Free Rad. Res., Vol. 34, pp. 263-283
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Nitroxide Radicals. Controlled Release from and Transport Through Biomimetic and Hollow Fibre Membranes

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Accepted for publication by Prof. B. Kalyanaraman

(Received 25 April 2000; In revised form 25 July 2000)

Stable nitroxide radicals have found wide applications in chemistry and biology and they have some potential applications in medicine due to their antioxidant properties. Nitrocellulose filters impregnated with lipid-like substances are used as an imitation of biomembranes and could be used as a controlled drug release vehicle, while experiments with hollow fibres can be useful in the modelling of a drug delivery via blood vessels. This paper describes mechanisms of the nitroxide transport in four different model systems, i.e. a) exit of nitroxide into aqueous solution from porous nitrocellulose filters, impregnated with organic solvents, b) transport of nitroxides through the impregnated membrane from one into another aqueous solution, c) transport of nitroxides from bulk phase of organic solvents through the impregnated membrane into aqueous phase with ascorbic acid, and d) transport of nitroxides from liquid organic phase into aqueous solution through porous hollow fibres. The results are analysed in terms of mass transfer resistance of a membrane, organic and aqueous phase, based on nitroxide diffusion and distribution coefficients. Ascorbic acid reduced nitroxides in water and enhanced the rate of their transfer due to the decrease of transport resistance of unstirred aqueous layers. It is demonstrated that in the case of biomembranes the rate limiting step could be the transport through unstirred aqueous layers and membrane/water interface.

Keywords: Nitroxides, transmembrane transport, control release, hollow fibre membranes, two-film theory, reduction and oxidation of stable radicals, interface resistance

1. INTRODUCTION

Stable nitroxide radicals have found a broad spectrum of applications in biology and chemistry as so called spin-probes due to the sensitivity of EPR spectra of these reporter molecules to the properties of microenvironment[1]. They can be used in analytical chemistry, for example in analysis of vitamin C in biological liquids[2], polymer science[3] and also used in biophysics, including investigations of tissues and living systems[4]. Mechanisms of drug release from liposomes and topical delivery through the skin in vivo were recently characterized using nitroxides[5]. Among different applications probably the most interesting are the characterisation of metabolic activity of biological tissues[6] and magnetic resonance imaging, where nitroxides

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can be used as metabolically active and hypoxia sensitive contrast agents[7].

Nitroxides are physiologically active substances[8]. They have antioxidant properties[9], can protect DNA in skin from illumination-induced damage[10] and even to be used as less toxic anticancer agents[8,11]. In general they are able to protect against oxidative stress in vivo, resulting in neuroprotection and decreasing ischemia-reperfusion injury of myocardium[12,13]. Unlike most of low molecular weight antioxidants, which are depleted while attenuating oxidative damage, nitroxides can be recycled due to the ability to be transformed from reduced to oxidised form with the nitroxide as an intermediate[14]. Superoxide dismutase mimic activity of some nitroxides can be used to modify activity of malaria parasites directly in human erythrocytes[15].

The purpose of this paper was to characterise some physico-chemical properties of nitroxides, especially their mass transport, to imitate some aspects of pharmacokinetics, to characterise the possibility of their delivery with a membrane type of drug delivery vehicles, etc. The availability of nitroxides with different hydrophobicity, possibility to measure their concentration in both organic and aqueous phases as well as their ability to be reduced in aqueous solutions combined with the sensitivity of the EPR spectra to the microenvironment makes nitroxides a very good model in the investigations of fundamental mechanisms of membrane transport and control release of different drugs.

It is well-known that nitroxides in water can be reduced by ascorbic acid[7,16] and this is one of the major mechanisms of the nitroxide metabolism in vivo[2]. Ascorbic acid is not surface active and at physiological pH it has very low solubility in organic phase. The reaction of nitroxides with ascorbic acid has found a lot applications in biophysics, including measurements of lipid flip-flop[17], rates of nitroxide transport through liposome and biomembranes[18,19] and many others[20,21].

Main experiments described below were conducted using simple artificial membranes, made by impregnation of nitrocellulose porous support with organic solvents. These and similar membranes can be used as an imitation of many barrier properties of biomembranes[22]. Comparison of different systems was conducted. In the first system the nitroxide was delivered from the biomimetic membrane into aqueous solution with and without ascorbic acid. In other systems the same membrane separated oil and water and two aqueous phases, respectively.

In one more group of experiments we have used the membranes in the form of porous hollow fibres, separating aqueous phase flowing inside and oil flowing outside of the hollow fibres. These experiments could be useful for the interpretation of the drug delivery mechanisms complicated by blood flow or using artificial artery.

Finally a brief comparison of permeability of biomimetic and biological membranes is given and possible role of oil/water interface resistance in biology is discussed.

2. THEORY OF MASS TRANSFER THROUGH THE LIQUID/LIQUID INTERFACE

If a substance diffuses from a bulk (superscript b) organic phase (subscript o) towards its interface (superscript i) and then reversibly penetrates through it into an aqueous (subscript a) solution, and does not participate in any chemical reaction, its steady state concentration in organic phase near the surface (C^i_o) can be described by the equation 1. Corresponding steady state concentration in aqueous solution near interface C^i_a can be described by similar equation 2:

$$\frac{D_o}{L_o}(C^b_o - C^i_o) - (k_o C^i_o - k_a C^i_a) = 0 \qquad (1)$$

$$\frac{D_a}{L_a}(C_a^b - C_a^i) - (k_a C_a^i - k_o C_o^i) = 0 \qquad (2)$$

k_a and k_o are the rate constants for mass transfer from aqueous (a) into organic (o) phase and *vice versa*, cm/s, C^i_o and C^i_a – are concentrations of the substance at the interface on the organic or aqueous side, and $L_o$and L_a are the effective thickness of organic and aqueous phases, respectively. The flux (mol/cm^{2}s) from organic phase into water in this case is described by the equation 3:

$$F = \frac{D_o}{L_o}(C_o^b - C_o^i) \qquad (3)$$

Diffusion in organic phase includes diffusion through organic unstirred layer and pores of membrane, impregnated with organic solution. The effective thickness of organic unstirred layer is:

$$L_o = L_u + \frac{L_m \zeta}{\varepsilon} \qquad (4)$$

L_u, L_m – thickness of unstirred organic layer near membrane and membrane, impregnated with organic liquid, respectively, ζ/ε – tortuosity of the membrane divided by its porosity.

Solving the system (1–3) and taking into account (4) we can write the next two equivalent equations for the flux:

$$F = MTCo \quad (C_o^b - K_d C_a^b) \qquad (5a)$$

or,

$$F = MTCa \quad \left(\frac{C_o^b}{K_d} - C_a^b\right) \qquad (5b)$$

In the first and the second cases mass transfer coefficients are calculated based on organic (MTCo) and aqueous (MTCa) phases as a reference, respectively:

$$MTCo = \frac{1}{\frac{L_u + L_m(\zeta/\varepsilon)}{D_o} + \frac{K_d L_a}{D_a} + \frac{1}{k_o}} \qquad (6a)$$

$$MTCa = \frac{1}{\frac{L_u + L_m(\zeta/\varepsilon)}{D_o K_d} + \frac{L_a}{D_a} + \frac{1}{k_a}} \qquad (6b)$$

It is clear that

$$MTCa = K_d\ MTCo, \qquad (7)$$

If we assume that both interface transfer constants are very big ($k_o$and $k_a \gg D/L$), MTCo and MTCa will be determined by the first and second terms in the denominator, which have the meaning of mass transfer resistances of the organic and aqueous phases, respectively. This is the usual case, described in the double film theory[23]. Further one can see that MTCo is not dependent on hydrophobicity (K_d), if the first or the third terms in the denominator are big in comparison to the second term. This is possible for slow transport in viscous media or slow exit from organic phase (D_o or $k_o$are small).

We can rewrite the equation (3) in terms of concentrations, membrane surface area (S) and aqueous phase volume (V_a):

$$F = \frac{V_a dC_a^b}{Sdt} = MTCo(C_o^b - K_d C_a^b) \qquad (8)$$

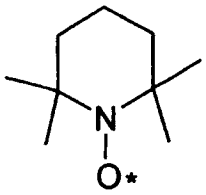
Using mass conservation law, after integration of equation (8) we have:

$$\ln A = \ln\left[1 - \frac{C_a^b(V_a + K_d V_o)}{M_t}\right] = -\left(\frac{MTCo}{V_o} + \frac{MTCa}{V_a}\right) St \qquad (9)$$

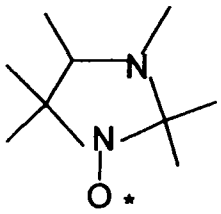
M_t – total content of the nitroxide. In the simple case when $V_a = V_o = V$ equation (9) is reduced to the one, described in literature[24].

If nitroxide after it leaves the organic phase is immediately chemically transformed (reduced by ascorbic acid or oxidised by a complex of Fe^{3+} in water) we can neglect the influence of backward transport from aqueous into organic phase. Besides that we have $C_a^b = 0$. After integration of (8) and using initial condition $C_o = C_o^o$ at t=0, we have usual equation, which allows to measure MTC_o:

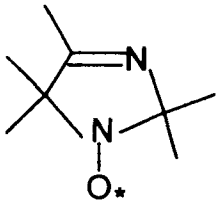
$$\ln\left(\frac{C_o^b}{C_o^o}\right) = -\frac{MTC_o}{V_o} St \qquad (10)$$



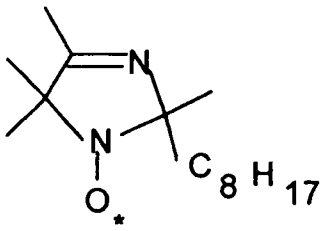
2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO)



2,2,3,4,5,5-Hexamethylimidazolidine-1-oxyl (Imidazolidine R·)



2,4,5,5-Pentamethyl-3-imidazoline-1-oxyl (Imidazoline R·)



Octyl-2,4,5,5-tetramethyl-3-imidazoline-1-oxyl (C8 imidazoline R·)

FIGURE 1 Structural formulas of nitroxides

Another way to calculate MTCo is based on initial rates of mass transfer of radicals from organic phase:

$$\mathrm{MTC_o} = \frac{\mathrm{F}}{\mathrm{C_o^b}} \qquad (11)$$

MTCo, calculated according to the equation (11) is often called permeability.

The above analysis demonstrates that even without assumption of equilibrium distribution through the liquid/liquid interface the governing equations are very similar to the described in literature[23]. The only difference is the additional term in the denominator of the equations 6, which reflects the role of possible interface resistance. It means that the simple demonstration of the experimental validity of equation 10 does not necessary mean that the distribution processes through the interface is fast and can be characterised by equilibrium constant, as it is usually assumed[23].

3. EXPERIMENTAL

Materials and methods

Structures of nitroxides used in different experiments are given in Figure 1. TEMPO (tetramethylpiperidine-1-oxyl, Sigma) and other nitroxides

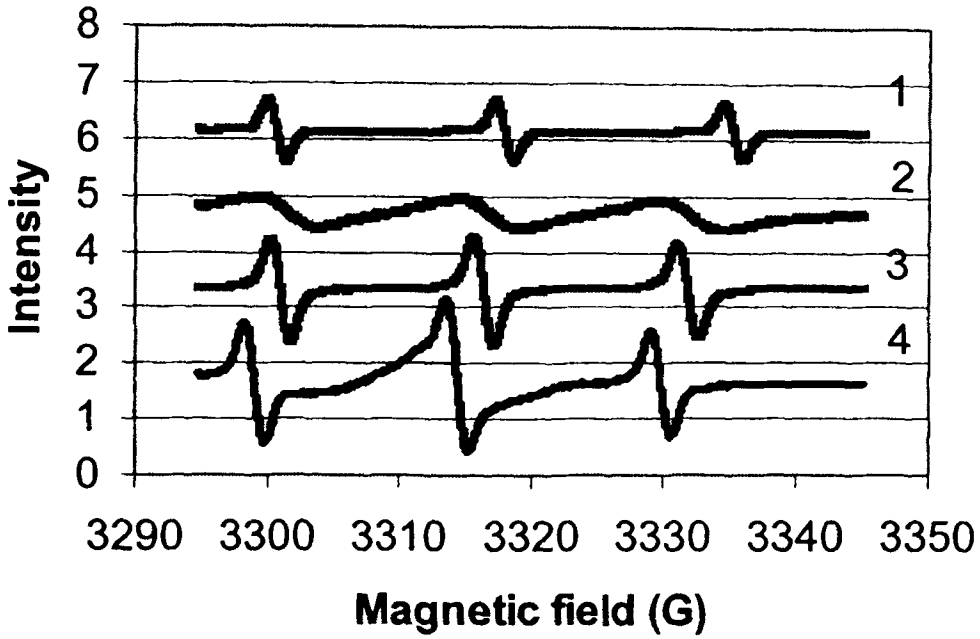


FIGURE 2 EPR spectra of TEMPO in different solvents (1,2,3- in water, octane and in mineral oil, respectively), and the spectra in a membrane impregnated by mineral oil after the experiment –4

(Ecology Inc., Novosibirsk, Russia, [25]), ascorbic acid (99.0%, Nacalai Tesque, Japan) and also cationic detergent N-cetyl-N,N,N-trimethylammoniumbromid (CTMA, Analytical Grade, from Merck) and anionic dodecylbenzenesulfonic acid, sodium salt (DBSA, Technical Grade, Aldrich) were used without further purification. Solution of Fe(III)-dipyridyl was prepared by mixing of 2,2'-dipyridyl and Fe(III) perchlorate hydrate (both from Aldrich), dissolved in water in molar ratio 3:1 with further adjustment of volume and pH. Viscosity of mineral oil (Mallinckrodt) at 25°C (55 cP) was measured using standard rotating-cylinder viscometer. Viscosity of octane (LAB-SCAN) at 25°C is 0.52 cP[26].

Initial concentration of nitroxides in organic solvents was 10^{-3}M in the experiments with flat membranes and membrane slabs and 10^{-4} M with the hollow fibre modules. The X-band Electron Paramagnetic Resonance (EPR) spectra were measured in quartz tubes periodically in both aqueous or organic phases without O_2 removal, using EPR spectrometer 8400, Resonance Instruments Inc., USA, with the internal Mn/MgO standard. Ratio between intensity of lower field component of spectrum of the radical and the third peak of Mn(II) was used as a measure of radical concentration. Modulation amplitude was 1G, centre field 3292G and power attenuation 15dB. Time for measurement of one spectrum was one minute. Usually six spectra were accumulated for each point and average was used in kinetics analysis.

Distribution coefficients K_d of nitroxide radicals were determined as a ratio of concentration of radicals in organic and aqueous phases. It was done both in the end of experiments with membranes and independently in liquid/liquid extraction experiments. For TEMPO this value at room temperature was near 15 for both octane and mineral oil. K_d values for other radicals are presented in the Table I.

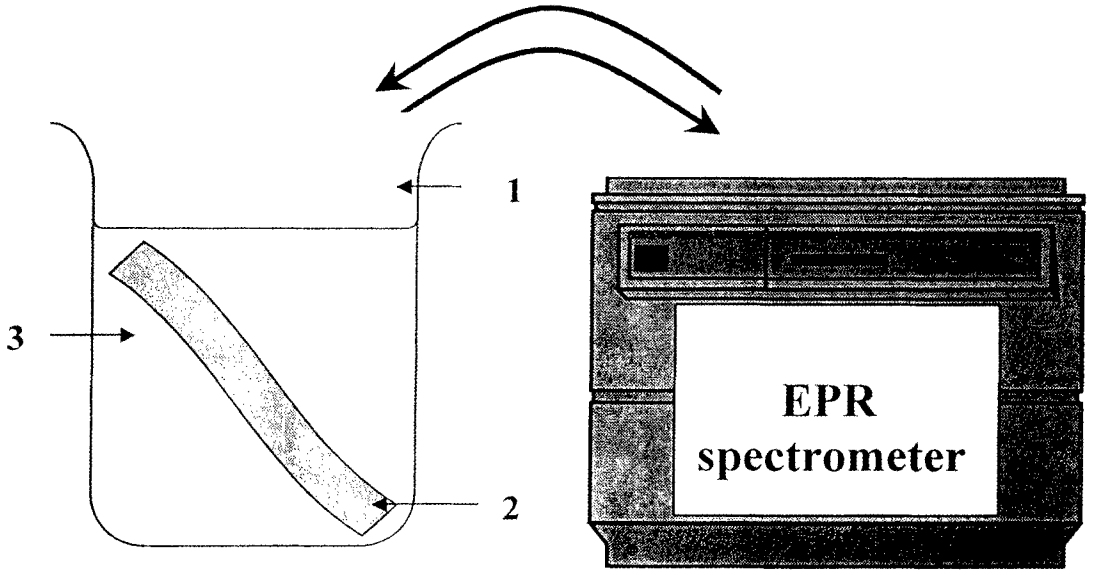


FIGURE 3 Schematic representation of experiment with transfer of nitroxides from membrane slabs. 1 – beaker, 2 – membrane, impregnated with solution of nitroxide in mineral oil, 3- aqueous phase with or without ascorbic acid

TABLE I Parameters of transport of nitroxides between mineral oil and phosphate buffer (pH=4) through the hollow fibre module

Radical	K_d	MTC_o 10^{-5} *cm/s*	MTC_a 10^{-5} *cm/s*
TEMPO	15	0.3	4.5
Imidazolidine R˙	0.5	0.9	0.5
Imidazoline R˙	0.1	0.5	0.05

Typical spectra EPR of TEMPO dissolved in water, mineral oil and octane are presented in the Figure 2 (spectra 1–3) in comparison with the spectrum of a mineral oil -impregnated nitrocellulose filter after the experiment, where the nitroxide transfer from the filter was measured for 90 hrs (spectrum 4). Less intensive and wider peaks of the spectra for the solution in the octane are due to the spin-spin exchange with dissolved oxygen. The spectrum of the membrane can be interpreted as a superposition of a spectrum of the radical immobilised on a polymeric support and the one dissolved in mineral oil[1].

Investigation of nitroxide exit from impregnated filters

Initially a strip of nitrocellulose filter (Millipore, Type GSWP, 0.3 cm wide and 14 cm length and the thickness near 150 μm, pore size 0.22 μm and average porosity 79%) was dipped into mineral oil, containing 0.001M TEMPO or C8-imidazoline nitroxide (Figure 1). Impregnated filter was then incubated in aqueous solution for a relatively short period (from 0.1 to 5 minutes). Then intensity of the EPR spectra of the stable radical in the membrane was measured after insertion of the strip into the quartz tube, fixed in the resonator of spectrometer. The cycle incubation + measurement was repeated several times (Figure 3). It was impossible to conduct similar measurements with a filter impregnated with less viscous octane because of its high volatility.

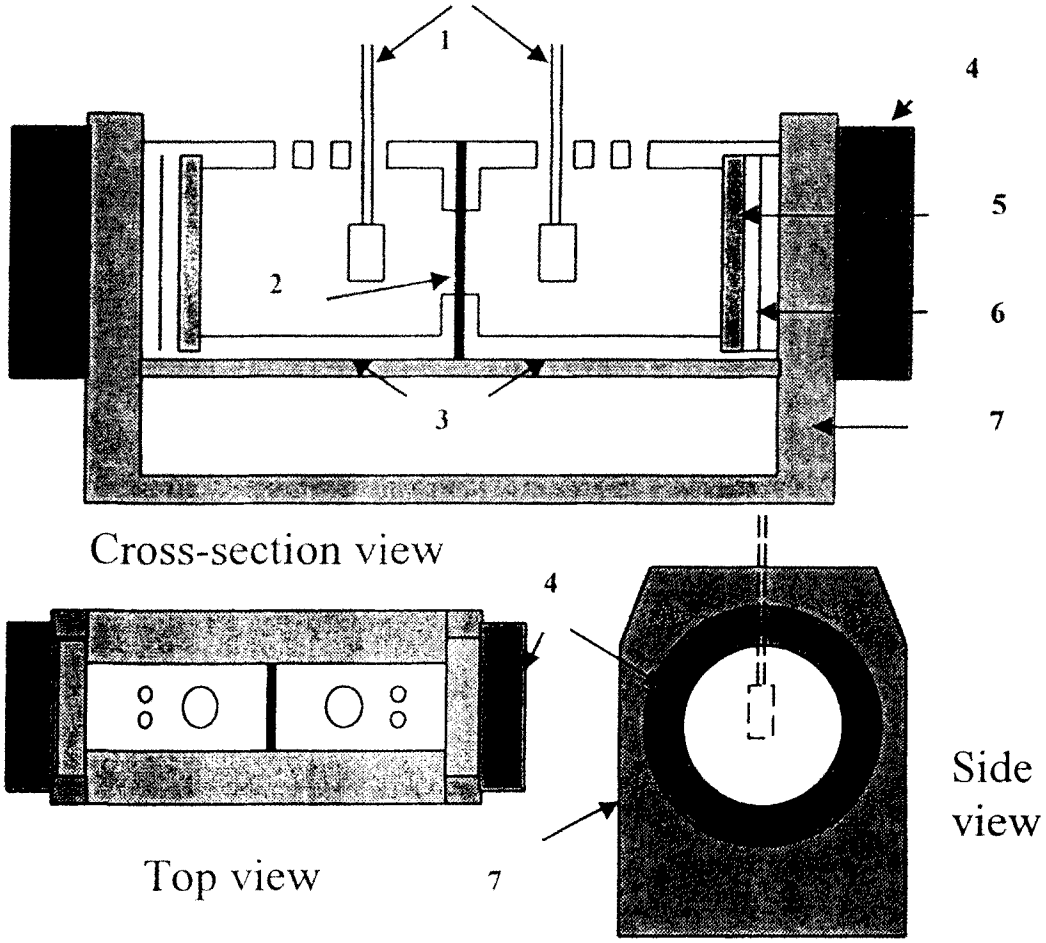


FIGURE 4 Schematic diagram of an experimental cell with vertical membrane. 1 – stirrers, 2 – membrane, 3 – Teflon semichambers, 4 – metal screw, 5 – glass window, 6 - Teflon ring, 7 – metal frame

Investigation of nitroxide transport from bulk organic phase through a flat membrane into an aqueous phase

Experiments were conducted in the cell with a vertical flat membrane (Figure 4), separating octane and aqueous phases. Usually it was nitrocellulose membrane Millipore, with the pore size 0.45, 0.22 or 0.05 μm and the thickness near 150μm. In some experiments PTFE membranes from Millipore, (pore size 0.2μm and thickness 50μm) and also having positive surface charge membranes Hybond-N^+ from Amersham (thickness 155μm and volume porosity 66%) were used. The membrane initially was impregnated with pure organic solvent and fixed in the chamber. Membrane surface area was 4.9 cm^2. Aqueous phase was added into one semichamber and then organic phase with dissolved nitroxide was added into another side. Volume of each liquid phase was 37 cm^3.

$5 \cdot 10^{-3}$ M phosphate buffer was used as aqueous phase. In the experiments with an ascorbic acid pH was 6.0. Both phases were mechanically

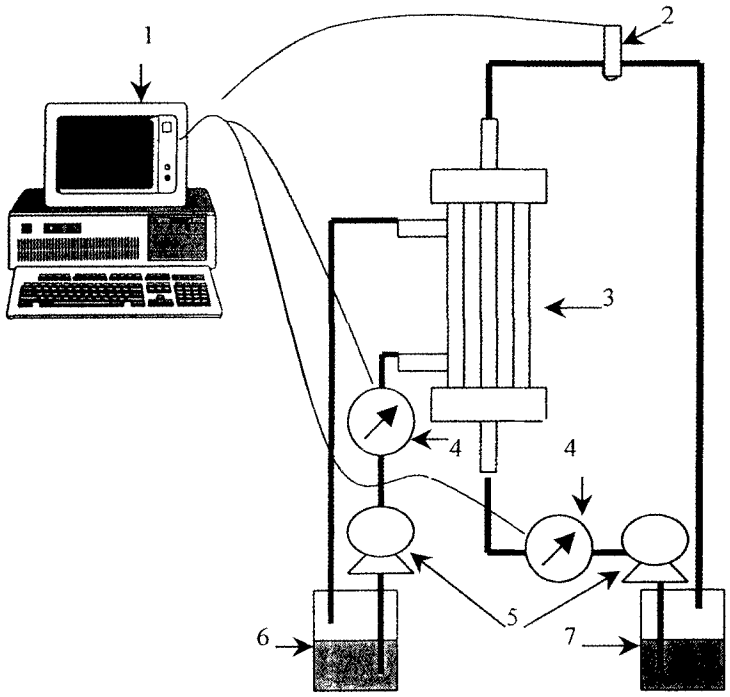


FIGURE 5 Schematic diagram of a set-up with hollow fibre module. 1 – computer, 2 – pH electrode, 3 – hollow fibre module, 4 – pressure transducers, 5 – peristaltic pumps, 6 -solution of nitroxide in organic phase, 7 – aqueous solution

stirred with a rotating mechanical stirrer at 120 rpm. If necessary the thickness of membrane was changed by using stacks of impregnated filters. Similar experiments were conducted also in water/membrane/water system and TEMPO, where the nitroxide was added into one aqueous solution and its concentrations in both aqueous phases were measured as a function of time. In some experiments a cell with a horizontal membrane and more effective magnetic stirrers was used. Construction of the cell is described in [27].

Investigation of nitroxide transport from liquid organic phase through hollow fibre walls into aqueous phase

Another group of experiments was conducted in the hollow fibre contactor module with microporous polypropylene fibres (Celgard 5PCM-102, Hoechst-Celanese). Usually this module is used for water deaeration and carbonation, hemoglobin processing, culture media oxygenation, etc. Schematic diagram of a system with hollow fibre membrane module is presented in Figure 5. The fibre walls with the effective pore size 0.05μm had the thickness 30μm, the inner diameter of fibre is 240 μm, fibre length was 18,4 cm. The module had 7500 fibres and the total membrane surface area was near $1m^2$. Aqueous phase was pumped through the inner tube side, while organic solvent was pumped outside. Volumes of both circulating phases were 0.5 L. Flow rate of aqueous solution was changed from 90 to 200 ml/min (linear rate from 0.5 to 1.1 cm/s), and was constant for oil (90 ml/min, linear rate 0.1 cm/s). In order to

prevent any leakage of organic liquid into water the pressure in the aqueous phase was higher than in organic solvent by 2–3 psi.

Values of both mass transfer coefficients for nitroxide transport from organic solvent into an aqueous buffer were calculated from linear anamorphoses of kinetic curves, plotted based on measurements of nitroxide concentration in water, and equations 7 and 9. In the case of reduction of nitroxides by ascorbic acid or its oxidation by Fe(III)-Dipyridyl complex, measurements of the nitroxide concentration in water were impossible and the analysis was based on the measurements of concentration decrease in the organic phase and the equation 10.

4. RESULTS

Nitroxide exit from impregnated filters

It is difficult to measure the changes of nitroxide concentration in the slab in contact with water continuously, because the aqueous phase in the resonator of EPR spectrometer decreases the sensitivity of measurements. That is why the measurements were conducted in a cyclic manner. Typical kinetic curves for the nitroxide exit from a membrane slab impregnated with solutions of TEMPO or C8-imidazoline nitroxide in mineral oil are presented in Figures 6a and 6b. X-axis corresponds to the total time of incubation in the aqueous solution. For example in the investigation of TEMPO exit into water without ascorbic acid the incubation time at each step was 1 minute and total number of cycles was 4, which corresponds to 4 minutes of incubation (Figure 6a, curve 1). Total time of the experiment was near 35 min due to the time necessary for the EPR measurements. Addition of 1M ascorbic acid to the aqueous solution (Figures 6a and 6b) increases the rate of transfer, especially for the more hydrophobic C8-imidazoline nitroxide. It means that after addition of ascorbic acid into aqueous solution we can both eliminate the nitroxide backward transfer from water into organic phase and also decrease the mass transfer resistance of the aqueous layer. This fact demonstrates an important role of diffusion of radicals in the aqueous phase, which increases overall mass transfer resistance.

Slower exit of more hydrophobic C8-imidazoline nitroxide also demonstrates the role of unstirred aqueous layers and can be interpreted for example based on equation 6a. Three terms in the denominator of this equation reflect resistances for mass transfer of unstirred organic and aqueous layers and resistance of interface, respectively. Much higher distribution coefficient of C8-imidazoline nitroxide results in the increase of resistance of unstirred aqueous layer, which becomes dominant for this hydrophobic radical.

It is important that increase of incubation time in every "incubation-measurement" cycle in the presence of high concentration of ascorbic acid, when role of diffusion in aqueous phase is not essential, resulted in the slower kinetics of exit for both radicals. During the measurement in the EPR spectrometer, which takes near 6 minutes, concentration profile of the nitroxide in the membrane becomes more uniform, but then it is formed again during the next incubation. This fact demonstrates the importance of non-steady and changing with time concentration gradients in the slab, which are formed due to transport of a nitroxide into water and result in a decrease of the exit rate till the steady state value. This also means that the resistance of interface mineral oil/water for the nitroxide exit is not high. If it were dominant, non-steady state diffusion and incubation time should not play an important role. Unfortunately, this set of cyclic experiments is not easy to describe quantitatively, especially in the presence of ascorbic acid. Diffusion coefficients in both organic and aqueous phases and corresponding mass transfer resistances were measured in the experiments with flat membranes, described in the next part.

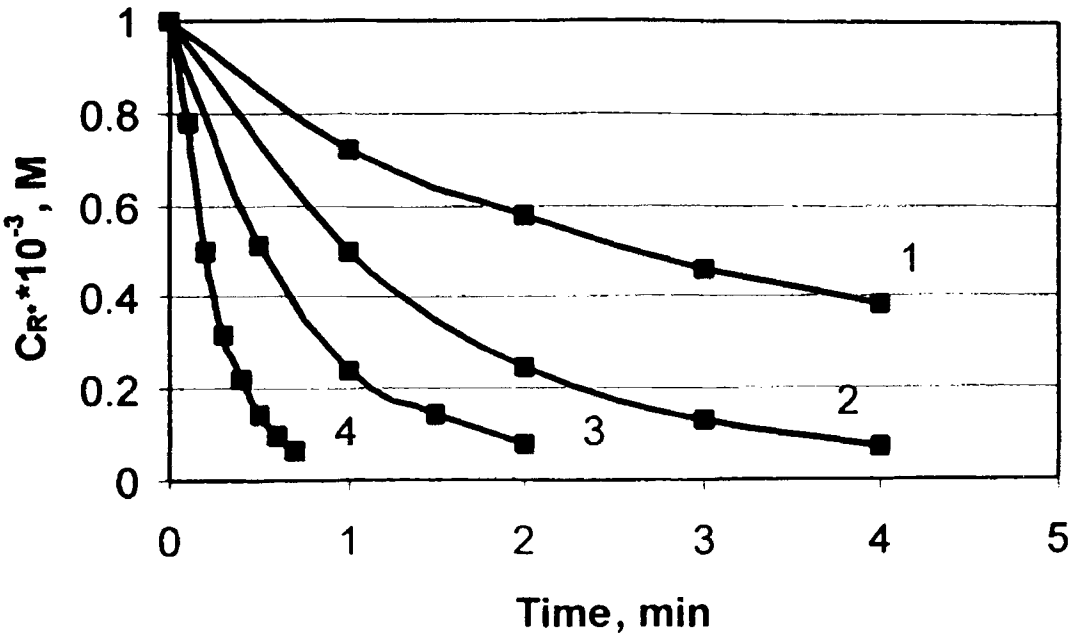


FIGURE 6A Kinetics of TEMPO transfer from a membrane slab into water (1, incubation time 1min.) or 1M ascorbic acid, pH 6 (2,3,4-incubation time 1; 0.5; 0.1 min, respectively)

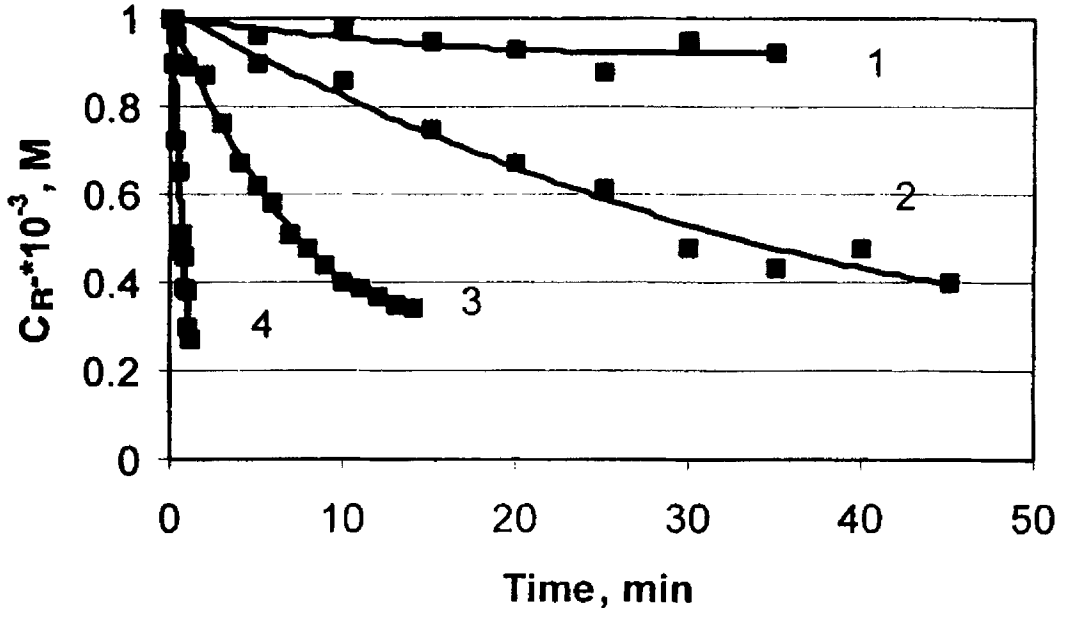


FIGURE 6B Kinetics of nitroxide C8-imidazoline radical transfer from a membrane slab into water (1, incubation time 5 min.) or 1M ascorbic acid, pH 6 (2,3,4-incubation time 5; 1; 0.1 min, respectively)

TEMPO transport from bulk organic phase through a flat membrane into aqueous phase

In the case of nitroxide transport through a flat membrane from bulk organic phase into aqueous solution the concentration of the nitroxide in the oil phase during the experiment was practically constant, but we were able to measure its increase in the aqueous phase. Kinetic curves for TEMPO transport from octane and mineral oil are presented in the Figure 7 -a for the membranes having the thickness of one and five filters. The linear anamorphoses are presented on Figure 7-b. Based on the slopes and distribution coefficients we calculated corresponding MTCo values. For transport through one filter it was 1.8×10^{-5}cm/s with octane and 0.44×10^{-5}cm/s with more viscous mineral oil. The relative changes are much lower than the ratio of viscosities, which can be explained by the different rate limiting steps for the TEMPO transfer from mineral oil and octane. In the separate experiment TEMPO was added not into the organic but the aqueous phase. Kinetics of its transfer from water into octane also gave a straight line in the semilog coordinates with similar mass transfer parameters.

Increase of membrane thickness in five times resulted in a significant decrease of flux in the case of mineral oil, which again demonstrates the role of diffusion resistance in the organic phase (Figures 7-a,b, curves 1,2).

In the case of membrane impregnated with less viscous octane the same variation of the membrane thickness almost did not change the flux (Figures 7-a,b, curves 3,4). To clarify the possible role of aqueous and interface resistances in this case we conducted the experiments with ascorbic acid. Though kinetics was slow it was possible to measure it based on the nitroxide concentration decrease in organic phase. Dependence of Ln C_o on time was a straight line for more than 200 min, but the effective first order rate constant increased only by a factor of four when 1M ascorbic acid was added in the solution (data not shown).

Transmembrane transport of TEMPO from one aqueous solution into another

In these experiments decrease of the radical concentration in one phase was equal to the increase in another and finally both concentrations were practically equal. Kinetics was analysed in the coordinates of the reversible first order process and the corresponding permeability for the membrane impregnated with octane was near 1.4×10^{-4} cm/s. Initial concentration of TEMPO in water in this case was 10^{-4}M. Corresponding equilibrium concentration in organic phase in this case is 1.5×10^{-3}M, which is similar to the typical concentrations used in the experiments described in the previous section. It is interesting that the value of MTCa in those experiments is equal to 2.7×10^{-4} cm/s ($K_d \times$ MTCo) and is approximately twice as high as the membrane permeability, which reflects the difference in the experiment arrangements.

If a filter was impregnated with mineral oil or water, the MTCa was 8.7×10^{-5} and 1×10^{-4} cm/s, respectively. In both cases initial concentration of the nitroxide was 10^{-3}M.

Nitroxide transport through hollow fibres

Hollow fibre modules have much higher surface/volume ratio than the chamber with a flat membrane, which gives much faster mass transfer processes. As the result without ascorbic acid it was possible to measure nitroxide concentration changes in both phases. For example in the case of Imidazolidine radical initially dissolved in mineral oil both the decrease in organic phase and increase of its concentrations in the aqueous phase practically finished after 250 min (Figure 8). Volumes of both oil and aqueous phases were equal and the decrease of the radical concentration in the oil was similar to its increase in water, which demonstrated negligible influence of adsorption on membrane. Final concentration of this nitroxide in aqueous phase

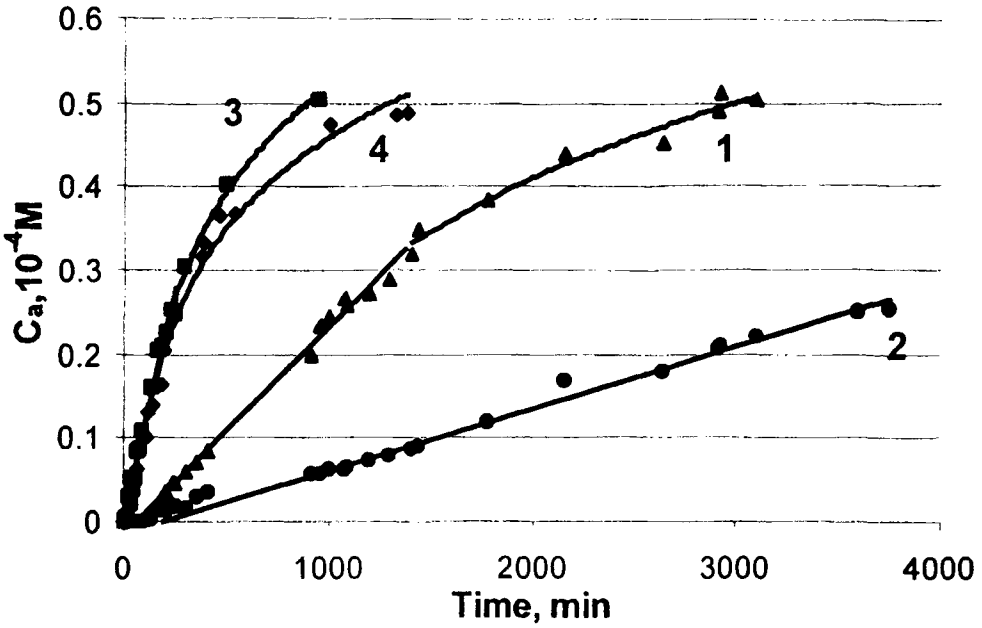


FIGURE 7A Kinetics of TEMPO transport through a membrane from organic solvent to an aqueous solution at pH4. 1,2-mineral oil, 3,4- octane, 1,3- one filter, 2,4- five filters. Initial concentration of TEMPO in the organic phase (C^0_o) is 10^{-3}M

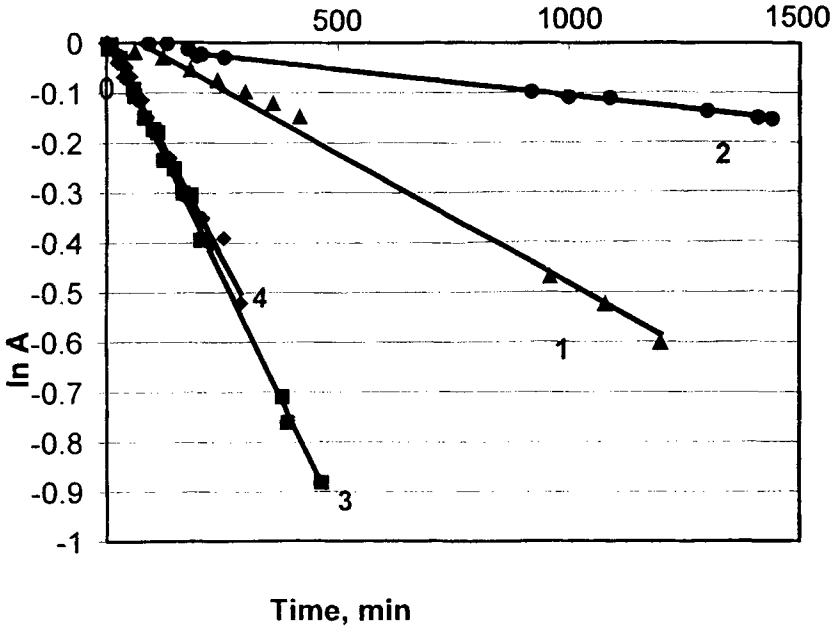


FIGURE 7B Linear anamorphoses for TEMPO transport through a membrane from organic solvent to an aqueous solution at pH=4: 1,2-mineral oil, 3,4- octane, 1,3- one filter, 2,4- five filters

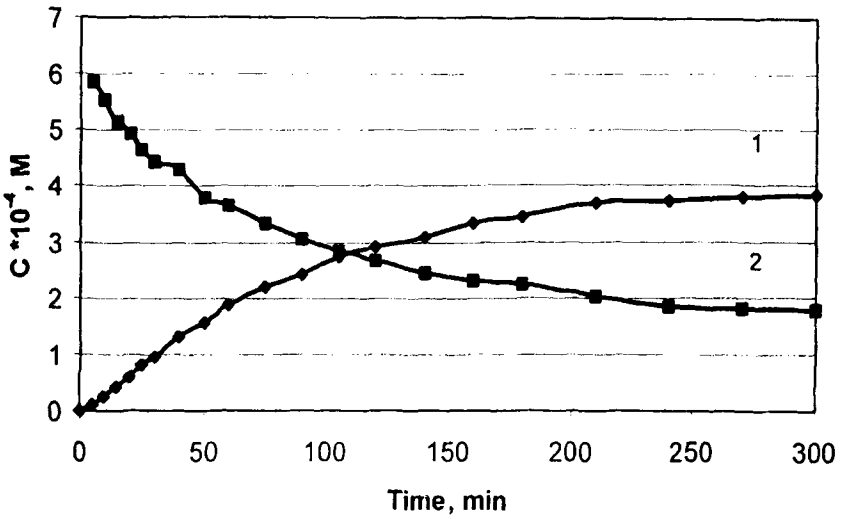


FIGURE 8 Kinetics of Imidazolidine radical distribution between mineral oil and water at pH 4 in hollow fibre module. 1 – phosphate buffer, 2 – mineral oil

was higher than in oil, which corresponds to the distribution coefficient K_d equal to 0.5.

For more lipophilic TEMPO changes of concentration in organic phase are small and accuracy of measurements is low. Due to this fact only kinetics of TEMPO concentration increase in the aqueous phase was used to characterise its mass transfer through the membrane. The linear anamorphose of this curve together with similar dependencies for two other more hydrophilic radicals plotted based on the equation 9 gave the straight lines. Correlation coefficient in all cases was higher than 0.99 with the changes of ln A(t) by more than unity. Values of MTCo and MTCa, calculated from the corresponding slopes, are given in the Table I together with the distribution coefficients K_d.

Another method of MTCo calculation was based on the values of initial rates of the radical transport, when nitroxide concentration in the aqueous phase is small and the backward transfer from aqueous solutions can be neglected. Though this method is less accurate, the results were in good agreement with the data calculated from the linear anamorphoses.

MTCo values with mineral oil are independent of K_d and are similar for different radicals. On the other hand the values of MTCa are parallel to the changes of K_d. This tendency can be explained based on the equations (6a and 6b) and means that unstirred organic layer plays a dominant role in mass transfer processes in mineral oil.

In the case of much more hydrophobic C8-imidazoline radical (K_d ~ 200) the limiting step is different. Its aqueous concentration is much lower and the resistance of unstirred aqueous layer (second term in the denominator) plays an essential role. As the result its MTCo is much lower (~10^{-7} cm/s).

If instead of mineral oil we use octane as a solvent, rate of TEMPO distribution through the hollow fibres into water is much higher and equilibrium was practically established in less than five minutes. The estimated MTCo and MTCa are ~2×10^{-5} cm/s and ~3×10^{-4} cm/s, respectively (K_d is 15). These values are only ~6–7 times bigger than with mineral oil. This difference is much less than the difference of the viscosity of these two solvents and reflects the

changes of the rate-limiting step from organic solvent to water, similar to the demonstrated for flat membranes.

In the presence of ascorbic acid it was possible to measure decrease of TEMPO concentration in organic phase. Concentration of ascorbic acid in these experiments was 0.1M. First order kinetic anamorphoses were the straight lines both for octane and mineral oil. The MTC_o value for TEMPO exit from octane was similar to the one for flat membranes and equal to 3.4×10^{-5} cm/s. Corresponding value in mineral oil was 3.9×10^{-6}cm/s. Again the relative change of the slope was only 9 times, which is much less than the difference of the corresponding viscosity and D_o values. Both values were only slightly bigger than those without ascorbic acid (Table I). It worth it to mention again that 1M ascorbic acid gave only 4 times increase of the rate in the experiments with flat membranes.

5. DISCUSSION

Nitroxide transfer from impregnated filters

Experimental results demonstrated that in the case of cyclic movement of the membrane from the incubation media to the EPR instrument and then back we are dealing with the non steady state diffusion of nitroxide in the liquid organic phase in the membrane and then in the unstirred aqueous layer. The last process can be made faster by nitroxide reduction with ascorbic acid. Quantitative description of the system in this case should be based on the numerical solution of the system of partial differential equations describing two- phase diffusion with the reaction in one of the phases. The cases where analytical solution of similar problem is available, are usually dealing with continuous, not cyclic, mass or heat transfer.

In our experiments the time between successive incubations in aqueous solution was near 6 min, which was necessary for the sample treatment and data acquisition. In more viscous mineral oil the D_o value is near $3^{*}10^{-7}cm^2/s$ (see later). This value allows calculations of the characteristic time necessary to reach the steady state profile. Based on the Einstein equation (12) we have around 100s. It means that we can assume the uniform concentration profile formed in the membrane after each

$$\tau_d = \frac{L_m^2}{D} \tag{12}$$

measurement. For octane the characteristic time is even lower.

Without reaction mass transfer from organic into aqueous phase for small incubation time τ can be described by the equation 13[24]:

$$F = \frac{C_o^o}{1 + K_d\left(\frac{D_o}{D_a}\right)^{1/2}}\left(\frac{D_o}{\pi\tau}\right)^{1/2} \tag{13}$$

We can assume that even in the presence of chemical reaction the equation for the average flux during each incubation will look like

$$\bar{F}_i = \frac{dC_o^n V}{Sdt} = \frac{AC_o^n}{\sqrt{\tau_0}}$$

where C_o^n is the initial concentration in the n-th cycle, τ_0 is the time of incubation in each cycle and t is total time of incubation.

For small incubation times in each incubation we can neglect the difference of initial and current concentrations and after integration we have:

$$Ln\frac{C_o}{C_o^0} = \frac{SA}{V\sqrt{\tau_0}}t$$

Experimental kinetics plotted in these coordinates for small incubation times (6s) gives good straight lines for both TEMPO and C8-imidazoline radical (Figure 9). Increase of temperature resulted in the acceleration of mass transfer in the presence of ascorbic acid and effective activation energy for the process was near 22 kJ/mol in the range from 276 to 321 K. Though this value is relatively small, it is difficult to interpret this result as long as the relationship of the

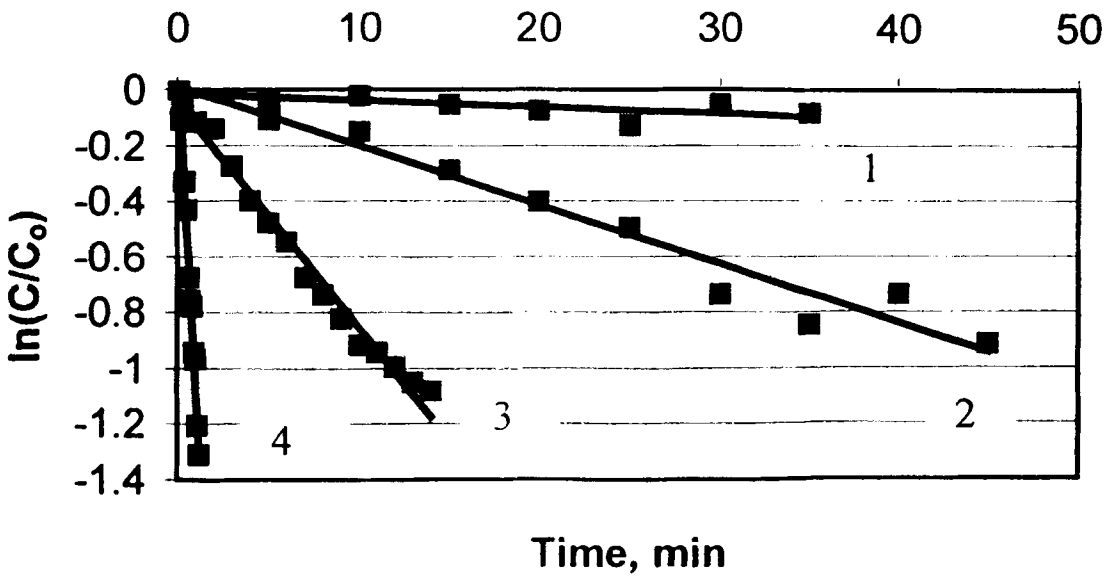


FIGURE 9 Linearization of C8-imidazoline radical transfer kinetics in the semilog coordinates. (All other conditions like in Figure 6b)

parameter A, diffusion coefficients and chemical reaction is not clear. At neutral pH Ea for the reaction of TEMPO and ascorbic acid is near 30 kJ/mol[7]. More quantitative analysis in terms of diffusion coefficients was possible in the case of flat membranes, separating two liquid phases, which is discussed in the next section.

Investigation of nitroxide transport from bulk organic phase through a flat membrane into aqueous phase

Based on the equations 6a and 8 and changing the membrane thickness we can determine the coefficient D_o multiplied by the ratio porosity/tortuosity of the membrane. Analysis of the slope of $Flux^{-1}$ dependence on the membrane thickness is presented in Figure 10. For mineral oil and octane the corresponding values are 7×10^{-8} and 5×10^{-6} cm^2/s, respectively. Calculation of $D_o$for TEMPO according to the well-known Wilke-Chang correlation equation[28] gives 2.8×10^{-7} and 1.8×10^{-5} cm^2/s for mineral oil and octane, respectively. One can see that the experimental D_o in the membrane are 4 times less then corresponding theoretical values. This decrease could be determined by both the solvent immobilisation in the membrane pores (see Figure 2, spectrum 4) and also by the influence of porosity and tortuosity of the membrane.

Rotational correlation times for TEMPO[29], characterising microviscosity for the rotational mobility of nitroxides, were 5×10^{-11}s and 2×10^{-11}s in mineral oil and water, respectively. These times are typical for so called "fast rotation" range[1]. Their relative changes from water to the mineral oil are much less than the relative difference for the translational diffusion. For long molecule of C8-imidazoline radical the difference of rotational correlation times was much bigger (near 10).

The value of the intercept with the Y-axis in Figure 10 can be explained by existence of an extra mass transfer resistance, which is not dependent on the membrane thickness. In general it could be determined by transport through three components: unstirred organic layer near

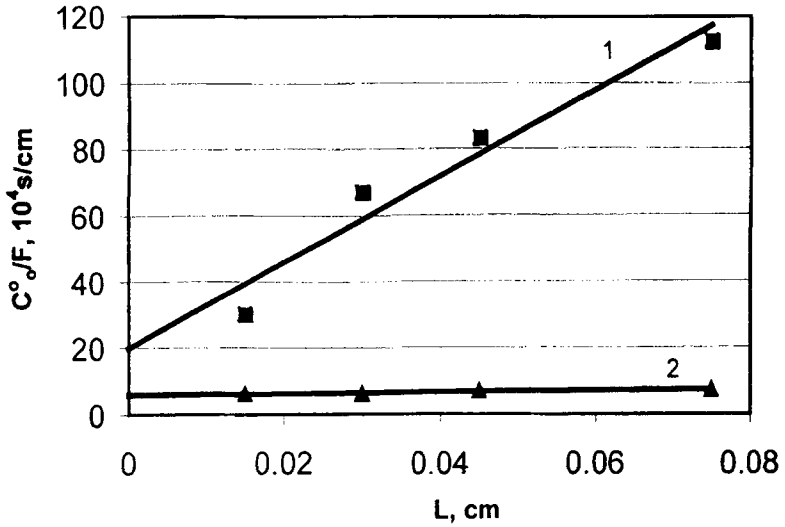


FIGURE 10 Transport resistance of flat membranes as a function of the membrane thickness: 1 – mineral oil, 2 – octane

the membrane, the interface organic solution /water and finally unstirred aqueous layer on another side of the membrane. This total extra resistance can not be neglected. In the case of mineral oil it is comparable to the transport resistance of one impregnated filter. In the case of octane the extra resistance becomes dominant and it is bigger than the inner resistance of five impregnated filters together. Though the value of this total extra resistance was different for the two solvents and equal to 2×10^5 and 6×10^4 s/cm for mineral oil and octane, respectively, the relative difference for the two solvents again is less than the difference of their viscosity. This demonstrates the essential role of either aqueous solution or interface or both of them.

In the experiments with a chamber with horizontal membrane we had much stronger stirring. As the result the effective thickness of unstirred aqueous layers was decreased, the process was 4 times faster and the effect of ascorbic acid was less evident than in the case with vertical membrane.

Aromatic solvents are able to form intermolecular complexes with TEMPO[7]. As the result in the experiments with toluene the Kd value was 167 and MTCo due to the increase of aqueous resistance was equal to only 0.3×10^{-5}cm/s. Viscosity of the toluene is only 10% higher than that of octane. In the presence of ascorbic acid we did not see any decrease of TEMPO concentration in the toluene during two days. Summary of the MTC values for the TEMPO transfer from different organic solvents is given in the Table II. The smaller rate of transfer in the case of toluene in comparison to octane demonstrates the role of intermolecular interactions in the liquid/liquid mass transfer kinetics.

In the case of octane mass transfer resistance due to diffusion through the organic solvent and membrane is relatively small. Both with and without ascorbic acid, the increase of membrane thickness by five times gives much smaller decrease of the exit rate for TEMPO. In 1M ascorbic acid MTC_0 values for one and five membranes were 6.4×10^{-5} and 4.1×10^{-5} cm/s, respectively, or in the terms of resistance it gives 1.6×10^4 and 2.5×10^4 s/cm or less then 2×10^3 s/cm per one filter. As the result the measurements of other components of total mass transfer resistance are more accurate than with the mineral oil.

TABLE II Transport parameters for TEMPO distribution between different organic solvents and phosphate buffer through a flat sheet membrane

Solvent	K_d	MTC_o 10^{-5} *cm/s*	MTC_a 10^{-5} *cm/s*
Toluene	167	0.3	50
Octane	15	1.8	27
Mineral oil	15	0.4	6

Role of aqueous resistance can be decreased due to the chemical reaction with ascorbic acid, taking place simultaneously with the nitroxide diffusion. The corresponding theoretical value of aqueous resistance with 1M ascorbic acid, calculated using expression 17, should be only 2.5×10^3 s/cm. This value is relatively small and is similar to the estimated earlier resistance for simple diffusion through one layer membrane with octane.

Still the experimental effect of ascorbic acid was less than expected based on the two-film theory. Usually the acceleration factor due to the fast irreversible reaction in an aqueous phase is described by equation 16[23]:

$$f = \sqrt{\frac{D_a k C_{asc}}{MTC_a{}^2}} \coth \sqrt{\frac{D_a k C_{asc}}{MTC_a{}^2}} \qquad (16)$$

C_{asc} is the concentration of ascorbic acid in aqueous solution. Using this equation and nitroxide reduction rate constant k equal to 5.8–7.5 $M^{-1}s^{-1}$[7] we should expect the acceleration factor, equal to 18–20 for the 1M ascorbic acid, which is much bigger then the observed experimental value, equal to 4. Besides that total mass transfer resistance was higher than a sum of calculated resistances of octane and aqueous phase with 1M ascorbic acid.

In the case of mass transfer with irreversible first order reaction in the aqueous phase its resistance as a function of concentration should be described as [23]:

$$\frac{K_d}{\sqrt{D_a k C_{asc.}}} \qquad (17)$$

In our experiments concentration of ascorbic acid was changed in the range from 1mM to1M. Though the accuracy of the point at low concentration is not high, still experimental dependence of F^{-1} versus $C_{asc}{}^{-0.5}$ gives a satisfactory straight line (Figure 11) and the value of the rate constant k estimated from the slope is (4,7 ± 1,9) $M^{-1}s^{-1}$, which is similar to the values described in literature and equal to 5.8–7.5$M^{-1}s^{-1}$ at pH 7 and room temperature[7].

It is interesting that in the presence of 10^{-2}M Fe(III)$(bipy)_3$, which is able to oxidize nitroxides with high rate constant ($5 \times 10^6 M^{-1}s^{-1}$ [30,31]), MTCo was 1.6×10^{-4} cm/s. Much higher efficiency of this reaction in comparison to that with ascorbic acid can be explained by the ability of bipyridine complex to bind with the organic/water interface, similar to its interactions with both anionic and cationic micelles[31, 32].

In the presence of 1M ascorbic acid there should not be appreciable amount of oxygen in the aqueous phase. Still we did control experiment in a sealed glow box with argon bubbled through all three solutions before the experiment starts. The rate of mass transfer was practically the same as without a glove box, which means that reoxidation of nitroxides in the acceptor aqueous solution cannot be the reason of the low efficiency of ascorbic acid.

Another possible explanation of the lower efficiency of the ascorbic acid at high concentration could be the influence of the negative change on the membrane surface. It is well known that the nitrocellulose filters have carboxylic and other anionic groups, which makes the surface negative and results in the formation of the transmembrane potential of impregnated filters in the presence of organic cations[22]. Electrostatic repulsion between negatively charged filter surface and ascorbate anions, reacting with nitroxides[7], could result in the decrease of ascorbate concentration in the reaction zone and effective decrease of the reaction rate between ascorbate and nitroxide. Still comparison of TEMPO transport from octane into 1M solution of ascorbic

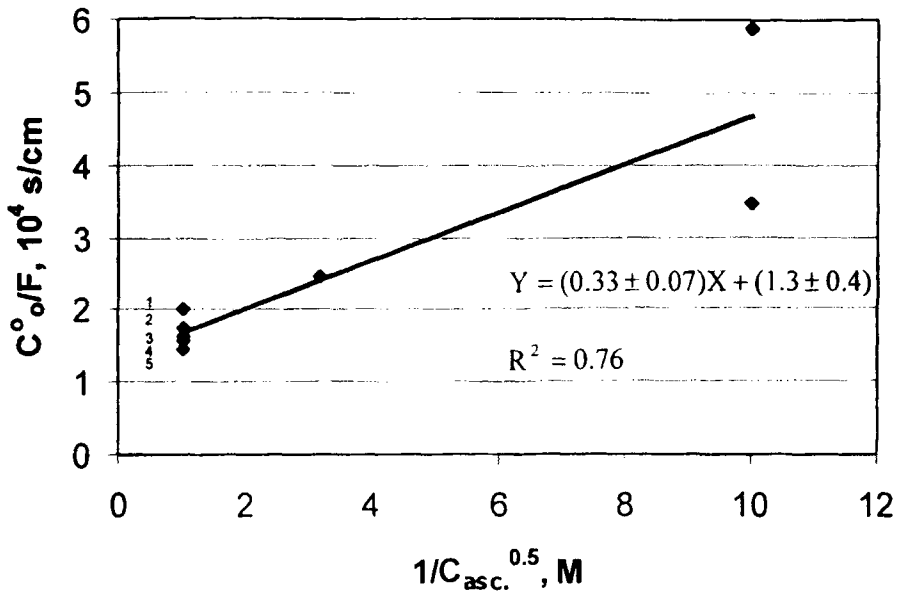


FIGURE 11 Relationship of mass transfer resistance and concentration of ascorbic acid, pH 6. for the 1M ascorbic acid points 1 -Nitrocellulose membrane, 2 – Teflone membrane, 3 – Hybond N^+, 4 – Nitrocellulose membrane in the presence of $2^{*}10^{-4}$M CTMA in the aqueous solution

acid at pH 6 through the membranes with different surface charge demonstrated that this effect does not play an essential role. We have compared impregnated nitrocellulose membrane, the same membrane in the presence of cationic detergent (2×10^{-4}M CTMA), and also impregnated Hybond N^+ and Teflon membranes. The nitrocellulose membrane with adsorbed CTMA and Hybond N^+ should have a positive surface potential, while Teflon should have no net charge or maybe a small negative potential[33].

In all these cases the difference of the experimental MTCo was not more than 10 %, CTMA resulted in the acceleration of the process only at concentration higher than critical micelle concentration (CMC), which is determined by the phenomenon of micellar catalysis[16]. Anionic detergent (14×10^{-2} M DBSA) practically did not influence the rates of process both with or without ascorbic acid.

Small effect of ascorbic acid can be explained by the fact that extrapolation of the experimental dependence does not go the origin of coordinates and we have to assume the influence of one more factor, for example interface resistance. The value of interface resistance is relatively small and it is only near 20% of that of the aqueous phase without reaction. The relative influence of the interface would be even lower in comparison to the total mass transfer resistance, especially in the case of viscous solvents like mineral oil. Still, it seems this is the first time when the oil/water interface mass transfer resistance was demonstrated experimentally.

Nitroxide transport through hollow fibres

Using the values of diffusion and distribution coefficients discussed earlier it is possible to compare the experimental value of MTCa for hollow fibre contactors and theoretical values of separate components of mass transfer resistance, calculated using the correlation criteria, given in [34].

For the process inside the hollow fibre, filled with water, we have:

$$\frac{\mathrm{MTCa_1}\, d}{\mathrm{D_a}} = 1.62 \left(\frac{\mathrm{d}^2 \nu}{\mathrm{ZD_a}}\right)^{1/3} \qquad (18)$$

ν – water velocity, 1.1 cm/s
Z – module length, 18.4 cm
Calculated $\mathrm{MTCa_1}$is 6×10^{-4}cm/s, which is twice as high as experimental MTCa for TEMPO in octane. It is much higher than with mineral oil and cannot play essential role in this case.

Transport resistance of the organic phase is mostly determined by diffusion through porous membrane impregnated by organic solution. Corresponding $\mathrm{MTCa_2}$ can be calculated using equation 19:

$$\mathrm{MTCa_2} = \frac{\mathrm{K_d D_o}}{\mathrm{L_m}} \left(\frac{\varepsilon}{\zeta}\right) \qquad (19)$$

$\frac{\varepsilon}{\zeta}$ – porosity / tortuosity, for Cellanese membrans it is equal to 0.1 [34]

Calculated $\mathrm{MTCa_2}$ values are 5×10^{-5}cm/c for TEMPO in mineral oil, which is similar to the observed in the experiment. The $\mathrm{MTCa_2}$ value for transport through the membrane with octane (8×10^{-3} cm/s) is much higher than the experimental.

Resistance of organic unstirred layers outside the fibres we can evaluate according to equation 20:

$$\frac{\mathrm{MTCa_3 d}}{\mathrm{D_o}} = 1.4 \mathrm{K_d} \left(\frac{\mathrm{d}\ \nu}{\mathrm{D_o}}\right)^{1/3} \qquad (20)$$

ν – liquid velocity, 0.1 cm/s

Though equation 20 gives very approximate results[34], calculated $\mathrm{MTCa_3}$ values are much bigger than those for diffusion through porous membrane, impregnated by organic solution, and it means this step is not rate determining in any case.

Summarising this analysis we can say that in the hollow fibres we have the same rate limiting steps for nitroxides transfer as in the experiments with flat sheet membranes. Biological blood vessels and other structures should be an essential barrier, determining the kinetics of drug distribution and blood circulation should not play an essential role at least on the first steps of drug delivery. It corresponds to the fact that blood flow in the experiments with rats did not change nitroxide delivery through skin of animals at least for one hr [5].

Mechanisms of nonelectrolyte transport through biological membranes

Qualitatively role of the interface resistance we can characterise using the equation 6b. In the case of biological membranes it is possible that the first and second terms in the denominator are small due to the small thickness of biomembranes and fast reactions in the cytoplasm or flow in blood vessels. In this case mass transfer can be determined by the interface resistance. In this case the flux dependence on concentration is similar to the usual dependence from double film theory[23], but the physical meaning of the MTCa is different.

Based on the equation 6a and the idea that the intercept with Y-axis on the Figure 11 reflects the interface resistance, we have $\mathrm{k_o}$equal to $(8.5 \pm 2.5) \times 10^{-5}$ cm/s or $\mathrm{k_a}$near 10^{-3} cm/s. These values could be qualitatively explained based on the theory of the absolute rates and gave reasonable activation energies. For example, diffusion of a small molecule through the homogeneous layer with the thickness 10Å (reasonable for the interface thickness) and with diffusion coefficient as in water should give

$$k_l = \frac{D_a}{L_i} = 50\ cm/s \qquad (21)$$

We can assume that the experimental $\mathrm{k_a}$ value for the interface transfer is described by equation

$$k_a = k_l \exp(-E_{act}/RT) \qquad (22)$$

In other words we add extra activation energy necessary to overcome the interface barrier due to the inhomogeneity of the media. Using $\mathrm{k_a}$ from the experiments with hexane, we have the additional activation energy equal to only

27 kJ/mol This value is probably determined by solvation / dehydration processes, when the molecule moves through the interface. The activation energy for the exit from organic phase is bigger (34 kJ/mol) and can be easily explained by van der Waals interactions in the organic phase. Bilayer of lipids in biomembranes is certainly more viscous than octane. Using D_o for mineral oil we have activation energy only 20 kJ/mol, which is similar to the experimental activation energy for the TEMPO transport from the membrane.

Finally we would like to note that the interface resistance can be very important in biological transport processes, where the membrane thickness is less then 0.01 μm, the transported molecules can be very hydrophobic, have a high molecular weight and are metabolised in cytoplasm.

NOMENCLATURE AND ABBREVIATIONS

C_{asc}	Concentration of ascorbic acid in aqueous solution, M
C_o, C_a	Concentrations of nitroxide in the organic (o) or aqueous (a) phases
d	Fibre diameter, cm
D_o, D_a	Diffusion coefficients of nitroxides in organic and aqueous phases, cm^2/s
F, $\bar{F}$	Flux, mol/cm^2s and average flux during one incubation
f	Acceleration factor due to chemical reaction
k_a, k_o,	Rate constants for the mass transfer from aqueous (a) into organic (o) phase and *vice versa*, cm/s
K	Second order reaction rate constant, $M^{-1}s^{-1}$
K_d	Organics/water distribution coefficient
L_o, L_a, L_m, L_i	Effective thickness of organic and aqueous phases, membrane and interface, cm
MTCo, MTCa	Mass transfer coefficients, based on organic and aqueous phases, respectively, cm/s
$MTCa_1$, $MTCa_2$,	Mass transfer coefficients for hollow fibre module,
$MTCa_3$	calculated using correlation criteria: 1,3- for the process inside and outside of the hollow fibres, 2 – for the diffusion through porous membrane, respectively.
M_t	Total content of the substance, mol
S	Membrane surface area, cm^2
V_o, V_a	Volumes of organic and aqueous phases, cm^3
Z	Hollow fibre module length, cm
ν	Liquid velocity in hollow fibre module, cm/s
ε	Membrane porosity
ζ	Membrane tortuosity
τ_d, τ_o	Characteristic time of diffusion and time of incubation in each cycle, respectively
superscripts^{i,b,o}	The interface(i), bulk(b) or initial(o), respectively

Acknowledgements

This work was supported by the grant from the National University of Singapore.

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