J. Pharm. Pharmacol. 1992, 44: 543–549
Received August 29, 1991

Epi-fluorescence Microscopy and Image Analysis Used to Measure Diffusion Coefficients in Gel Systems

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Abstract—A method using epi-fluorescence microscopy and image analysis has been developed to follow and quantify the diffusion of fluorescent compounds through gels. Two mathematical approaches were employed to calculate diffusion coefficients. The spatial resolution provided by the fluorescence microscope allowed diffusion to be followed over very short distances; accordingly diffusion coefficients were obtained within minutes, even for slowly diffusing systems. The method was successfully applied to the diffusion of macromolecules into agar, carbopol and mucus gel systems.

The diffusion coefficient (D) is a fundamental parameter for describing the mass transport of compounds through different media (Flynn et al 1974). It is of considerable pharmaceutical interest since diffusion coefficients can be used to describe and predict drug release, transport and absorption. The release of drugs from topical applications (Addicks et al 1989), polymeric controlled release systems (Peppas & Lustig 1985; Ritger & Peppas 1987; Harland et al 1988; Courraze et al 1989), gels (Muhr & Blanshard 1982), across synthetic membranes (Colton et al 1971; Sato & Wan Kim 1984) and through mucus gels (Peppas et al 1984) have all been characterized using diffusion coefficients.

Many methods for determining diffusion coefficients have been reported. The most frequently used consists of a three compartment cell with donor and acceptor cells separated by a diffusion barrier. If passage across this barrier is Fickian, D can easily be calculated from the flux (Smith et al 1986; Lee & Nicholls 1987). The time required to reach a constant flux is often substantial, especially for slowly diffusing systems. Degradation of the medium or diffusing substance must then be considered.

A number of novel techniques have been used to follow the diffusion of compounds between two different phases and to obtain diffusion coefficients in polymeric gels. The movement of radiolabelled compounds in silicon gels has been followed using linear TLC radioactive counters (Conrath et al 1989). UV absorbance has been used to examine boundary spreading of a loaded phase into a receptor phase (Muramatsu & Minton 1988; Bain et al 1990).

Nuclear magnetic resonance (NMR) has also been used to determine diffusion coefficients in solutions, polymer solutions, gels and emulsions (Stilbs 1987).

Fluorescent compounds can be detected even within complex media and this property has been used to follow their movement. Recovery after photo-bleaching was used to calculate diffusion coefficients for fluorescent markers in phospholipid multilayers (Lopez et al 1988). More recently fluorescence microscopy quantified by image analysis has been used to follow the movement of fluorescent particles in gel systems (Griess & Serwer 1990), on cell surfaces (Morrison et al 1990) and in the release of macromolecules from a polymer matrix into unstirred solutions (Radomsky et al 1990).

We have reported a method for observing penetration of macromolecules into mucus gels using fluorescence microscopy and image analysis (Henry et al 1990). This technique has been advanced to allow rapid determination of diffusion coefficients in a variety of gels.

Materials and Methods

Apparatus

Fig. 1. shows the apparatus used to follow diffusion. Gels were packed into hollow microslides (0·2 mm depth, 5 mm width, Camlab, UK) and placed on a purpose built temperature controlled stage (set at 25 or 37°C) on the microscope (Labophot, Nikon, UK). The tip of a second microslide (0·05 mm depth, 1 mm width) was advanced to within 100–200 μm of the interface with a micromanipulator. Fluorescent compounds in solution were pumped at low flow rates (2–10 μL min^{-1}, Varioperpex pump, LKB UK), providing a constant concentration at the interface. Diffusion of the

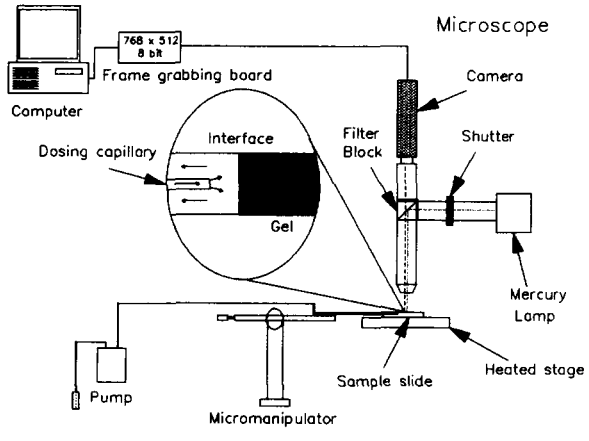

FIG. 1. Apparatus used to follow the diffusion of fluorescent compounds in gel systems.

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fluorescent compound into the gel was visualized by epi-fluorescent illumination (100 W mercury lamp, B2-A filter block Nikon, UK). Exposure to fluorescent light was carefully controlled, to minimize photo-bleaching, using a specially constructed shutter. This consisted of an aperture in the light path covered by an aluminium flap, movement of which was controlled by a rotary solenoid (342-329, RS, UK). An electronic timing circuit allowed variable exposures (0·05–0·35 s), a variable delay between exposures and synchronization with the computer.

Images of the interface were recorded by a camera (Cohu 4722, Synoptics, UK) and digitized by the computer (Dell 310, UK), with an 8 bit 768 × 512 framestore (Synergy, Synoptics, UK). The captured images were analysed using software based on Semper 6+ kernal (Synoptics, UK).

Calibration of apparatus

The response of the microscope, camera and framestore was calibrated with fluorescein (0·1–100 μg mL^{-1}, sodium salt, BDH, UK) dissolved in phosphate-buffered saline (PBS) and sealed in hollow microslides.

Fluorescent photo-bleaching of fluorescein was quantified by observing fluorescein-loaded (10 μg mL^{-1}) agar gels (1% w/v). The fluorescence intensity was monitored under both continuous exposure and intermittent exposure using the automatic shutter.

Diffusion studies

Diffusion studies were conducted with three different gel systems, as follows: 1% w/v agar was dispersed in PBS by heating for a short time. The molten gel was pulled up into the microslide by capillary action and then solidified on cooling to room temperature (21°C). Three per cent w/v carbopol (Goodrich, USA) solution in distilled water was converted into a gel by adjusting its pH to 7·4. The gel was then centrifuged into the microslide (5 min at 3000 *g*, Centaur 2, LKB, UK). Sections of jejunum from freshly-slaughtered pigs were stored under ice during transport. Mucus scraped from the small intestinal sections was centrifuged into microslides.

In each case the gel end of the microslide was sealed and PBS placed on the gel to relieve interfacial tension and prevent dehydration.

Fluorescein- and fluoresceinisothiocyanate-labelled dextrans (FITC-dextran, Sigma, UK) with a range of mol. wt were used in diffusion studies. FITC-dextrans have been shown not to contain any free FITC and to be stable in mucus under the conditions used (Henry et al 1991). Fluorescein was used at a concentration of 10 μg mL^{-1} and FITC-dextrans at 0·5–2 mg mL^{-1} in PBS. These compounds were initially pumped onto the interface at 10 μL min^{-1} for 10 s to flush away buffer; timing was started (t = 0) once fluorescent compound met the gel interface. The flow rate was then reduced to 2 μL min^{-1} for the duration of the experiment. Sequential images of 0·25 s exposure were captured.

Processing of images.

A small field (768 × 10 pixels) perpendicular to the interface was extracted from every picture. This reduced the volume of data stored and the processing time. The image was corrected for background, uneven illumination and camera response. Background illumination was removed by a simple subtraction of a background image, of an illuminated gel without fluorescent material, from all subsequence images. Uneven illumination is a particular problem of fluorescence microscopy. Correction used a fluorescent image of an evenly distributed fluorophore. Each pixel in subsequent images was multiplied by the mean of the whole standard picture and then divided by the corresponding pixel of the standard picture.

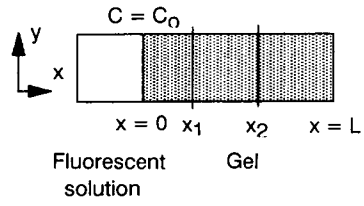

FIG. 2. Diagrammatic representation of the diffusion cell.

Calculation of diffusion coefficients.

Two mathematical approaches were used to calculate diffusion coefficients within the gels, from concentration changes at two separate points in the gel and from penetration profiles.

Method 1. This was briefly reported by Hibberd et al (1990); a more complete derivation is given here.

Fig. 2. is a diagrammatic representation of the diffusion cell. If concentration, C, at the interface, $x = 0$, remains constant, $C = C_o$, and the length of the cell, L, is sufficiently large that end effects at $x = L$ on the concentration measured at x_1 and x_2 are negligible, then the concentration, $C(x_1)$, at distance, x_1, can be described by:

$$C(x_1,t) = C_o \operatorname{erfc}\left(\frac{x_1}{2\sqrt{Dt}}\right) \quad (1)$$

Provided the diffusion is Fickian, with constant diffusivity (Crank 1974), then t denotes time, D is the diffusion coefficient and erfc is the complementary error function. Similarly the concentration, $C(x_2)$, at distance x_2 is given by;

$$C(x_2,t) = C_o \operatorname{erfc}\left(\frac{x_2}{2\sqrt{Dt}}\right) \quad (2)$$

A third concentration profile can be constructed, which is the difference between these two concentration profiles:

$$C(x_1,x_2;t) = C(x_1,t) - C(x_2,t) \quad (3)$$

$$= C_o\left(\operatorname{erfc}\left(\frac{x_1}{2\sqrt{Dt}}\right) - \operatorname{erfc}\left(\frac{x_2}{2\sqrt{Dt}}\right)\right) \quad (4)$$

All three concentration vs time profiles are shown in Fig. 3. It can be seen that $C(x_1,x_2;t)$ has a maximum value at $t = t_{max}$. By differentiation of equation 4 with respect to time, we obtain:

$$\frac{dc}{dt} = C_o\left(\frac{2}{\sqrt{\pi}} e^{\frac{-x_1^2}{4Dt}} \cdot \frac{x_1}{4\sqrt{D}\cdot t^{3/2}} - \frac{2}{\sqrt{\pi}} e^{\frac{-x_2^2}{4Dt}} \cdot \frac{x_2}{4\sqrt{D}\cdot t^{3/2}}\right) \quad (5)$$

$$= \frac{C_o}{2\sqrt{\pi}\sqrt{D}\cdot t^{3/2}}\left(x_1 e^{-\frac{x_1^2}{4Dt}} - x_2 e^{-\frac{x_2^2}{4Dt}}\right) \quad (6)$$

When $t = t_{max}$, $dC/dt = 0$, so that:

$$x_1 e^{-\frac{x_1^2}{4Dt_{max}}} = x_2 e^{-\frac{x_2^2}{4Dt_{max}}} \quad (7)$$

or

$$\frac{x_2}{x_1} = e^{\frac{(x_2^2 - x_1^2)}{4Dt_{max}}} \quad (8)$$

Taking logarithms,

$$\ln \frac{x_2}{x_1} = \frac{x_2^2 - x_1^2}{4Dt_{max}} \quad (9)$$

and hence D is given by,

$$D = \frac{x_2^2 - x_1^2}{4 \ln\left(\frac{x_2}{x_1}\right)} \cdot \frac{1}{t_{max}} \quad (10)$$

Thus by measuring t_{max} for chosen distances from the interface, x_1 and x_2, D can be calculated. The major advantage of this method is that absolute concentrations and the concentration at the interface, C_o, are not required. However, a large number of penetration profiles is required for accurate estimation of t_{max}.

Method 2. The second approach is to use equation 1 directly. At each observation time, t, the penetration profile of fluorophore into the gel produces values for C and C_o at known values for x and t. Values for erfc can be obtained from tables or calculated (Abramowitz & Stegam 1970). A calculation method was used to allow simple inclusion within the software. This allowed D to be readily determined.

Unlike method 1, this approach requires concentration at the interface, C_o. However, D can be obtained from a single penetration profile.

Results

Fig. 4 shows a typical calibration line produced using fluorescein standards. The response was linear ($r^2 = 0{\cdot}9992$, $n = 6$) until saturation of the camera. Higher concentrations can be brought on scale either by reducing the amount of light reaching the sample using neutral density filters or by altering the camera's sensitivity. At the most sensitive camera settings, the detection of unwanted IR light increased significantly; this was reduced by including an IR filter (MXA20390, Nikon, UK) between the filter block and the camera.

Fig. 5a illustrates photo-bleaching of fluorescein at low concentrations. A 50% reduction in fluorescent intensity was observed within 10 s of continual exposure. A shutter on the microscope was used to obtain intermittent illumination. Manual operation of this shutter proved unreproducible. The use of a specially constructed automatic shutter allowed

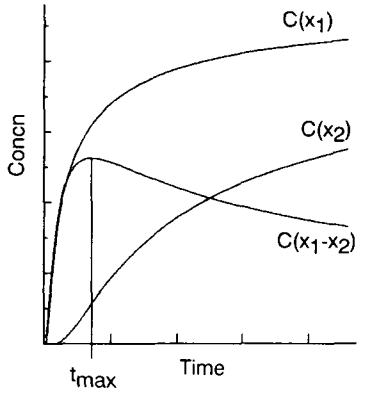

FIG. 3. Concentration/time profiles for a diffusing substance measured at x_1 and x_2.

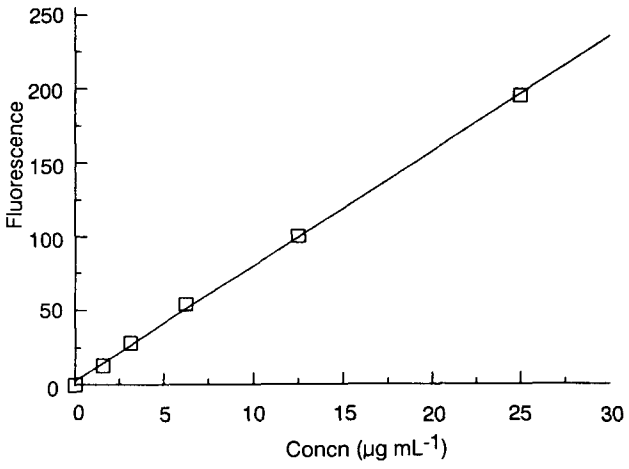

FIG. 4. Calibration of fluorescence microscope and image analysis systems after correction for background and uneven illumination.

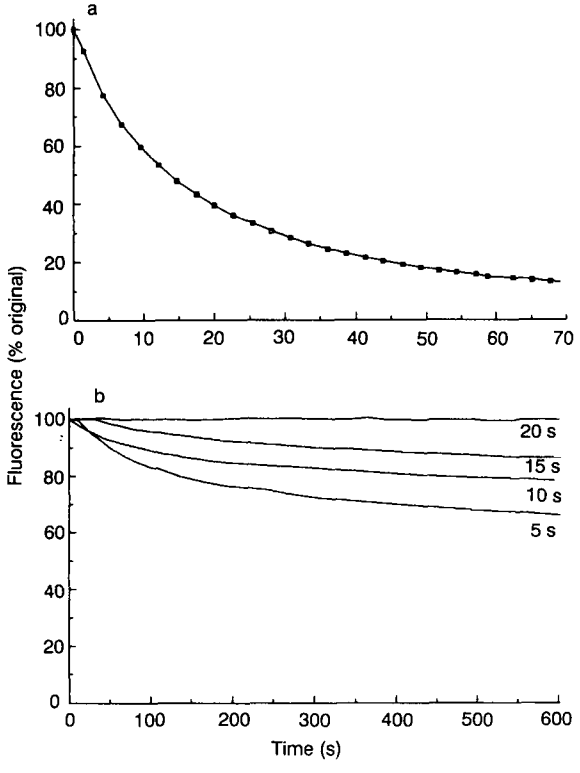

FIG. 5. Photo-bleaching of fluorescein (10 μg mL^{-1}) in the fluorescence microscope when exposed to (a) continuous and (b) intermittent UV light.

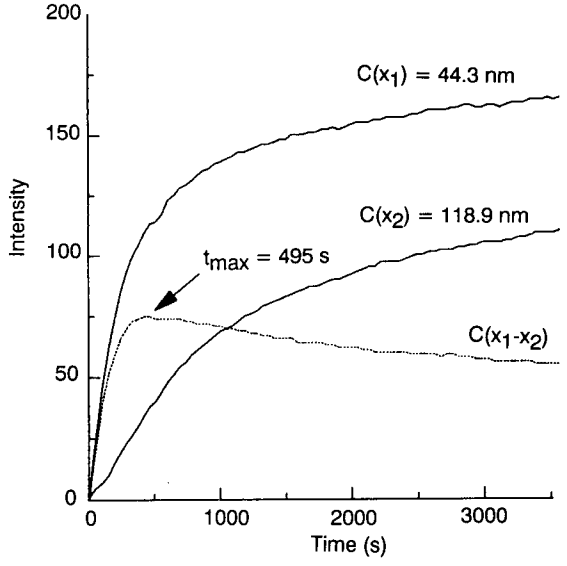

FIG. 6. The concentration/time profiles for FITC-dextran (mol. wt 506 000) diffusing through a 1% w/v agar gel at 25°C, when measured at two distances from the interface.

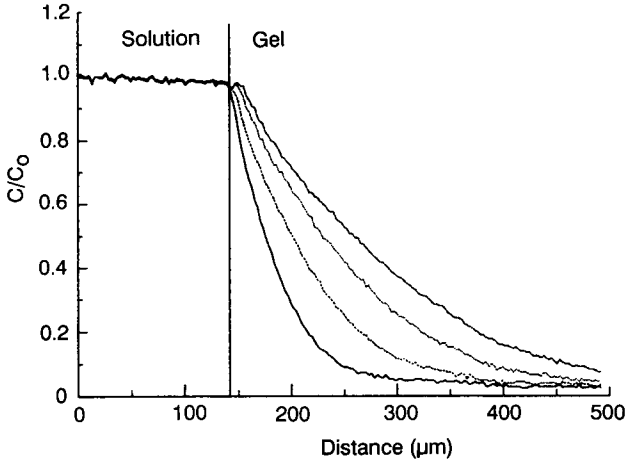

FIG. 8. Penetration profiles for FITC-dextran (mol. wt 148 000) diffusing through a 3% w/v carbopol gel (pH 7·4, 37°C) at different time intervals, —— 1 min, - - - - 3 min, · · · · 6 min, — · · — · · — · · 10 min.

well defined exposure conditions. Variation in picture frequency, with a constant shutter opening (0·25 s) altered photo-bleaching (Fig. 5b). An interval of 15–20 s allowed a series of images to be obtained with minimal photo-bleaching.

Two techniques were used to pack gels into the hollow microslides. Gels that collapsed reversibly when heated (agar) produced well defined interfaces when pulled into the microslide by capillary forces. Gels that deformed under the stresses developed in a centrifuge, were centrifuged into the slides, producing flat interfaces, and were not disturbed by the pumping of liquids onto them. This was used for mucus and carbopol. Other gel systems successfully packed into slides include gelatin, various hydroxypropylmethylcellulose gels, polycarbopol and polyacrylamide gels.

Solutions of FITC-dextran (mol. wt 4000 and 506 000) were pumped onto a 1% w/v agar gel interface. Images were obtained every 15 s and the diffusion of the FITC-dextran into the gel was followed. Fig. 6 shows how concentration changed at two selected distances ($x_1 = 44{\cdot}3$ μm and $x_2 = 118{\cdot}9$ μm), for mol. wt 506 000 FITC-dextran. The difference profile $\{C(x_1,x_2;t)\}$ is shown and t_{max} (495 s) indicated, D was calculated to be $6{\cdot}23 \times 10^{-8}$ cm^2 s^{-1}. Similar profiles for mol. wt 4000 FITC-dextran are shown in Fig. 7. Since diffusion is rapid, points were selected further from the interface ($x_1 = 105{\cdot}4$ μm and $x_2 = 296{\cdot}3$ μm), when t_{max} was 195 s and D calculated to be $95{\cdot}1 \times 10^{-8}$ cm^2 s^{-1}. Software allowed points x_1 and x_2 to be varied and D calculated.

For mol. wt 4000 FITC-dextran it can be seen that t_{max} occurred at 195 s. This was calculated from only 13 data points, which may lead to poor estimation of t_{max} and hence D.

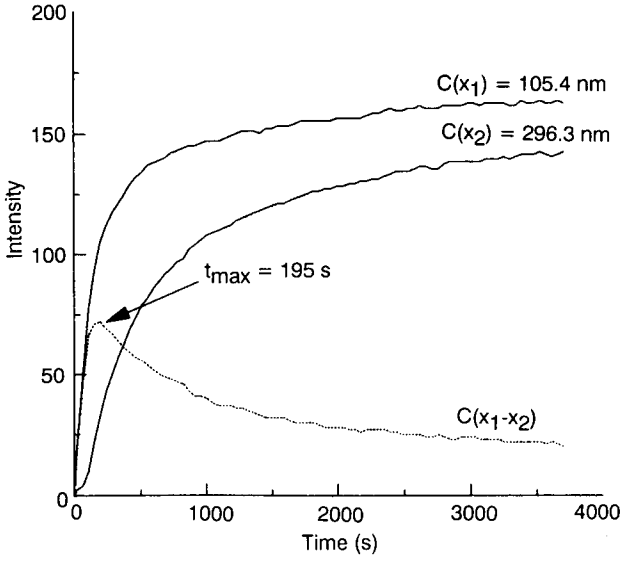

FIG. 7. The concentration/time profiles for FITC-dextran (mol. wt 4000) diffusing through a 1% w/v agar gel at 25°C, when measured at two distances from the interface.

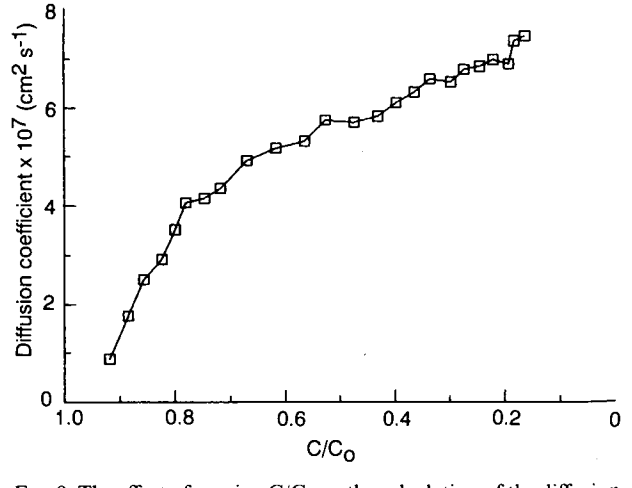

FIG. 9. The effect of varying C/C_0 on the calculation of the diffusion cofficient for FITC-dextran (mol. wt 506 000) diffusing through a 1% w/v agar gel at 25°C (measured at t = 209 s).

Table 1. The diffusion coefficient of macromolecules into mucus, as determined by two different methods.

Compound	Mol. wt	Diffusion coefficient ($cm^2 s^{-1}$) $\times 10^7$
Diffusion through pig small intestinal mucus at 37°C mean ± s.d. (n = 3)		
Fluorescein	332	42·9 ± 7·01
FITC-dextran	71 200	4·40 ± 4·9
FITC-dextran	148 000	1·90 ± 7·9
Diffusion through human mid-cycle human cervical mucus at 25°C[a]		
Fluorescein	332	55·0
BSA	68 000	5·90
IgG	146 000	1·20

[a] Radomsky et al (1990).

Method 2 was developed to allow the determination of D from a single penetration profile and avoid inaccuracies in estimating t_{max} from a small number of data points. Fig. 8 shows a series of penetration profiles of mol. wt 148 000 FITC-dextran diffusing into 3% w/v carbopol gel (pH 7·4, 37°C). Each profile permitted the calculation of D using method 2. When $C/C_o = 0{\cdot}5$ each profile produced values for D between 20·6 and $23{\cdot}9 \times 10^{-8}$ cm^2 s^{-1}. The diffusion coefficient declined slightly with time. This variation was also apparent when different points along a single profile were used. Fig. 9 shows how the diffusion coefficient varies at different values of C/C_o for 506 000 FITC-dextran diffusing into 1% w/v agar. Points further from the interface which have lower values of C/C_o produced higher diffusion coefficients.

Using method 2 the diffusion of fluorescein and FITC-dextrans (mol. wt 71 200 and 148 000) through pig small intestinal mucus was followed at 37°C. In a similar method Radomsky et al (1990) followed the release of macromolecules into mid-cycle human cervical mucus from polymeric controlled release systems. The results are shown in Table 1 for comparison. Both methods gave similar diffusion coefficients for compounds of comparable mol. wt. A relationship between mol. wt and the diffusion coefficient was observed. The variation of results is indicated in Table 1. Mucus gels were heterogenous and produced uneven penetration profiles, this may account for the variability observed. Increased sample numbers are required to improve reproducibility.

Discussion

Diffusion coefficients provide vital information on the movement of compounds in many systems. Our methods allow the rapid calculation of diffusion coefficients for fluorescent compounds within gels.

The whole system was calibrated with fluorescein standards and responded in a linear manner to changes in concentration. The fluorescent intensity was used directly as a measure of concentration. At higher concentrations the system became saturated and background illumination limited the use of dilute concentrations. However, by manipulation of microscope and camera settings it was possible to use a range of concentrations. The use of more sensitive cameras would extend the usable concentration range.

The movement of fluorophore into the gel was followed by taking and storing a series of pictures (every 15–20 s to minimize photo-bleaching). Images could be corrected for uneven and background illumination. Extraction of concentration profiles from stored images allowed D to be calculated.

Method 1 allowed the diffusion coefficient to be obtained within 10 min even for slowly diffusing compounds. However, insufficient data could be collected for rapidly diffusing compounds. Using mol. wt 4000 FITC-dextran, t_{max} occurred within 195 s; this was estimated from just 13 data points. This problem could be overcome by either recording more images over the initial stages of diffusion (but was precluded by photo-bleaching) or by following diffusion over greater distances. The diffusion of FITC-dextrans into agar was followed, with a × 10 objective, over a distance of 400–500 μm. A lower magnification would allow diffusion to be followed further, i.e. × 4-1250 μm, but higher concentrations of FITC-dextran or a more sensitive camera would be required to overcome the loss in fluorescent intensity.

The second method of calculating diffusion coefficients overcomes these limitations. The concentration at a single point on the penetration profile, at a known t and C_o are all that is required to calculate D. In practice, three or four time intervals and a range of points around $C/C_o = 0{\cdot}5$ were used to calculate diffusion coefficients.

Diffusion coefficients calculated by method 2, varied with both the time interval and the value of C/C_o. This was less marked with fluorescein, suggesting that it is a property of FITC-dextrans.

Dextrans are long chains of α-1,6-linked glucose units (Virnik et al 1975) and the shape of the polymers can be described as rods (Colton et al 1971). As they diffuse into the gel, molecules preferentially orientated may be able to diffuse faster through the gel network. At shorter time intervals and lower values of C/C_o the correctly oriented molecules will cause a higher value for D to be measured. Furthermore, dextrans are polydispersed around a mean mol. wt. The lower mol. wt fractions would diffuse faster through the gel network and increase D calculated at shorter time intervals and lower values of C/C_o. As FITC-dextran diffuses through the gel, it may be fractionated by its size or by the shape of the molecules in the gel network. At different times and values of C/C_o, D will vary according to the fraction of FITC-dextran being measured.

In comparing the two methods for calculating diffusion coefficients, method 1 is more applicable to slowly diffusing systems ($D < 1{\cdot}0 \times 10^{-6}$ cm^2 s^{-1}), where large amounts of data can be used to provide D in less than 10 min. Method 2 is more appropriate for following diffusion in fast diffusing systems ($D > 1{\cdot}0 \times 10^{-6}$ cm^2 s^{-1}), as single images in the early stages of diffusion can provide sufficient data to calculate D.

Carbopol has been studied as a bioadhesive polymer (Harris et al 1990; Mikos & Peppas 1990). The diffusion coefficient of macromolecules in these gels will allow assessment of their ability to control the release of such compounds in bioadhesive formulations.

Mucus secreted from epithelial goblet cells forms a protective layer over mucosal membranes (Neutra & Forstner 1987). This can hinder the absorption of drugs (Kearney & Marriott 1982) and macromolecules (Edwards

1978). This technique will allow diffusion coefficients of compounds moving through mucus to be calculated and the extent of the mucus barrier to be studied.

The diffusion coefficients of FITC-dextrans within mucus are comparable with values obtained by Radomsky et al (1990) obtained using similar sized macromolecules. However, the use of different experimental conditions and mucus samples do not allow for direct comparison. This method (Radomsky et al 1990) followed penetration over a distance of 3–4 mm and took 6–7 h to complete. From the available data it can be seen that, for large mol. wt compounds, the mucus layer forms a significant barrier to absorption across mucosal membranes. A model for diffusion through a cross-linked mucus gel network has been established (Peppas et al 1984). This predicts that diffusion will be affected by mucus glycoprotein concentration, the effective cross-linking density of the glycoprotein network and the size of the diffusing species. Further studies by the present method will allow this model to be tested.

Many methods have been devised to follow diffusion from penetration profiles. The duration of experiments depends on the spatial resolution of the detector. Using a scanning absorbance system and resolution of 17 data points mm^{-1} (Muramatsu & Minton 1988), the diffusion of proteins in solution was followed over 400 μm and diffusion coefficients were obtained in 20 min. In a similar method the diffusion of verapamil was followed in hydroxypropylmethylcellulose gels (Bain et al 1990) for 7 days with measurements made at 8 mm intervals. In the present method, the spatial resolution depends on the magnification used. Most experiments were conducted with x10 objective which allowed 768 data points per 500 μm to be collected. This resolution allowed ample data to be collected in short time intervals. A limitation is the quality of the interfaces. Using objectives above $\times 20$ the mucus and carbopol interface appeared uneven, making definition of the interface difficult. Agar gels produced a meniscus which made location of the interface difficult and introduces a 3 dimensional component.

This technique has been successfully applied to a number of gel and penetrating molecules. Conversely, the release of molecules from loaded gels or individual microparticles could be quantified.

Preliminary investigations (Adler & Cheema 1990; Cheema & Adler 1990) with a confocal microscope suggest that our approach can be applied to determine diffusion coefficients into and within tissue in-vivo. The increased spatial resolution and high sensitivity of the photomultiplier tubes would overcome the problems encountered with method 1.

Conclusions

A method has been developed for the determination of diffusion coefficients by combining the spatial resolution of fluorescence microscopy and quantification with image analysis. This allows diffusion coefficients to be determined over short time intervals, even for systems exhibiting slow diffusion.

Two mathematical approaches have been used to obtain diffusion coefficients. Either could be used where the concentration of a diffusing substance can be followed at known distances from a constant source.

Acknowledgements

B. T. Henry is supported by an SERC CASE award in conjunction with Upjohn Laboratories–Europe. J. Adler is supported by an SERC grant.

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