organic dye, anionic, wholly acid, with both COOH and OH groups. It is highly soluble in distilled water (over 20% at 20 °C) and moderately soluble in glycerol (slightly more than 1%, and thus largely more than 3 μM).[36] Water contribution, which becomes sufficient enough from 0.7 water activity, could play a double role. On one hand, water can plasticize the double helices of carrageenan and render the chains more flexible, and on the other hand, an increased amount of water can disjoin some group of helices making more open space for fluorescein to diffuse through. Both mechanisms lead to an increase of the diffusion process of fluorescein.

The system could thus be described with a population of fluorescein molecules which always diffuse quickly (D is at least about 10^{-13} m^2 s^{-1}), maybe through microscopic open connected channels within the film matrix, a second population with a slow diffusional motion and a third population which is

retained among the mesh of the film while the water concentration is not large enough in the system to give complete mobility. Increasing water content could favor creation of flow channels and facilitate fluorescein diffusion. The system then exhibits a complete diffusion breakthrough as the film water content increased, or said differently when the polymer concentration decreases. It arises as a ternary system, the polymer, the diffusing solute, and the solvent, in which the solvent appears to play a preponderant role. The gel state arises on the asymptotic part of the isotherm to aw = 1. For 0.98 water activity, the polymer concentration still remains at a high level. The diffusion of 4-ethyl guaiacol, a two times smaller molecule than fluorescein (diffusion coefficient of both molecules in pure water are comparables), has been studied in iota-carrageenan gels by Rondeau-Mouro et al.,[37] using the widely method of pulsed field gradient spin−echo nuclear magnetic resonance, which also allows a direct measure of small molecules diffusion coefficients, but for values above 10^{-14} m^2 s^{-1}. They observe a small decrease of the aroma and water self-diffusion coefficients in gel (less than two times), compared to those obtained in pure water. They suggest the possible effect of water microviscosity changes on the retardation of molecules diffusion, with hydrodynamic interactions between the aroma molecules and the polymer chains. In our case, a hypothesis that could explain the observed results is retention of water in double helices of carrageenan polymer that render water unavailable for fluorescein motion. Carrageenan polymer could indeed present more affinity for water than the diffusant, fluorescein, leading it to sorb more water at first than fluorescein until a certain level of hydration. Therefore, this preferential sorption mechanism should affect the increase of fluorescein diffusion coefficient with a threshold effect, corresponding to the carrageenan threshold of hydration.

3.2.2. Effect of Glycerol Content on Fluorescein Diffusion. The presence of this plasticizer is suspected to enhance the diffusion of other molecules present in the matrix such as fluorescein as it usually improves flexibility of the whole polymer chains in the film.[22] Results displayed in Figure 6a, which present the evolution of the fast relaxing species as glycerol concentration is increasing, tend to confirm this hypothesis: fluorescein diffusion coefficient increases by 3 orders of magnitude (from about 10^{-13} to 10^{-10} m^2 s^{-1}) from the lowest to the highest water activity and glycerol concentration. These values of diffusion correspond to the most mobile population of fluorescein in the system. As seen previously, the diffusion is independent of water concentration for a water concentration below 0.2 g/g dry matter, and it appears also to be independent of glycerol concentration for a glycerol concentration below 45% (corresponding to 0.31 g/g dry matter). Therefore, it appears that both glycerol and water (mainly at high water activities) present a great impact on fluorescein diffusion from a certain threshold. These values are much higher than those obtained for fluorescein diffusion in hydroxypropylcellulose solutions (most of FRAP experiments concerning biopolymers have been done on solutions or gels media): 4.9 up to 2.9 × 10^{-12} m^2 s^{-1} at 25 °C when the polymer gel concentration varied from 0 to 3.871% in water,[38] which are only two times more than the highest value found for the most hydrated carrageenan film. Thus, the influence of glycerol seems really important, and the following mechanism for this large increase of the fluorescein diffusion coefficient can be proposed at first. The glycerol may act by disrupting the double helices aggregates of carrageenan, leading to a higher diffusion coefficient for fluorescein because of the increase of the overall mobility of the system.

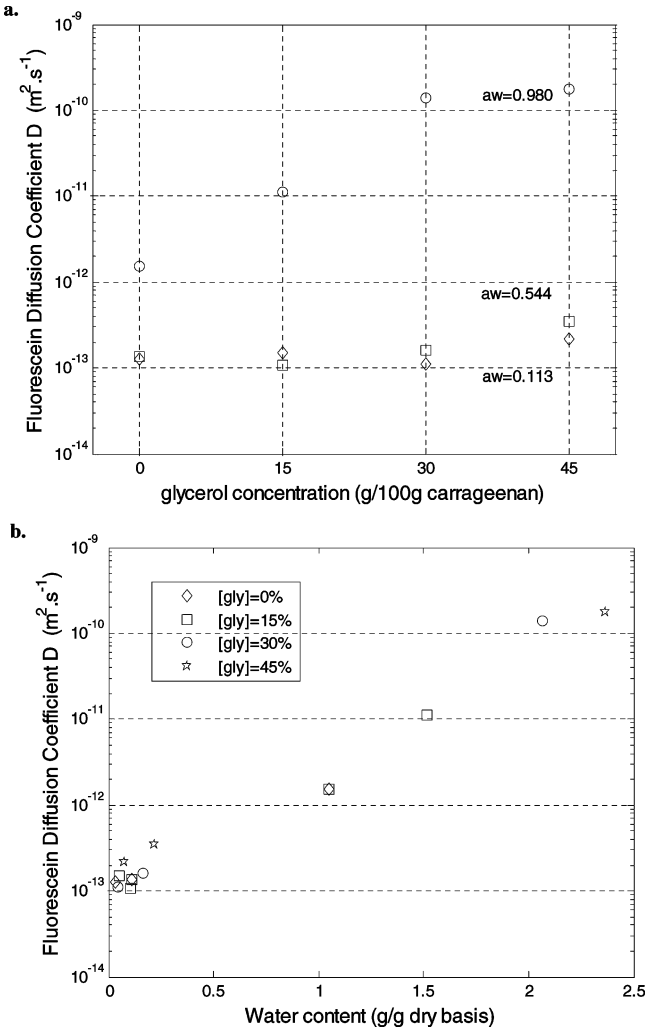

Figure 6. a. Fluorescein diffusion coefficient *D* in a 3% carrageenan film containing different amounts of glycerol (0, 15, 30, and 45% of the carrageenan dry matter). *D* has been measured at three different level of hydration, for aw = 0.113 (◊), 0.544 (□), and 0.980 (○). b. *D* as a function of film water content whatever the glycerol concentration.

Nevertheless, from Figure 6b, the predominance of water in the increase of the diffusional process of fluorescein in the film appears clearly. Therefore, the glycerol only increases the amount of sorbed water, by acting as a water activity depressor, thus favoring moisture sorption.

A lot of studies have already been done on the effects of polyol plasticizers added in edible film materials. Most of them concern mechanical and thermal properties as well as permeability properties.[10,22,25] The barrier properties are related to the permeation mechanism, which is governed by a kinetic parameter, the diffusion of the permeant through the film, and a thermodynamic parameter, its solubility in the matrix network. The effects of plasticizers on both these parameters are largely documented in the literature for various permeants, especially water vapor and oxygen, and less often aroma compounds.[39] Thus, the permeability of biopolymer film often refers to its permeability to gas transfer across the film. However, the physical state of water is also found to affect hydrophilic films, inducing a higher liquid water transfer due to interactions with the polymer.[40] The addition of plasticizers to biopolymers aims to increase their workability, flexibility, or extensibility.[23] Plasticization results in lowering the glass−rubber transition

temperature of the polymer, concomitant to the reduction of the viscosity, with a positive impact on the gas permeability of the film to small molecules, which is attributed to a reorganization of the polymer network and increase of free volume.[24,41] This phenomenon is indeed thought to be related to the ability of the plasticizer to establish favorable hydrogen bonds with the polymer thereby disrupting intermolecular polymer interactions (this mechanism being favored for high relative humidities), with consequent increase of polymer chain mobility and therefore decrease of the film barrier properties.[42−44] Although there is a lot of work on gas permeability and mechanical properties of biopolymer films, there is little research on molecular mobility within the film. Ozdemir et al. [45] reported a value on the order of 10^{-11} m^2 s^{-1} for the diffusion coefficient of potassium sorbate (an antimicrobial agent of about 150 g mol^{-1} molecular weight) in whey protein films plasticized with sorbitol. The results obtained in this study show interesting findings regarding the impact of the plasticizer on the increase sorption of water (for the glycerol concentration range tested), which is the only one and real ubiquitous plasticizer that affects the molecular mobility in edible barriers.

As FRAP involves a low fluorescein concentration with a small amount of bleached molecules, the diffusion of the probe is very close to Brownian motion. Assuming Brownian motion, the fluorescein diffusion coefficient can then be described by the Stokes−Einstein relation

$$D = \frac{kT}{6\pi\eta r} \tag{7}$$

where D = translational diffusion coefficient (m^2 s^{-1}), k = Boltzmann constant (m^2 kg s^{-1} K^{-1}), T = temperature (K), η = medium viscosity (Pa s), r = hydrodynamic radius of the spherical diffusing molecule (m).

The hydrodynamic radius of the fluorescein molecule has been estimated to be 5.02 Å by Mustafa et al. using FRAP measurements.[26] From the highest diffusion coefficient corresponding to a carrageenan film containing 45% glycerol and equilibrated at 0.98 water activity, the viscosity of the medium surrounding fluorescein molecules can thus be estimated to be 0.24 Pa s. To compare with a pure glycerol environment, where the viscosity is 1.50 Pa s,[46] fluorescein diffusion should be 2.85×10^{-11} m^2 s^{-1}. The viscosity of the medium in which fluorescein molecules are moving (0.24 Pa s) could be related to the viscosity of a 90% glycerol−10% water mixture.[46] Thus, carrageenan molecules play an important role in the viscosity of the medium. This consideration could be in favor of the hypothesis of a preferential hydration of the carrageenan molecule that renders water less accessible for other molecules in the system. An increase in molecular mobility of other species should thus need a larger amount of sorbed water to be effective.

In such a system composed of a polymeric matrix of carrageenan, the following mechanism can be involved in the diffusing molecule mobility as a function of water content in the film. First, water would mainly be sorbed by the carrageenan polymer until a water threshold of about 0.2 g/g dry matter. The water fixation sites of the polymer are then saturated, which makes the excess amount of water available for the expansion of the matrix, inducing an exponential increase in the diffusion of small molecules. This mechanism thus implies a stronger interaction energy between the polymer with water molecules rather than with fluorescein. According to this hypothesis, glycerol is nothing more than a water activity depressor. Moreover, different sites of the carrageenan molecule could be implied in the fluorescein interaction, giving rise to different populations with a proper mobility. Concerning the two mobile populations, the fast diffusing molecules should never be in interaction with carrageenan, whereas the slow diffusing ones should present weak interactions with carrageenan leading to a slower diffusional motion. The immobile population should be more strongly adsorbed on the carrageenan molecule, until a high level of hydration (>1 g/g dry matter) from which water makes all fluorescein molecules mobile.

4. Conclusions

The application of the FRAP technique to solid films composed of a iota-carrageenan matrix containing various amounts of glycerol, in which fluorescein was added, has enabled us to study the motion of this small molecule as a function of relative humidity conditions. The investigation of diffusion into the material by FRAP analysis provides a useful tool to assess materials properties in different environmental conditions and leads to the following conclusions concerning the studied system.

1. In a pure carrageenan film, a threshold of water content exists from which mobility in the matrix is enhanced. The water quantity available in the system directly controls the diffusion of small molecules in the polymeric film. This gives direct information on the functional characteristic of this film as a coating for its resistance to the water sorption and its concomitant permeability to small molecules.

2. In such films, complex diffusion mechanisms are engaged, with partial fluorescein retention by the matrix and a total release at the highest water activity. This fluorophore immobilization may arise from either physical (steric hindrance) and/or weak physicochemical interactions (electrostatic, hydrogen bonding). That cannot be assessed by the technique, used but it could be the starting point of another work at a molecular scale. Studying the influence of temperature on the diffusion coefficient of fluorescein could give some information on the energy of binding of the immobile species with the matrix. Nevertheless, these results are of great interest for understanding the barrier properties of a film to a molecule of interest for various intrinsic and extrinsic conditions, for example to optimize drug release in pharmaceutical research or to select active substances incorporated in biopolymer coatings or films for food packaging applications.

3. The action of glycerol on diffusion is not a direct plasticization. It enables larger amounts of water to be adsorbed, which in fact is the only plasticizer having a huge impact on diffusion.

Working at a microscopic scale through FRAP analysis thus enables us to obtain information about the molecular-scale structure and diffusion processes involved in the studied system of dried carrageenan film under various concentrations ranges and relative humidity conditions. It allows a better understanding of the permeation process occurring when edible barriers are applied.

Acknowledgment. We gratefully acknowledge ANRT (Association Nationale de la Recherche Technique), France, and Ministère de l'Enseignement Supérieur et de la Recherche and Industries for financial support of this work that is part of the national program CANAL "Conception Assistée d'Aliments Composites". We thank in particular Pr. Trystram (ENSIA, Massy), Dr. Daudin, and Tania Rougier (INRA, Clermont-Ferrand) and Pr. Guilbert, Pr. Gontard, and Elisabeth Roca (UTCA, Montpellier) for their constructive and enriching

discussions regarding experimental results during meetings of this program.

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BM060179R