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Simple and Cost-Effective Eggshell Membrane Model for Diffusion Characteristics of Biochemical Materials

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Abstract: Understanding of diffusion of solute particles across the biological partition is very well studied both in vitro and in vivo. Diffusion is the movement of solute molecules by random thermal or Brownian motion across the barriers. Various experimental and mathematical models were employed to study the diffusion characteristics which have also helped to design the experiments and interpretation of data. In the present investigation, we have developed a simple, cost-effective eggshell membrane model for understanding diffusion characteristics. The passive diffusion (in vitro) of sugar molecules through a natural (Chicken egg shell) membrane model was studied. The diffusion coefficient (D), diffusion rate (J), and permeability coefficient (P) in relation to temperature, viscosity of solution, surface area of the membrane, and the molecular size of diffusing molecules have been quantitatively determined. Fick's first law of diffusion is used for the evaluation of experimental data. All the experiments were carried out at a physiological pH. The diffused solute particles in the donor compartment were measured using UV visible spectroscopy at three different time intervals. Diffusion rate and the permeability coefficient were found to increase with the higher temperature of the solution as well as for the larger surface area of the membrane; however, it decreased with the viscosity and size of the diffusing solute particles. The possible mechanisms and characteristics of the diffusion of the molecules have been elucidated. We conclude that such a model may be useful as a teaching and learning model for diffusion characteristics.

Keywords: Diffusion, Eggshell Membrane, Sugars, Surface Area, Temperature, Viscosity

1. Introduction

Diffusion is the most fundamental and important phenomenon that occurs through cell membranes, and therefore it has been extensively studied in biology and medicine [1]. It is a key process that takes place in all eukaryotes, prokaryotes, and plant cells; moreover, it is a core part of the physiological concept [2]. Diffusion is the net movement of the solute particles such as oxygen, nutrients, waste products, etc., in animal systems from an area of higher concentration to an area of lower concentration. It is due to random/Brownian motion induced by temperature where striking of one particle to others imparts movement. This movement is restricted by the viscosity of the medium; however, a rise in temperature reduces the viscosity and lowers the resistance of particle motion. Diffusion is one of the main concepts that helps us to understand cells and their relation to the environment. The exchange of materials between living organisms during equilibrium and water transportation is an essential operation of life and biological events in living systems [3]. Diffusion explains functional events in biology both at the micro and macro level i.e. at

cellular and organ level. Diffusion also relates to structures of biomolecules, physicochemical properties, energy and biological organization etc.

The transport of molecules across the biological membrane is generally expressed in fluxes [4]. The flux simply defines a mass number of a molecule moving through a cross-sectional area during the giving period of time expressed mathematically J = m/At where J is the flux of mass 'm' moving through cross sectional area 'A' during time 't' which is express in mol cm^{-2} min^{-1}. According to the Adolf Ficks law, diffusion rate (*J*) is expressed as J(x,t) = -Ddc(x,t)/d(x) where dc(x.t)/dx describes the concentration gradient and D is the proportionality constant known as Diffusion coefficient. A negative sign in the equation indicates that the diffusion is down the concentration gradient [5].

The diffusion coefficient is one of the most important characteristics of biological molecules/solute particles and has a close association with velocity. The diffusion coefficient depends upon the size of particles and viscosity of the medium; according to the Stokes-Einstein expression $D = RT/6\pi nrN_0 = KT/6\pi nrN_0$ where R is the universal gas constant, T is absolute temperature, r is radius of spherical solute particle, N_0 is Avogadro's number, K is Boltzmann constant (1.38×10^{-23} $m^2kgs^{-2}K^{-1}$), n is the viscosity of the solvent. The Stoke-Einstein equation indicates that the diffusion coefficient decreases with the increasing size of the solute particle and increasing viscosity of the solvent.

The cell membrane serves as a barrier, they are semipermeable, which means some molecules can cross the cell membrane and others do not. Small hydrophobic molecules and gases like oxygen, and carbon dioxide can cross the membrane rapidly; similarly small polar molecules like water, and ethanol can also pass through the membrane but very slowly. On the other hand cell membranes restrict the diffusion of highly charged molecules like ions, large molecular weight, sugars, amino acids, etc. Therefore, the permeability of the cell membrane plays a major role. The following Fick's law can be adopted for permeability quantification. $J = P\ C_{donar} \leftrightarrow P = J/C_{donor}$ where C_{donor} is the concentration of the donor compartment and *P* is the permeability coefficient [5].

In past, different membranes, natural and synthetic, were employed to understand the process of diffusion. Ryotaro et al. have studied the diffusion of lipophilic and hydrophilic compounds from a membrane (mimicking human skin) composed of poly (dimethylsiloxane)/poly(ethylene glycol) (PEG) 6000 copolymer [6]. Boglarka and the group have investigated the role of penetration enhancers (transcutol and sucrose esters) on the diffusion of the drug (ibuprofen) through human skin and skin mimic artificial membranes [7]. Similarly, diffusion properties of various compounds (niacinamide, ascorbic acid 2-glucoside, retinol, and polyethoxylated retinamide) were evaluated through various hydrophilic and hydrophobic synthetic membranes [8].

The aim of the present investigation is to provide a simple and cost-effective membrane model for the diffusion characteristics of biomolecules. The chicken eggshell membrane was selected as a model membrane. The chicken egg consists of an inner shell membrane and an outer calcified shell. The inner eggshell membrane is the interwoven organic fibers mainly composed of proteins as major constituents with small molecules like lipids and carbohydrates. The inner eggshell membrane consists of two membranes – the inner membrane and the outer membrane. The inner membrane is 20µm thick and it is in direct contact with the albumen. The next layer is the outer membrane which is about 50µm thick and is placed in between the inner shell membrane and the calcified part of the shell. This imparts a thickness of 70µ to the inner shell membrane [9]. Diffusion of sugar molecules across chicken egg shell membrane was also investigated under varying conditions such as temperature, size, and viscosity of the solvent; and surface area of the membrane.

2. Materials and Methods

2.1. Eggshell Membrane Preparation and Experimental Setup

Fresh egg shells were used in the experiment. The broken eggshell was washed with double distilled water (ddw) and kept under saline to prevent it from any physiological changes. The inner eggshell membrane was manually peeled carefully without damaging it. The membranes were again kept in saline for further use. The diffusion cell setup was comprised of a donor compartment and a receiver compartment. 10 ml of the test sample was placed in the donor compartment. The eggshell membrane was fixed carefully on the brim of the donor compartment. The egg membrane was washed with distilled water before putting it on the donor compartment. The receiver compartment was filled with 5ml distilled water. The donor compartment was placed in such a way that only the egg membrane surface touched the distilled water kept in the receiver compartment as shown in figure 1.

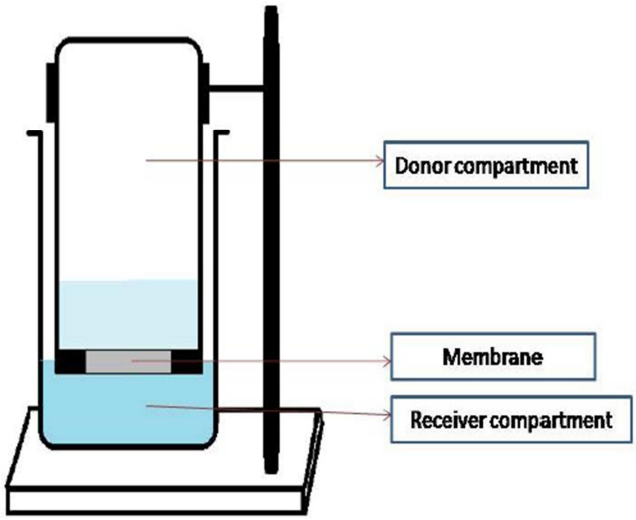

Figure 1. *Schematic Diagram of experimental set up.*

2.2. Preparation of Solutions

Sugar molecules – Glucose (Himedia) and Sucrose (SD Fine) were used for diffusion studies; polyethylene glycol 300 (PEG) (SD Fine) was used as a viscosity agent; Hydrochloric acid (HCl) (SD Fine), Sodium hydroxide (NaOH) (Loba chemical), and 3,5-dinitrosalicylic acid (3,5-DNSA) (SD Fine) was used for the estimation of sugar in the solution.

Stock solution of glucose (0.0277M) was prepared using ddw. Varying concentrations of glucose were prepared to obtain the calibration curve. Separate solutions of 0.5M NaOH and 0.09M 3,5-DNSA were prepared using ddw as solvent; they were used for the calorimetric estimation of glucose solutions.

The study was performed at 280.13K and 303.13K to analyse the effect of temperature on the diffusion of glucose across membrane. The effect at 280.13K was performed by placing the whole setup inside refrigerator set at 280.13K. The solutions were also pre cooled before arranging the set up.

To understand the effect of viscosity of solution on diffusion properties, two solutions of glucose containing different solvents were prepared, viz., pegylated glucose solution prepared in 20% PEG and non-pegylated glucose solution prepared in ddw.

Similarly, the effect of surface area of the membrane was studied using two different donor compartments; one of radius 0.5cm and other of radius 1cm covering the respective surface area of egg membrane of 0.785 cm^2 and 3.14 cm^2. The amount of solutions in the donor compartment and receiver compartment remained unchanged in both the set up.

In addition to glucose diffusion studies, separate study on sucrose diffusion was also performed to understand the effect of molecular size on the diffusion properties. Stock solution of 0.0277M sucrose was prepared using ddw as solvent. Different lower concentrations of sucrose solution were prepared to obtain the calibration curve. For the detection of sucrose content in the solution, another additional solution of 5M NaOH, 0.045M 3,5-DNSA and 11M HCl was prepared using ddw. Color imparted by the addition of these solutions was estimated using UV visible spectroscopy.

The observations of the diffused sample from the receiver compartment were noted at 1,2 and 3-hour intervals. For each observation, new sets were used. In the case of glucose diffusion, 100µl of the diffused sample was mixed with 1ml of 0.5M NaOH and 0.09M of 3,5-DNSA solution. The mixture was mixed thoroughly and kept in the boiling water bath for 15 minutes. The solution was then placed in ice for 5 minutes to bring the mixture to room temperature. The absorbance was measured at wavelength of 517nm using a UV visible spectrophotometer with an appropriate blank. All the experiments were performed thrice in triplicates. Similarly, 100µl of a diffused sample of sucrose solution from the receiver compartment was mixed with 10µl of 11M HCl. This solution was well mixed and kept in a boiling water bath for 5 minutes. The solution was removed and 60µl of 5M NaOH and 1ml of 0.045M DNSA was added and mixed thoroughly. The solution was again heated in a boiling water bath followed by cooling of a sample on ice for 5 minutes. After attaining room temperature the absorbance of the sample was measured at wavelength of 510nm using UV-visible spectrophotometer. An appropriate blank was used. The experiment was performed thrice in triplicates.

2.3. Quantification of Diffusion Data

The diffusion rate (J), Permeability constant (P), and Diffusion coefficient (D) of the solute molecule was calculated using the following equations [5].

$$J(x,t) = -\frac{Ddc\,(x,t)}{d(x)} \quad (1)$$

Diffusion coefficient of the solute is given by equation 2.

$$D = \frac{KT}{6\pi r\eta} \quad (2)$$

where K is the Boltzmann constant (1.38 x 10^{-23} $m^2kgs^{-2}K^{-1}$), T is the temperature, r is the radius of the molecule, η is the viscosity of the solution.

Viscosity was measured for each molecule by Ostwald viscometer and calculated using the following formula [10].

$$\eta_2 = \frac{\eta_1 \times \rho_2 \times t_2}{\rho_1 \times t_1} \quad (3)$$

Where $\eta_1$is viscosity, ρ_1 is density of distilled water, t_1 is the time taken by the distilled water to flow between the regions A and B marked on the viscometer. $\rho_2$is density of the test sample and t_2 is the time taken by the test sample to flow between the regions A and B marked on the viscometer.

The permeability coefficient (P) was calculated according to Fick's first law of diffusion, based on the steady-state flux and the concentration of the donor phase as shown in equation 4 [5].

$$P = \frac{J}{C_{donor}} \quad (4)$$

3. Results and Discussion

In our previous literature, eggshell membranes were examined using a scanning electron microscope (SEM) at different magnifications [11]. Higher magnification SEM images revealed the arrangement of interwoven fibers made up of organic materials mainly collagen proteins lying parallel to the egg surface [9]. Pores ranging from 2.28 to 5.62 microns were observed which are supposed to be formed due to inter-crossing of organic fibers. Similar observations were observed in previous reports [12, 13]. Diffusion of the molecules could be taking place through these pores present in the inner eggshell membrane.

The transport of sugars across the shell membrane was determined in fluxes which depend on a number of factors such as the number of solutes, size of solutes, temperature of the system, surface area of the membrane, etc. Fick's first law was useful in analyzing all diffusion parameters.

This study clearly indicates the influence of the varying factors on the diffusion of sugars across the membrane. From Tables 1-3, the trends are visible; Table 1 shows that as the surface area of the membrane increased form 0.785cm^2 to 3.14cm^2, the rate of diffusion and the permeability coefficient of the glucose molecules was found to increase.

Table 1. *Diffusion rate (J), Permeability coefficient (P), and Diffusion coefficient (D) of glucose molecule through egg shell membrane of two different surface areas.*

Surface area of membrane ($\times 10^{-4}$ m^2)	Time (hr)	D ($\times 10^{-10}$ m^{2}s^{-1})	J ($\times 10^{-5}$ molm^{-2}s^{-1})	P ($\times 10^{-6}$ ms^{-1})
0.785	1	8.025	3.337 ± 0.315	1.20 ± 1.138
	2		7.818 ± 0.478	2.82 ± 1.727
	3		12.087 ± 0.792	4.36 ± 2.866
3.14	1	8.025	7.982 ± 0.275*	2.88 ± 0.994$^{\#}$
	2		12.582 ± 0.075*	4.54 ± 0.271$^{\#}$
	3		15.704 ± 0.596*	5.67 ± 2.152$^{\#}$

The results are expressed as Mean ± SD. At respective hours, *Indicates the significant difference in diffusion rate of solute, *$p < 0.05$ and $^{\#}$Indicates the significant difference in the permeability coefficient of membrane, $^{\#}p < 0.05$.

Similarly, when the diffusion experiment was performed at a lower temperature i.e. at 280K, a significant drop in the diffusion rate and permeability was observed (Table 2). The results can be attributed to the energy of the molecules; at lower temperatures molecular motion gets retarded due to lower available energy to the molecule. Thus this lower energy also affects the solute molecule to cross the membrane efficiently. Upendra et al. have studied the effect of temperature on the permeation of compounds (buspirone, bupivacaine, antipyrine, and caffeine) across porcine buccal mucosa. This study has shown a considerable increase in the permeability with temperature with the increment of 7°C [14].

Table 2. *Diffusion rate (J), Permeability coefficient (P), and Diffusion coefficient (D) of glucose molecule through egg shell membrane at two different temperatures.*

Temperature (K)	Time (hr)	D ($\times 10^{-10}$ m^{2}s^{-1})	J ($\times 10^{-5}$ molm^{-2}s^{-1})	P ($\times 10^{-6}$ ms^{-1})
280.15	1	3.91	1.388 ± 0.031	0.50 ± 0.119
	2		2.163 ± 0.376	0.78 ± 1.354
	3		3.402 ± 0.981	1.23 ± 3.543
303.15	1	8.025	7.982 ± 0.275*	2.88 ± 0.994$^{\#}$
	2		12.582 ± 0.075*	4.54 ± 0.271$^{\#}$
	3		15.704 ± 0.596*	5.67 ± 2.152$^{\#}$

The results are expressed as Mean ± SD. At respective hours, *Indicates the significant difference in diffusion rate of solute, *$p < 0.05$ and $^{\#}$Indicates the significant difference in the permeability coefficient of membrane, $^{\#}p < 0.05$.

The effect on diffusion through membrane was also checked with viscous solution (Table 3). The viscosity of the medium is a measure of the thickness of the solvent. As viscosity of solution increases, the diffusion rate of molecules decreases as shown in the equation. As the viscosity of the medium increases from 0.709mPa.s to 1.85mPa.s, the diffusion coefficient of the solute dropped to 3.0759×10^{-10}m^{2}s^{-1} from 8.0254×10^{-10}m^{2}s^{-1}. As a result of this, the rate of diffusion and permeability dropped considerably in the presence of 20% PEG. In another report, Sandip et al. have shown a similar effect of the viscosity of medium on the transport of ketorolac through a synthetic membrane; in 10%of viscous gel, flux of ketrolac decreased significantly compare to drug in an aqueous solution [15].

Table 3. *Diffusion rate (J), Permeability coefficient (P) and Diffusion coefficient (D) of glucose molecule through egg shell membrane at two different viscosities.*

Viscosity ($\times 10^{-3}$ Pa.s)	Time (hr)	D ($\times 10^{-10}$ m^{2}s^{-1})	J ($\times 10^{-5}$ molm^{-2}s^{-1})	P ($\times 10^{-6}$ ms^{-1})
0.709	1	8.025	7.982 ± 0.275*	2.88 ± 0.994$^{\#}$
	2		12.582 ± 0.075*	4.54 ± 0.271$^{\#}$
	3		15.704 ± 0.596*	5.67 ± 2.152$^{\#}$
1.85	1	3.075	1.378 ± 0.081*	0.49 ± 0.294$^{\#}$
	2		2.609 ± 0.210*	0.94 ± 0.760$^{\#}$
	3		4.055 ± 0.145*	1.46 ± 0.526$^{\#}$

The results are expressed as Mean ± SD. At respective hours, *Indicates the significant difference in diffusion rate of solute, *$p < 0.05$ and $^{\#}$Indicates the significant difference in the permeability coefficient of membrane, $^{\#}p < 0.05$.

To understand the effect of solute size the study was performed with two sugars of different molecular size. The hydrodynamic size of glucose and sucrose are 0.39nm and 0.49nm respectively [16-18]. The observed results indicate that as the size of the solute increases, the diffusion coefficient and subsequent rate of diffusion and permeability

coefficient decreases (Table 4). Recent revived literature by Nicole and Marlon have compared the permeability coefficient of small molecules across artificial membranes which clearly indicate that effect of size on the permeability coefficient [19].

Table 4. *Diffusion rate (J), Permeability coefficient (P) and Diffusion coefficient (D) of glucose and sucrose molecule through egg shell membrane.*

Size (nm)	Time (hr)	D ($\times 10^{-10}$ m^2s^{-1})	J ($\times 10^{-5}$ $molm^{-2}s^{-1}$)	P ($\times 10^{-6}$ ms^{-1})
0.39	1	8.025	7.982 ± 0.275*	2.88 ± 0.994#
	2		12.582 ± 0.075*	4.54 ± 0.271#
	3		15.704 ± 0.596*	5.67 ± 2.152#
0.49	1	3.07	2.875 ± 0.056*	1.04 ± 0.204#
	2		3.889 ± 0.035*	1.40 ± 0.127#
	3		4.499 ± 0.012*	1.62 ± 0.041#

The results are expressed as Mean ± SD. At respective hours, *Indicates the significant difference in diffusion rate of solute, *$p < 0.05$ and #Indicates the significant difference in the permeability coefficient of membrane, #$p < 0.05$.

Diffusion across the membrane (natural as well as synthetic) plays an important role in pharmaceutical studies. Passive diffusion/permeation of drugs and drug like molecules is of fundamental biological importance. A recent review on passive transport has highlighted the role of membrane properties and the effect of permeants on the permeation process [20]. Oung and group have used the computational approach to study the diffusion of cocaine through a model membrane. Change of the protonation state of the membrane affected the diffusion properties of the molecule [21]. Other report by Volkova and Perlovich focused on the permeation of antifungal fluconazole derivatives through a lipophilic membrane. Investigators have focussed on the effect of the structure of linker fragments and different derivatives on the permeation [22]. Other group has reviewed the gases (carbon dioxide) diffusion through membranes [23]. The drug diffusion studies post encapsulation through designed drug delivery vehicles is also important to understand the release of drug through it. Zhang et al. have studied the release of anticancer drug doxorubicin hydrochloride through polymeric vesicles [24]. Diffusion covers huge range of field in experimental studies and hence, requires systematic studies.

4. Conclusion

This manuscript represents a conceptual understanding of the diffusion process across the biological membrane. The diffusion rate, diffusion coefficient, and permeability constant of glucose were evaluated for different experimental conditions such as different temperatures, viscosity, size of biomolecules, surface area of membrane etc. The characteristics of diffusion govern according to Fick's law of diffusion and various models were studied in past. It is interesting to understand basic/ fundamental concept of diffusion by simple and cost effective membrane. It is simple and effective method for quantification of diffusion characteristics and ideally suited to train the graduate and under graduate students.

Conflict of Interest

The authors declare that they have no conflict of interest.

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