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Solubility and *in vitro* transdermal diffusion of riboflavin assisted by PAMAM dendrimers

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ABSTRACT

PAMAM dendrimers of full generation (**Gn**) and half generation (**Gn.5**) were used as solubility enhancers of riboflavin (**B_2** vitamin) in methanol. They were found to weakly enhance solubility of **B_2** (7.2–10.3 times) according to the order: **G2** ≫ **G2.5** > **G3** ≫ **G3.5** > **G4**. The homogeneous mixtures of **Gn** (or **Gn.5**) with **B_2** of 1:1 molar ratio were obtained by removal of methanol to form oily host-guest complexes. The complexes were released from o/w emulsions and the transdermal permeation of **B_2** through polyvinyldifluoride (PVDF) and pig ear skin (PES) membranes was estimated. PAMAM dendrimers were demonstrated to promote permeation of **B_2** according to the order: **G2** > **G3** ≫ **G2.5** > **G3.5** > **G4** (none). The permeation of fluorescein labeled dendrimers is faster than **B_2**; the diffusion of **G2** dendrimer through PES was the slowest of all studied **Gn** dendrimers, presumably due to absorption inside the skin. On the other hand the **G2** was the best permeation enhancer for **B_2**. The water soluble PAMAM dendrimers **G2** and **G3** can be successfully applied in cosmetic and dermatologic emulsions for this weakly water soluble vitamin.

1. Introduction

Riboflavin (vitamin **B_2**) is dermatologically important factor and nutrient, which is the precursor of flavinadenine dinucletide (FAD) and flavin mononucleotide (FMN), the important coenzymes for oxidoreductases (Stryer, 1995). Although it belongs to water soluble vitamins of group B its solubility is low and skin permeation properties were not widely studied, mainly because it is present in cosmetic creams or gels together with other vitamins of group B, as so-called complex B. These vitamins are added to cosmetic creams as not worked-up mixtures obtained from plants or yeasts and usually not extra amount of **B_2** is added to creams for cosmetic use except only those used to prevent *pellagra* and treatment of *keratoconus* and *keratoectasia* by corneal collagen crosslinking with riboflavin (Wollensak et al., 2003).

Low water solubility of **B_2** (equal to 2.65×10^{-5} mol dm^{-3}) is limiting factor for high skin load of this vitamin. Therefore we have undertaken an attempt to overcome the problem using water soluble polyamidoamine (PAMAM) dendrimers as transporting platform. The PAMAM dendrimers are widely used as transdermal carriers for drugs (Svenson, 2009). They can be used as vehicle for bioconjugates with prodrugs or as hosts for encapsulated or adsorbed drugs (Menjoge et al., 2010).

The dendritic molecules have strictly defined molecular weight, shape and size. The polyamidoamine dendrimers (PAMAM) were synthesized at the beginning of this century (Tomalia et al., 2003). The PAMAM dendrimers were obtained by alternate addition of methyl acrylate to amine groups and condensation of ester group with diamine to form amide bond, beginning from diamine core. Thus the first generation PAMAM dendrimer; **G1** has 8 surface amine groups, which are able to bind 16 equivalents of methyl acrylate to give dendrimer **G1.5**. The alternate condensation and addition sequence leads to full generation **Gn** and half-generation **Gn.5** globular molecules of increasing size of macromolecules and number of surface groups. The conversions of surface amine groups provide the possibility of changing the solubility and permeability properties of dendrimers (Jevprasesphant et al., 2003; Imae et al., 2000).

PAMAM dendrimers were proven to be transdermal and able to pass the cell membrane barrier and therefore they were used as carriers to transport several water-insoluble drugs into cell, like *ibuprofen* (Kolhe et al., 2003), *indomethacin* (Chauhan et al., 2004), *flurbiprofen* (Asthana et al., 2005), *methotrexate* (Kukowska-Latallo et al., 2005), *tamsulosin* (Wang et al., 2003), *niclosamide* (Devarakonda et al., 2005a), *doxorubicin* (Papagiannaros et al., 2005), *8-methoxypsoralene* (Borowska et al., 2010), and in topical gels as skin permeation enhancers for *nipedipine* (Venuganti and Perumal, 2009) and *5-fluorouracil* (Devarakonda et al., 2005b). The PAMAM dendrimers themselves are biodegradable and non-toxic (Hans and Lowman, 2002).

Here we present the results on PAMAM assisted solubilization of **B_2** in solution and in neat dendrimers **G2**, **G2.5**, **G3**, **G3.5**, and **G4** studied by the ^{1}H NMR and UV–Vis methods and *in vitro* studies on diffusion of **B_2** absorbed in PAMAM dendrimers through artificial

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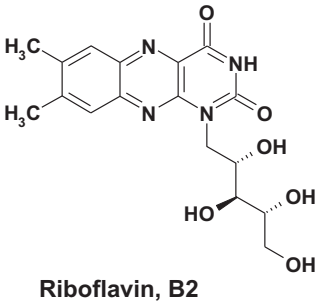
model membrane (polyvinydifluoride, PVDF) and pig ear skin (PES).



Riboflavin, B2

2. Materials and methods

2.1. Reagents and methods

The ^{1}H and ^{13}C NMR spectra were recorded with Bruker 500 MHz UltraShield^{TM}Plus instrument. UV–Vis spectra were recorded on Hitachi U-1900 spectrophotometer. The permeation of **Gn–1** and **Gn.5–1** complexes was studied using Franz diffusion assembly (Thermo Scientific (UK) model DC 600) equipped with 6 cm^3 acceptor compartments. Polyvinydifluoride (PVDF, 0.125 mm thickness) or prepared pig ear skin (PES, 0.55 mm thickness) model membranes were used for permeation studies.

Riboflavin (MW = 376.36 g mol^{-1}, CAS no 93-88-5) and fluorescein isothiocyanate (**FITC**, MW = 389.38 g mol^{-1}, isomer I, 90% purity) as well as common reagents and solvents were used as received from Aldrich.

2.2. Syntheses of PAMAM dendrimers

PAMAM dendrimers of generation 2, 2.5, 3, 3.5, and 4 (**G2**, **G2.5**, **G3**, **G3.5**, and **G4**, respectively) on ethylenediamine core were synthesized according to the published method by alternate addition of methyl acrylate to **Gn** and condensation of ethylenediamine with **Gn.5** (Tomalia et al. 2003). The dendrimers were characterized by the ^{1}H and ^{13}C NMR spectra in deuterium oxide and in methanol-d_4 to confirm their purity. For the spectral assignments the standard 1-D and 2-D COSY, NOESY, HSQC and HMBC measurements were performed.

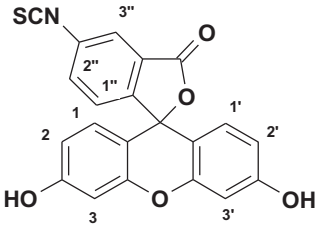
2.3. Syntheses of fluorescein labeled PAMAM dendrimers

The labeling of **G2**, **G3**, and **G4** dendrimers with fluorescein was performed by addition of 43 μmol of fluorescein isothiocyanate in acetone (as stock 0.1 M solution) to 43 μmol of dendrimer dissolved in 20 cm^3 of methanol (Jullian et al., 2009). The reaction of addition of **FITC** to amine groups took place immediately to obtain the conjugates **Gn–F**. The solvents were removed on rotary evaporator. The products were orange oils. The resonances of fluorescein moiety attached through thiourea group are shifted upfield in comparison with **FITC** as listed below.

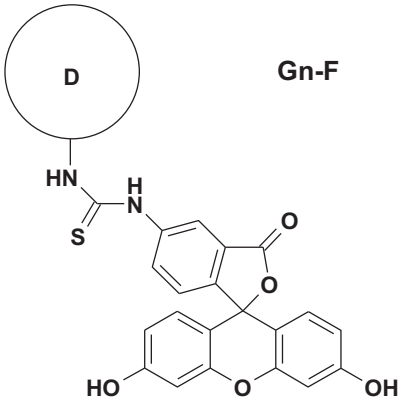
^{1}H NMR (**FITC**): 7.91 ppm (1H, d, J = 3.0 Hz, $H^{3''}$); 7.76 (1H, dd, J = 3.0 Hz, J = 7.0 Hz, $H^{2''}$); 7.37 ppm (1H, d, J = 7.0 Hz, $H^{1''}$); 6.74 ppm (2H, d, J = 3.0 Hz, H^{3} and $H^{3'}$); 6.71 ppm (2H, d, J = 7.0 Hz, H^{2} and $H^{2'}$); 6.62 ppm (2H, d, J = 7.0 Hz, H^{1} and $H^{1'}$).

^{1}H NMR (**G3–F**; flurescein resonances only): 7.24 ppm (1H, broad d, $H^{2''}$); 7.11 ppm (2H, unresolved broad signal, $H^{3''}$ and $H^{1''}$); 6.52–6.47 ppm (6H, unresolved broad signal, H^{1}, $H^{1'}$, H^{2}, $H^{2'}$, H^{3} and $H^{3'}$).

UV–Vis spectra: **G2–F**: 242 nm ($\varepsilon = 3.56 \times 10^4$ mol^{-1} dm^3 cm^{-1}); 501 nm ($\varepsilon = 4.50 \times 10^4$ mol^{-1} dm^3 cm^{-1}); **G3–F**: 242 nm ($\varepsilon = 2.55 \times 10^4$ mol^{-1} dm^3 cm^{-1}); 501 nm ($\varepsilon = 2.95 \times 10^4$ mol^{-1} dm^3 cm^{-1}); **G4–F**: 242 nm ($\varepsilon = 2.26 \times 10^4$ mol^{-1} dm^3 cm^{-1}); 501 nm ($\varepsilon = 2.28 \times 10^4$ mol^{-1} dm^3 cm^{-1}).



FITC



2.4. Solubilization of B_2 in methanol containing host dendrimers

The solubility of $\mathbf{B_2}$ in methanol-d_4, estimated by addition of reference chloroform into the saturated solution of $\mathbf{B_2}$ is 2.73×10^{-4} mol dm^{-3}, which was also confirmed spectrophotometrically. The UV–Vis spectrum in methanol (Fig. 1, dashed line) showed four maxima at: 444 nm ($\varepsilon = 5.60 \times 10^3$ dm^3 mol^{-1} cm^{-1}); 355 nm ($\varepsilon = 2.90 \times 10^3$ dm^3 mol^{-1} cm^{-1}); 269 nm ($\varepsilon = 1.51 \times 10^4$ dm^3 mol^{-1} cm^{-1}) and 222 nm ($\varepsilon = 1.50 \times 10^4$ dm^3 mol^{-1} cm^{-1}). The

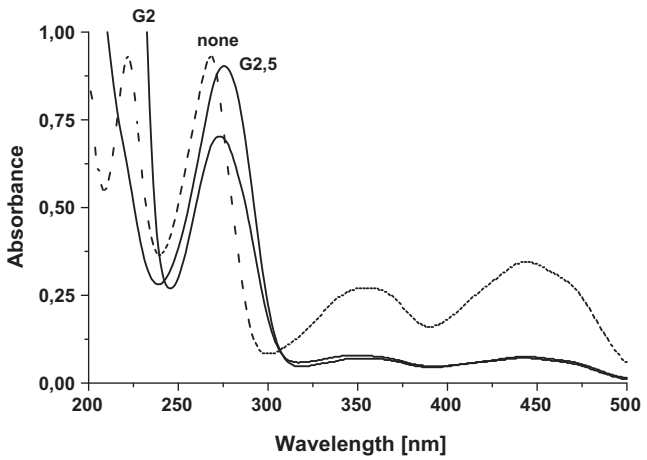


Fig. 1. The UV–Vis spectra of $\mathbf{B_2}$ in methanol ($[\mathbf{B_2}] = 6.15 \times 10^{-5}$) in the absence (dashed line) and in the presence of **G2** ($[\mathbf{G2.5}] = 7.9 \times 10^{-4}$ mol dm^{-3}) and **G2.5** ($[\mathbf{G2.5}] = 6.3 \times 10^{-4}$ mol dm^{-3}) (solid lines).

Table 1
The estimated solubility of $\mathbf{B_2}$ in the presence of dendrimers extrapolated exponentially to infinitive concentration of dendrimer and ratio of solubility in the presence of dendrimer to solubility of $\mathbf{B_2}$ in the absence of dendrimer (solubility enhancement factor).

Dendrimer	Limiting solubility	Solubility enhancement factor
G2	$1.98\ (\pm 0.13) \times 10^{-3}$	7.2
G2.5	$1.75\ (\pm 0.11) \times 10^{-3}$	6.4
G3	$1.68\ (\pm 0.04) \times 10^{-3}$	6.1
G3.5	$3.20\ (\pm 0.14) \times 10^{-3}$	11.7
G4	$2.82\ (\pm 0.25) \times 10^{-3}$	10.3

spectrum of $\mathbf{B_2}$ in the presence of dendrimers changed considerably. The third band was shifted to longer wavelengths, for example to 276 nm for the mixture $\mathbf{B_2}$ and **Gn.5** (half-generation dendrimers, Fig. 1 dotted line for **G2.5**) and to 273 nm for the mixture of $\mathbf{B_2}$ and **Gn** (full-generation dendrimers, Fig. 1 solid line for **G2**). Simultaneously the intensity of two bands at 444 and 355 nm decreased by a factor of 10.

The solubility of $\mathbf{B_2}$ in the presence of dendrimer was examined spectrally. The saturated solutions of $\mathbf{B_2}$ were obtained by addition of solid $\mathbf{B_2}$ into a dendrimer solution in methanol-d_4. No shift of two singlet aromatic resonances of $\mathbf{B_2}$ at 7.96 and 8.04 ppm was observed in the ^{1}H NMR spectrum of mixtures independently on kind and concentration of dendrimer. The saturated solutions were then filtered off in order to remove undissolved $\mathbf{B_2}$ and the filtrates were diluted with methanol to determine the concentration of $\mathbf{B_2}$ spectrophotometrically. No precipitation of $\mathbf{B_2}$ occurs upon dilution of the saturated solutions. The concentration of $\mathbf{B_2}$ increased exponentially with concentration of dendrimers (exemplified at Fig. 2a–c for **G2**, **G2.5**, and **G3** dendrimers, respectively). The non-linear fitting into exponential decay represented by $y = y_0 + A \cdot \exp(-(x - x_0)/t)$ using χ^2 method based on based on Levenberg–Marcquardt algorithm was performed with the help of ORIGIN program to give final x_0 as limiting values of [$\mathbf{B_2}$], which are collected in Table 1 together with SD. The output parameters are in given in boxes at Fig. 2a–c.

2.5. *In vitro permeation of **Gn–B₂** and **Gn.5–B₂** complexes*

Permeation of $\mathbf{B_2}$ in the presence of **Gn** and **Gn.5** complexes was studied using Franz diffusion assembly. The o/w emulsion was used as donor. The emulsion was prepared using cetearyl alcohol (1.5 g), Brij 72 (1.2 g), Brij 58 (0.3 g) as emulsifiers and vaseline (5.0 g), stearine (0.5 g), glycerin (1.5 g) and water (40.0 g). The samples for permeation studies were prepared by dissolving 4.0 mg of $\mathbf{B_2}$ and 66 mg of **G2** (or 106 mg of **G2.5**), 7.4 mg $\mathbf{B_2}$ and 111 mg of **G3** (or 135 mg of **G3.5**) to maintain *ca* 1:1 molar ratio of components in methanol, followed by evaporation of the solvent. Such prepared homogeneous mixtures were then added to 1.0 g of emulsion. For permeation study *ca* 250 mg samples were mounted over commercial PVDF membrane (0.125 mm thickness) or prepared PES membrane (0.55 mm thickness). The receptor medium was 0.067 M phosphate buffer pH = 7.4: ethanol 7:3 (v/v). The progress of diffusion was monitored spectrophotometrically at 266 nm using the extinction coefficient calculated for the solution of **1** in methanol (the same value of extinction coefficient was obtained with 5 times diluted sample of $\mathbf{B_2}$ in receiving solution). The receiving solution was stirred magnetically with 1000 rpm at 32 °C. The 10 ml aliquots of receptor solution were taken at 0.5 h or longer time intervals and the receiver compartment was filled with new 6 ml portion of receptor solution. The experiments were conducted until at least 3% of initial amount of $\mathbf{B_2}$ was received in receptor solution. The results were analyzed calculating the flux in μmol h^{-1} cm^{-2}. The active area of membrane determined by size of the ring in Franz cell was 0.176 cm^2. The cumulated amount of $\mathbf{B_2}$ received in function

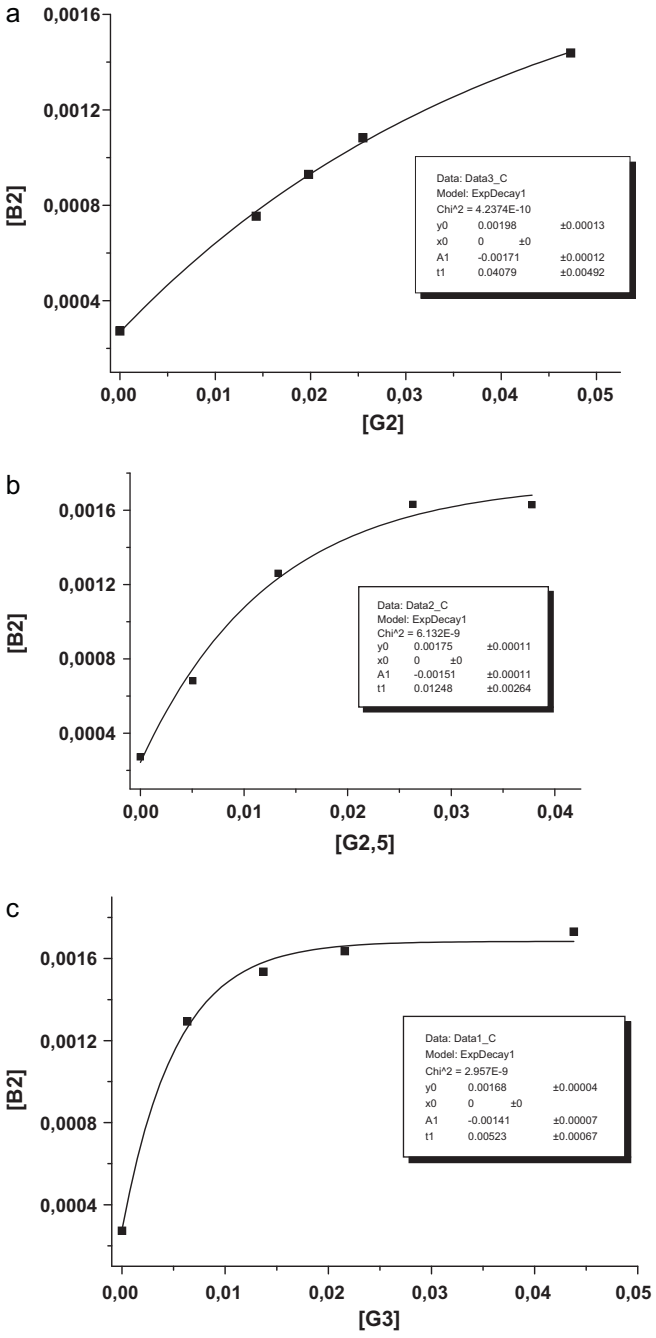


Fig. 2. (a) The dependence of concentration of $\mathbf{B_2}$ in function of **G2** PAMAM concentration. (b) The dependence of concentration of $\mathbf{B_2}$ in function of **G2.5** PAMAM concentration. (c) The dependence of concentration of $\mathbf{B_2}$ in function of **G3** PAMAM concentration.

of time of diffusion was crucial to determine the diffusion properties of **Gn–B₂** mixtures. For comparison of diffusion efficiency the time of 3% diffused $\mathbf{B_2}$ ($\tau_{3\%}$) was used as quantitative parameter. The measurements were repeated 7 times for every system. The raw data were corrected by multiplying the current flux by a factor of n_{in}/n_{temp}, where n_{in} is the number of micromoles of $\mathbf{B_2}$ at the beginning of the experiment (time = 0) and n_{temp} is the number of micromoles of $\mathbf{B_2}$ at the beginning of the aliquot (current "concentration" in emulsion). The corrected graphs of cumulated amount of $\mathbf{B_2}$ were then used to calculate the slopes using regular least squares method and final averaged values of time of 3% transfer were determined (Table 2).

Table 2
The time of 3% transfer of $\mathbf{B_2}$ ($\tau_{3\%}$) from emulsions containing dendrimers.

Sample (o/w)	$\tau_{3\%}$ [h]	
	PVDF	PES
$\mathbf{B_2}$	10.2 (±0.3)	4.1 (±0.1)
$\mathbf{B_2}$**–G2**	2.5 (±0.2)	1.5 (±0.1)
$\mathbf{B_2}$**–G2.5**	3.1 (±0.2)	2.1 (±0.1)
$\mathbf{B_2}$**–G3**	2.5 (±0.2)	1.5 (±0.1)
$\mathbf{B_2}$**–G3.5**	2.5 (±0.2)	2.9 (±0.2)

The permeation of fluorescein labeled dendrimers **G2**, **G3** and **G4**, was performed using the same o/w emulsion as previously. The **Gn–F** load in 250 mg sample was 10–12 mg of **G2–F**, 18–20 mg of **G3–F**, or 28–30 mg of **G4–F**. The same procedure of workup of raw data was used (vide supra).

3. Results and discussion

3.1. Solubilization of B_2 in the presence of dendrimers

The solubility of $\mathbf{B_2}$ in methanol estimated by UV–Vis is 103 mg dm^{-3}. When solid $\mathbf{B_2}$ was added to 0.01–0.04 M solution of dendrimers and the heterogeneous mixture was equilibrated at elevated temperature (55 °C) and cooled down, the filtered solution was diluted until the UV–Vis spectral determination of concentration of $\mathbf{B_2}$ could be done accurately. It has been found that the best solubilization enhancement showed **G4** and **G3.5** dendrimers, while **G2**, **G2.5** and **G3** dendrimers had slightly lower solubilization effect (Table 1). Thus, the larger size (and molecular weight) dendrimer was applied as solubilizer, the more pronounced was the solubilization effect. However, the dependence was not straight proportional neither to the molecular radius of dendrimer, nor to molecular weight. It seems that at least three different factors played the role: the packing of surface functional groups of dendrimer, which is most loose in case of lower generation **G2** and becomes more dense for **G3** (and **G2.5**) and then **G4** (and **G3.5**), the amount of accessible cavities which decreases in opposite direction (**G4** ≅ **G3.5** > **G3** ≅ **G2.5** > **G2**), and the chemical character of surface groups (–NH_2 vs –$COCH_3$ for full generation vs half-generation dendrimers). The latter factor influenced the solubility the least, if compare similar values of solubility within **G4** vs **G3.5** and **G3** vs **G2.5** pairs. Thus, if recalculate the solubility for one surface-available cavities (branch cells (Tomalia, 2005): 8 for **G2**, 16 for **G3** and 32 for **G4**), the solubility effect can be put in order: 0.90 (for **G2**) > 0.38 (for **G3**) > 0.32 (for **G4**) which leads to the conclusion on dominating impact of cavity size directed solubilization.

3.2. Permeation studies

The elucidation of permeation rate of dendrimers is essential for further analysis of data for transdermal diffusion of the guest (here: $\mathbf{B_2}$, and previously vitamin C and *psoralene*; Laskowski et al., to be submitted; Borowska et al., 2010) in the presence of PAMAM dendrimers.

Fluroscein, which is widely used for labeling the proteins by reaction of fluorescein isothiocyanate with terminal amine groups (Jullian et al., 2009) was chosen as fluorescent label for PAMAM **Gn** dendrimers in order to study further the permeation *in vivo*. Here the diffusion of **Gn–F** conjugates was studied *in vitro* using PVDF and PES membranes. The labeled dendrimers had attached one fluorescein molecule per macromolecule of **G2**, **G3** and **G4**.

The results of diffusion efficiency were compared by plotted dependences of percentage of transfer vs time for experiment on PVDF (Fig. 3a) and on PES (Fig. 3b). The diffusion profile and rate was different in case of experiments performed with PVDF membrane in comparison with those obtained with PES membrane. The rate efficiency in case of PVDF for dendrimers can be put in order: **G2–F** ≅ **G3–F** > **G4–F**, although time of 10% molar transfer was *ca* 5 h for all dendrimers (Fig. 3a). For experiments performed on PES membrane the order is: **G4–F** ≅ **G3–F** ≫ **G2–F** (Fig. 3b). The 10% molar transfer in case of PES experiments is two times shorter for **G3–F** and **G4–F** than that for PVDF, while for **G2–F** it is two times longer (10 h for PES in comparison with 5 h for PVDF). Thus the dendrimer **G2** is probably well absorbed inside the PES membrane and diffuses with delay through PES, while larger **G3** and **G4** diffuse without delay. These results were surprising, especially because further experiments on diffusion of $\mathbf{B_2}$ in the presence of any dendrimer, including half-generation **G2.5** and **G3.5** showed much lower rate of diffusion of $\mathbf{B_2}$ (Fig. 4a and b).

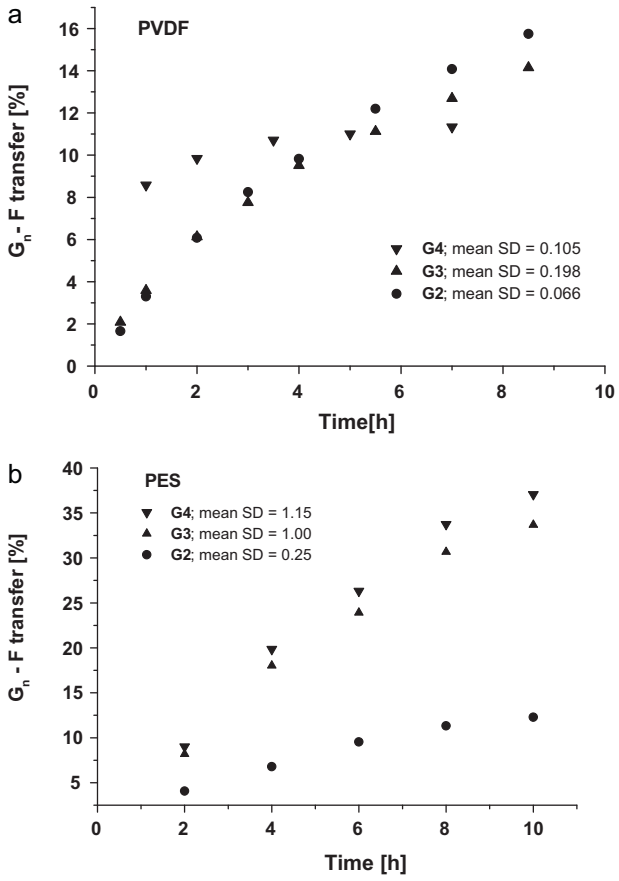


Fig. 3. (a) The percent of cumulated amount of **Gn–F** diffused through PVDF membrane vs time. The values of mean standard deviation of % of transferred $\mathbf{B_2}$ are given in inset at Fig. 4b for every system measured seven times. (b) The percent of cumulated amount of **Gn–F** diffused through PES membrane vs time. The values of mean standard deviation of % of transferred $\mathbf{B_2}$ are given in inset at Fig. 4b for every system measured seven times.

Although diffusion of dendrimers tested for fluorescein labeled macromolecules **Gn–F** showed relatively fast permeation of this potential drug carrier, the diffusion rate of their guests; vitamin $\mathbf{B_2}$ was much slower, especially without carrier. It seems that limiting factor resulting in slow release of $\mathbf{B_2}$ was the low solubility of the vitamin in delivering emulsion resulting in low efficiency of transfer both through PVDS and PES membranes (below 10% within 6 h in every case). Therefore for comparative purposes in these experiments the available parameter a time of 3% transfer ($\tau_{3\%}$) was chosen. It has been found, that: the first $\mathbf{B_2}$ diffused very slowly both through PES and PVDF when released from o/w emulsions (Table 2 presents collected values of $\tau_{3\%}$), *ca* three times slower through PVDF than through PES, and the second the dendrimers

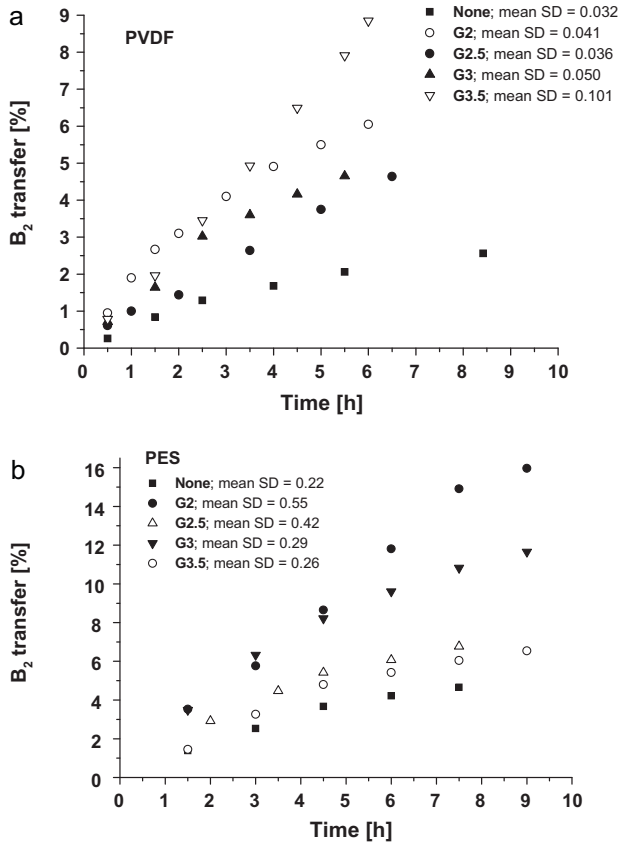


Fig. 4. (a) The percent of cumulated amount of $\mathbf{B_2}$ in the presence of dendrimers diffused through PVDF membrane vs time. The load of $\mathbf{B_2}$ was between 2.5 and 3.3 (±0.1) mg. The values of mean standard deviation of % of transferred $\mathbf{B_2}$ are given in inset at (a) for every system measured seven times. (b) The percent of cumulated amount of $\mathbf{B_2}$ in the presence of dendrimers diffused through PES membrane vs time. The load of $\mathbf{B_2}$ was between 1.0 and 3.1 (±0.1) mg. The values of mean standard deviation of % of transferred $\mathbf{B_2}$ are given in inset at (b) for every system measured seven times.

showed well pronounced enhancement of permeation; in case of PVDF the dendrimers **G2**, **G2.5**, **G3**, and **G3.5** increased the rate at least by factor of 3, while in case of PES the diffusion was speed up by factor of two in case of full-generation dendrimers **G2** and **G3**. There was some consistency of the result on outstanding solubilization effect of **G2**, the lowest diffusion rate of **G2–F** and efficiency of **G2** enhancer through PES. It seems that **G2** dendrimer plays the role of solubilizer not only in methanol or water, but also, when absorbed inside the skin tissue. Therefore it is the best candidate for application as $\mathbf{B_2}$ permeation enhancer of all **G2**, **G2.5**, **G3**, and **G3.5** dendrimers studied. The separate issue was the faster diffusion of $\mathbf{B_2}$ through PES in comparison with that through PVDF (compare columns 2 and 3 in Table 2). It seems reasonable that the multicomponent tissue of PES, including fatty components acts itself as slight solubilizer of $\mathbf{B_2}$. Our quantitative determination of $\mathbf{B_2}$ extracted from the membrane after permeation experiment confirms that speculation.

4. Conclusions

1. PAMAM dendrimers of generations **G2**, **G2.5**, **G3**, **G3.5** and **G4** are weak solubilizers for vitamin $\mathbf{B_2}$. Their solubilizing ability can be put in order: **G2** > **G2.5** ≅ **G3** > **G3.5** ≅ **G4**.
2. The diffusion of fluorescein labeled dendrimers: **G2**, **G3** and **G4** through PVDF is at the level of 10% transfer within 5 h, while the rate of diffusion through PES is 3 times larger for **G3** and **G4** and 1.5 lower for **G2** in comparison with those for PVDF. The **G2** diffusion delay was attributed to absorption of **G2** in PES.
3. The diffusion of $\mathbf{B_2}$ in the presence of **G2**, **G2.5**, **G3** and **G3.5** dendrimers both through PVDF and PES membranes is enhanced 2–3 times. The best permeation enhancer for $\mathbf{B_2}$ was hydrophilic, small-sized (29 Å hydrodynamic diameter) **G2** dendrimer.
4. Finally, the **G2** and **G3** dendrimers can be applied as transdermal carriers to control the kinetics of release of $\mathbf{B_2}$ from emulsions or hydrogels.

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