

UTILITY OF THE FLUORESCENCE RECOVERY AFTER PHOTBLEACHING TECHNIQUE TO CHARACTERIZE MOLECULAR DIFFUSION IN KONJAC GLUCOMANNAN SOLUTIONS.

Felipe Alvarez-Manceño¹, Mariana Landin¹, Ramón Martínez-Pacheco¹, Kevin Braeckmans², Stefaan C. De Smedt², Joseph Demeester²

¹Departamento Farmacia y Tecnología Farmacéutica. Facultad de Farmacia. Universidad de Santiago de Compostela. Santiago de Compostela, España. E-mail: mlandin@usc.es

²Laboratory of General Biochemistry and Physical Pharmacy, University of Gent, Harelbekestraat 72, 9000, Gent, Belgium.

Resumen / Abstract

En este estudio se llevó a cabo la determinación de los coeficientes de difusión de macromoléculas fluorescentes en disoluciones de Glucomanano de Konjac (KGM) de distintos orígenes geográficos, utilizando de forma combinada microscopía láser confocal y FRAP (fluorescente recovery after photobleaching). Los resultados obtenidos permiten concluir la utilidad y sensibilidad de la técnica de FRAP para caracterizar la difusión de macromoléculas de diferente peso molecular en disoluciones del polisacárido KGM.

In this study, diffusion coefficients of fluorescent macromolecules in solutions of Konjac Glucomannans (KGM) of different geographic origin were determined by Confocal Scanning Laser Microscopy (CSLM) in conjunction with Fluorescence Recovery After Photobleaching (FRAP). Results indicate that FRAP is a useful and sensitive technique in order to characterize diffusion of different molecular weight macromolecules through solutions of KGM.

Introduction

In the last years an important work has been carried out within the pharmaceutical field directed to establish the possible utility of polysaccharides as base excipients for the elaboration of controlled release dosage forms. Konjac Glucomannan, a polysaccharide obtained from the tubers of *Amorphophallus Konjac*, due to its thickening, film forming and gelling

properties, alone or synergistically with other polysaccharides, seems a promising candidate. When a solid dosage form based on KGM is placed into an aqueous environment it absorbs water from the medium and forms a gel through which drug is released. Understand how diffusion of the drug it is influenced by the structure of the polymer network it is a key aspect from a pharmaceutical point of view if we like to be able to modulated drug delivery.

There is a tradition in pharmaceutical research of performing release experiments to study diffusion. Nevertheless also other techniques like NMR, light scattering or fluorescence recovery after photobleaching (FRAP) have been proved very useful for the characterization of diffusion phenomena.

FRAP denotes a method for measuring the motion of fluorescently labeled molecules. A small spot on the fluorescent surface is photobleached by a brief exposure to an intense focused laser beam. Recovery occurs by replenishment of intact fluorophore in the bleached spot by lateral transport from the surrounding surface. Interesting information obtainable from FRAP experiments includes determination of the diffusion coefficient and total fraction of fluorophore which is mobile (1)

Nowadays, most confocal scanning laser microscopes (CSLM) are equipped with the feature to bleach user defined regions within fluorescent samples. This allows FRAP experiments to be easily carried out.

The aim of this study was to evaluate if CSLM in conjunction with FRAP can be useful for

measuring diffusion coefficients in Konjac Glucomannan (KGM) solutions and to assess the influence of molecular weight macromolecules on diffusion coefficients.

Materials and Methods

FRAP experiments were performed on a CSLM (model MRC1024 UV, Bio-Rad, UK) modified to be able to bleach arbitrary regions. Bleaching experiments have been performed with the 488-nm line of a 4 W Ar-ion laser model Stabilite 2017; Spectra-physics, Germany). A 10× objective lens (CFI Plan ApoChomat; Nikon, The Netherlands) with a numerical aperture of 0.45 was used. Typical photobleaching and measuring powers in the sample were 2 mW and 5,5 μW respectively.

The diffusion coefficient measurements were performed with fluorescein isothiocyanate dextran (FITC-dextran) probes (Sigma-Aldrich) of different averaged molecular weights (M_w): 7.7×10^4 g/mol, 1.3×10^5 g/mol, $5,11 \times 10^5$ g/mol and approximate $M_w = 2 \times 10^6$ g/mol. Solutions with a concentration range from 0.45 mg/mL to 20 mg/mL of probes were prepared in 20 mM HEPES buffer at pH 7.4 for linearity tests.

Diffusion experiments were performed on solutions of three Konjac Glucomannans (KGM) from different suppliers and geographical origins: American (Triple Crown America Inc. Lot: 3500C), European (Escuder, Spain, Lot: 019) and Japanese (Propol A®, Lot: AKG07). Solutions of the polysaccharides in 20mM HEPES buffer pH 7.4 (0.5% p/v) were prepared by mechanical stirring for one hour at 85 °C in a hermetic container. The fluorescent probes were added to an aliquot of the KGM solutions, stirred and left to cool at room temperature to obtain FITC-dextran / KGM homogeneous solutions.

Results and Discussion

Before performing FRAP measurements on KGM solutions, the concentration range in which a linear relation exists between the fluorophore observed fluorescence and its concentration has to be determined. FITC-Dextran solutions with a concentration range from 0.45mg/mL to 20 mg/mL were prepared and its fluorescence

measured. For concentrations up to 2 mg/mL the correlation coefficient for all the probes was bigger than 0.99 and a linear relation could be established between fluorescence and fluorophore concentration. However, at high concentrations the linear relation it's not valid. This could be attributed to saturation of the detector or to an increase in the probability that fluorescence emitted by one molecule is absorbed by a neighbouring molecule in a phenomenon known as "inner filter effect" (2). As a result all FRAP measurements have been done with a concentration of 1.5 mg/mL.

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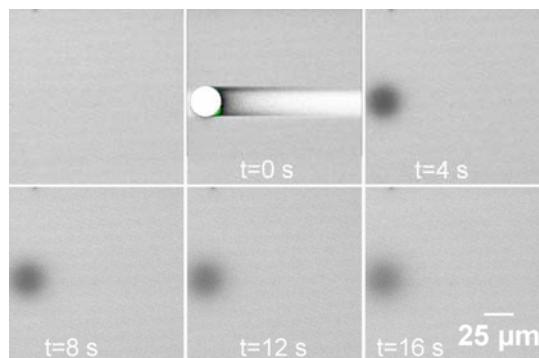


Figure 1. Sequence of the first six images of the experiment with 500000 g/mol FITC-dextran in the Japanese-KGM solution.

The first image corresponds to the FITC-dextran /KGM solution before bleaching, the second image was taken at the moment of bleaching the region of interest (disk), and subsequent images show the recovery of the fluorescence in the bleached area of the KGM solution

Images were processed to obtain the fluorescence recovery curve.

Figure 2 shows an example of the obtained recovery curves corresponding to the 500000 g/mol FITC-dextran in the KGM solutions. Points

correspond to the experimental values and lines to its fitting to the uniform disk model (3).

The uniform disk model developed by Braeckmans and co-workers (3) describes accurately the experimental recovery profile for FITC-dextrans probes in KGM solutions and permits to calculate diffusion coefficients. As American KGM has the faster fluorescence recovery the higher diffusion coefficient can be expected.

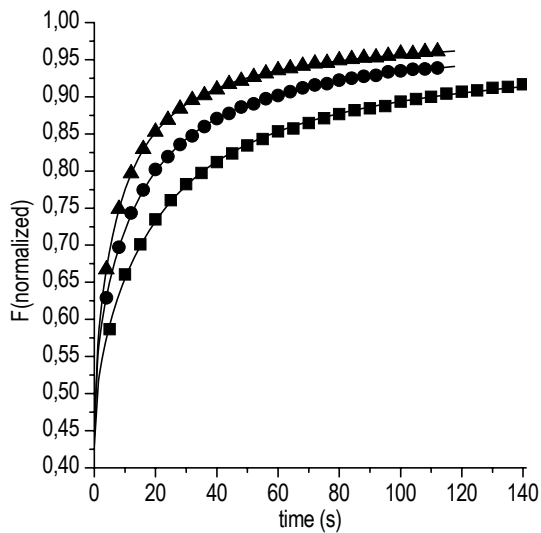


Figure 2. Fluorescence recovery curves for the 500000 g/mol FITC-dextran in japanese-KGM solution (■), european-KGM solution (●), american (▲)-KGM solution and fitting to the uniform disk model (-)

Mean and standard deviation of at least five diffusion coefficient measurements in different sample locations as a function of the probe molecular weight are presented in Figure 3.

KGM solutions have a large effect on the FITC-dextran diffusion. Polysaccharides act as a barrier to the probe diffusion producing reductions of D values higher than 40% against buffer. Variations in diffusion coefficients between KGM solutions for a specific probe are statistically significant showing the Scheffé test four subsets.

The results could be explained according to Stokes-Einstein equation in which diffusion coefficient D and dynamic viscosity η are indirectly related.

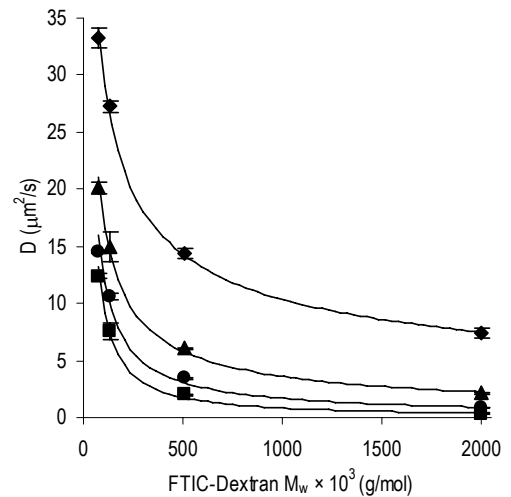


Figure 3. Diffusion coefficients (D) of FITC-dextrans in 0,5% KGM solutions from different origin: japanese(■), european (●), american (▲), and in HEPES buffer (♦) as a function of molecular weight and fitting to $D \sim M_w^a$ (-).

$$D = \frac{kT}{6\pi\eta r_H}$$

The variety with the lowest viscosity, the American KGM, shows the lowest reduction for all the probes. On the contrary, Japanese KGM has a high viscosity presenting the greater reductions because in this case there is a higher viscous drag.

FITC-Dextran chains diffuse slower as increasing probe molecular size. Dependence of FITC-Dextran diffusion coefficient as a function of probe molecular weight was analyzed and found that it can be described by the potential relationship:

$$D \sim M_w^a$$

Data of the fitting obtained are summarized in table 1

Table 1. Exponents and correlation factors for the $D \sim M_w^a$ fitting to the experimental data.

	a	R ²
HEPES buffer	-0,46	0,999
Japanese KGM	-1,08	0,996
European KGM	-0,88	0,993
American KGM	-0,69	0,998

Table 2. Reduction percentages of diffusion coefficients and standard deviations (SD) of FTIC-dextrans in the varieties of KGM with respect to diffusion coefficients in buffer

	<i>Japanese-KGM</i>	<i>European-KGM</i>	<i>American-KGM</i>
<i>FTIC-Dextran Mw</i>	<i>Reduction ± SD %</i>	<i>Reduction ± SD %</i>	<i>Reduction ± SD %</i>
<i>77000</i>	<i>37.3±1.2</i>	<i>43.5±1.2</i>	<i>60.6±2.1</i>
<i>130000</i>	<i>27.7±2.6</i>	<i>39.0±1.0</i>	<i>55.1±4.8</i>
<i>511000</i>	<i>13.8±0.6</i>	<i>24.0±1.0</i>	<i>42.2±1.5</i>
<i>2000000</i>	<i>4.8±0.6</i>	<i>11.3±0.6</i>	<i>29.0±1.7</i>

As can be derived from the exponent values the dependence is not the same for the KGM studied. If the increasing in viscosity produced by KGM was just the mechanism involved in the reduction of diffusion coefficients, having in mind Stokes Einstein equation, the same exponent for FTIC-dextran in buffer and KGM solutions and the same relative reduction at a specific molecular weight probe should be expected. On the contrary, reduction percentages of the probe diffusion coefficients (table 2) are different as a function of its molecular weights indicating an additional effect of the polymer network.

These findings could be explained by sterical hindrance of KGM polymer. so that larger the molecule, the stronger the sterical hindrance.

In conclusion we have shown the utility of FRAP in conjunction with CSLM in order to characterize diffusion of different molecular weight macromolecules through solutions of KGM polysaccharide. FRAP has been shown as a very sensitive technique allowing differentiation between varieties of KGM. This technique seems very promising for characterizing diffusion through polysaccharide solutions and gels based on konjack glucomannan polisaccharide.

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This work was supported in part by grant SAF2002-03440, from the Spanish Ministry of Science and Technology.

Autor de contacto:

Nombre y apellidos: Mariana Landin Pérez

e-mail: mlandin@usc.es

Institución: Facultad de Farmacia. Universidad de Santiago de Compostela.

Dirección: Campus Universitario Sur

Ciudad: Santiago de Compostela

Telf.:981-563 100

Fax: 981-547 148

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