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PAMAM dendrimers as solubilizers and hosts for 8-methoxypsoralene enabling transdermal diffusion of the guest

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ABSTRACT

PAMAM dendrimers form host–guest complexes with 8-methoxypsoralene (8-MOP) – the photosensitizer for PUVA therapy. The stoichiometry of complexes was studied by ^{1}H NMR spectroscopy in solution and by differential scanning calorimetry in neat mixtures containing 8-MOP and dendrimers of generations **G2.5**, **G3**, **G3.5**, and **G4**. The dendrimers showed solubilization effect for 8-MOP resulting in increase of 8-MOP concentration in methanol up to 15 molecules of 8-MOP per **G2.5** and **G3** and 30 molecules of 8-MOP per **G3.5**, and **G4**. Isolation of oily host–guest complexes containing 3 or 7 molecules of 8-MOP per **G3** and **G4**, respectively corroborate well with DSC results; glass transition temperature of neat host–guest complexes increases with number of host molecules in comparison with **G3** or **G4**, until the capacity of host is exceeded. The oily host–guest complexes of stoichiometry 3:1 and 7:1 of 8-MOP to **G3** and **G4**, respectively are well soluble in water. The 3:1 host–guest complexes diffused slowly through polyvinyldifluoride and pig ear skin membranes, when released from o/w emulsion. The host–guest complex 8-MOP-**G3** was proposed as convenient formulation for psoralene skin administration in PUVA therapy.

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1. Introduction

Dendrimeric polymers are molecules of strictly defined molecular weight, shape and size. The polyamidoamine dendrimers (PAMAM) were synthesized at the beginning of this century (Tomalia et al., 2003; Tomalia, 2005). 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 latter can be converted into **G2** by condensation with excess of diamine. When diamine used in synthesis is ethylenediamine, the **G3** and **G4** are globular molecules of *ca.* 7 and 14 kDa molecular weight, 3.5 and 4.6 nm diameter and posses 32 and 64 surface amine groups, respectively, which render them perfectly soluble in water. PAMAM dendrimers were proven to be transdermal and able to pass the cell membrane barrier and therefore they were attempted as vehicles to transport several water-insoluble drugs into cell (Svenson, 2009) 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., 2005), *doxorubicin* (Papagiannaros et al., 2005), and many others (D'Emanuele and Atwood, 2005). The PAMAM dendrimers are transdermal, biodegradable macromolecules which can be used as drug carriers (Hans and Lowman, 2002).

Therefore we are attempting to use them for deep skin administration of *psoralens*, which are photosensitizers for treatment of hyperproliferative skin diseases like psoriasis, vitiligo, mycosis fungoides, atopic eczema and many others (Saïd et al., 1997). The treatment of skin by UVA irradiation after oral or topical administration of *psoralene* (in PUVA therapy the 8-methoxypsoralene, **1** is used mostly) causes phototoxic and other side-effects such as gastrointestinal, glaucoma and increased risk of carcinogenesis (Stern et al., 1997). Thus the decrease of skin load of *psoralene* is critical for PUVA therapy. Here we present the results on simple host–guest chemistry between PAMAM dendrimers and **1** in solution and in neat dendrimers **G2.5**, **G3**, **G3.5**, and **G4** studied by the ^{1}H NMR and differential scanning calorimetry (DSC) methods in order to establish the proper formulation of the drug (Craig and Reading, 2007). Preliminary in vitro studies of diffusion through artificial model

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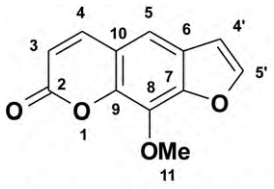
membrane (polyvinyldifluoride) and pig ear skin membrane of **1** absorbed in **G3** were also performed.



8-Methoxypsoralen, 1

2. Material and methods

2.1. Reagents

Psoralene (**1**, MW = 216.2 g/mol, m.p. = 142–148 °C, Xanthotoxin, 99% purity from Fluka) was used as received. All solvents and reagents were of reagent grade purity (Aldrich) and used without purification prior to synthesis or measurements.

2.2. Syntheses

2.2.1. PAMAM dendrimers

PAMAM dendrimers of generation 2.5, 3, 3.5, and 4 (**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 methanol-d_4 using standard 1D and COSY, HMBC, and HSQC 2D methods with 500 MHz Bruker UltraShield spectrometer.

2.3. Solubilization of *1* in methanol and water containing host dendrimers

The solubility of **1** in methanol-d_4 estimated by addition of reference chloroform into the saturated solution of **1** is 0.0198 mol dm^{-3}. The assignment of ^{1}H and ^{13}C resonances has been done based upon 1D and 2D NMR experiments.

^{1}H NMR: 8.00 (1H, H^{4}, d, $J_{3,4}$ = 9.6 Hz), 7.87 (1H, H$^{5'}$, d, $J_{4',5'}$ = 2.2 Hz), 7.52 (1H, H^{5}, s), 6.93 (1H, H$^{4'}$, d), 6.36 (1H, H^{3}, d), 4.23 (3H, H^{11}, s).

^{13}C NMR: 162.9 (C^{2}), 149.2 (C^{7}), 148.7 (C$^{5'}$), 146.8 (C^{4}), 144.3 (C^{9}), 134.0 (C^{8}), 128.2 (C^{6}), 118.2 (C^{10}), 115.2 (C^{3}), 115.0 (C^{5}), 108.1 (C$^{4'}$), 61.9 (C^{11}).

The solubilization of **1** in methanol was studied by simple addition of solid **1** into the 700 μL solution of **Gn** or **Gn,5** of variable concentration in methanol-d_4 in the presence of known amount of chloroform as concentration reference or 700 μL solution of Gn in D_2O with *tert*-butanol reference. Solubilization of **1** in water was time consuming and occurred within 4 h only when elevated temperature (80 °C) and vigorous mixing of crystalline **1** and 0.1 molar aqueous solution of **G3** were applied. The examination of the ^{1}H NMR spectrum showed the chemical conversion of **1** and partial decomposition of **G3**.

2.4. Differential scanning calorimetric studies on PAMAM-*1* stoichiometry

Glass transition temperature T_g was examined using standard mode of heat-flux differential scanning calorimeter DSC, Q1000^{TM} from TA Instruments, Inc., equipped with a mechanical refrigerator from temperatures 183.15 K (−90 °C) to 393.15 K (120 °C) (dry nitrogen gas with a flow rate of 50 mL min^{-1} was purged through the DSC cell in the instrument. Cooling was accomplished with a refrigerated cooling system). Samples of host–guest complexes were prepared by dissolving of **Gn** or **Gn,5** in methanol followed by addition of known amount of **1** and extensive vacuum evaporation of the solvent. The oily samples exhibited T_g higher than neat dendrimers until capacity of host was exceeded. At that moment the separation of **1** and dendrimer occurred, accompanied by appearance of crystalline **1** after evaporation of solvent and return of T_g of sample into original value of dendrimer. The values of obtained T_g are collected in Table 1.

Table 1
Glass transition temperatures [K] for **Gn** dendrimers and mixtures of **Gn** and **1**.

1:Gn ratio	**G2.5**	**G3**	**G3.5**	**G4**
0:1	229	253	224	248
1:1	237	270	244	257
2:1	239	272	245	258
3:1	241	274	246	259
4:1	243	253	251	260
5:1	238		243	262
6:1	239		229	265
7:1	238		223	269
8:1	228			250

2.5. In vitro permeation of *G3–1* complexes

Permeation of **G3–1** complexes was studied using Franz diffusion assembly (Thermo Scientific (UK) model DC 600 equipped with 6 cm^{3} acceptor compartments). The o/w emulsion was used as donor. The emulsion was prepared using cetyl alcohol (2.0 g) and polysorbate 60 (1.0 g) as emulsifiers and liquid paraphine (1.0 g), vaseline (5.0, g), and water (15.0 g). The emulsion samples containing **G3–1** complex were prepared typically as follows: **G3** (200 mg) was dissolved in 10 ml methanol, then 17.2 mg of **1** dissolved in 10 ml methanol was added, the solvent was removed under reduced pressure, and resulting oil was added to 1.0 g of emulsion, and stirred firmly to homogenize sample. For permeation study *ca.* 250 mg samples were mounted over commercial polyvinyldifluoride (PVDF) membrane or prepared pig ear skin (PES) membrane. The receptor medium was 0.1 M phosphate buffer pH = 7.4: ethanol 7:3 (v/v) as in (Fang et al., 2008). The progress of diffusion was monitored spectrophotometrically at 302 and 246 nm wavelength using the extinction coefficients calculated for the solution of **1** in receptor solution (Fig. 1). 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 25% of initial amount of **1**

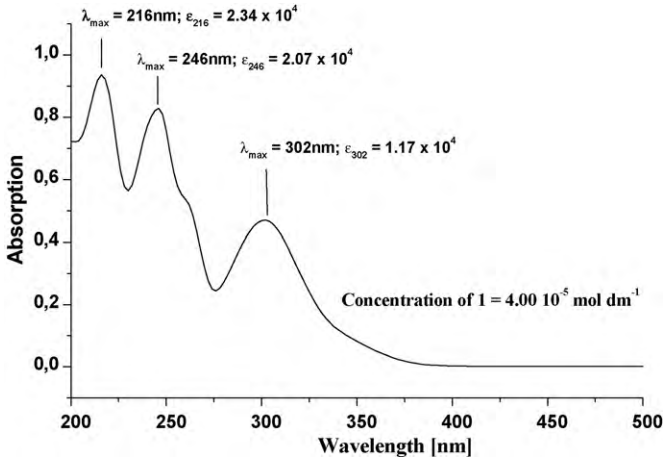


Fig. 1. The UV–vis spectrum of **1** in receptor solution.

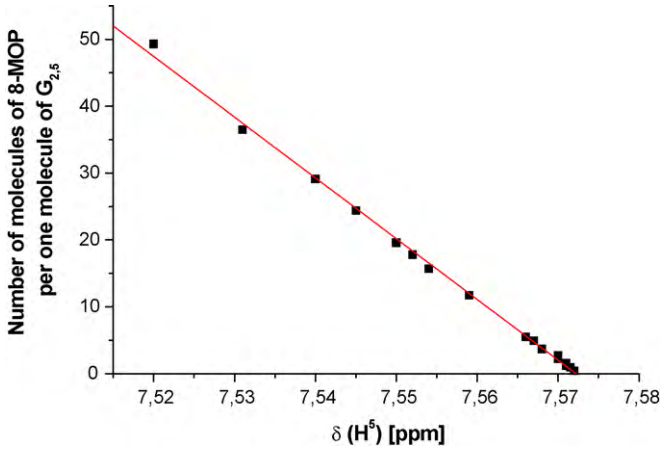


Fig. 2. The dependence of chemical shift of H^5(**1**) in methanolic-d_4 solutions of 2 mM **G2.5** PAMAM dendrimer on concentration of **1** in the ^{1}H NMR spectra.

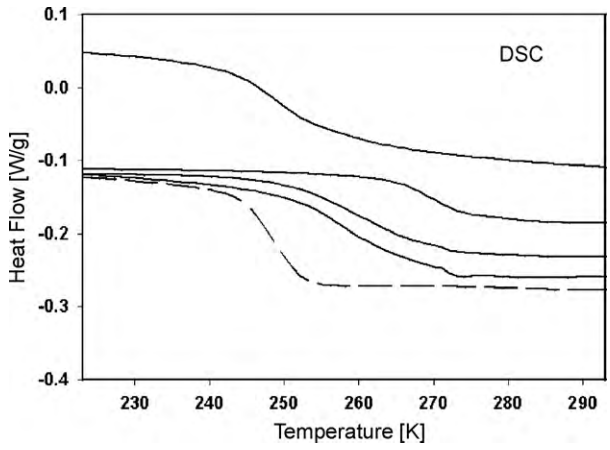


Fig. 3. The DSC curves (for the second runs of heating) for **1**:**G4** mixtures plotted as the dependence of heat flow against temperature [K] for the samples (from bottom to top): **G4** (dashed line), 2:1, 6:1, 7:1, and 8:1 **1**:**G4** mixtures.

(between 4 and 5 mg) 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 **1** received in function of time of diffusion was crucial to determine the diffusion properties of **G3**–**1** complexes. For comparison of diffusion efficiency the time of 10% diffused **1** ($\tau_{0.1}$) was used as quantitative parameter.

3. Results and discussion

8-Methoxypsoralene (**1**) is almost insoluble in water (<4×10^{-4} M concentration can be achieved). Nevertheless it is used as suspension in PUVA bath or as a cream (Grundmann-Kollmann et al., 1999) and therefore its dosing is not well controlled. According to the concept of dendrimer vehicle for drug (Svenson, 2009) we have studied the solubilization of **1** in water and methanol and PAMAM dendrimers of third and fourth generation: **G3** and **G4** and solubilization of **1** in methanol with **G2.5** and **G3.5** dendrimers.

3.1. Solubilization of 1 in the presence of dendrimers studied by the ^{1}H NMR

The solubility of **1** in methanol-d_4, estimated on the basis of integration of resonance H^5(**1**) at 7.52 ppm related to the intensity of added reference chloroform C*H*Cl$_3$ signal at 7.24 ppm, was equal to 0.0198 mol dm^{-3}. The number of molecules in PAMAM–**1** host–guest complex were determined by addition of solid **1** into the methanol-d_4 solution of PAMAM dendrimer (**G2.5**, **G3**, **G3.5** or **G4**) of variable concentration (0.001–0.002 M) with ^{1}H NMR monitoring until **1** dissolved. The resonances of **1** shifted downfield of about 0.05 ppm for the solution containing the dendrimer and 0.001 M **1** (1 or less molecule of **1** per one molecule of PAMAM dendrimer). Titration of dendrimer with **1** resulted in continuous shifting of H^5(**1**) into the position of free **1** (Fig. 2). When the equilibrium between the host–guest complex and free **1** reached the saturation of methanol with **1** (0.0198 M concentration), the precipitation of **1** took place. At this point the total concentration of **1** was calculated and number of molecules of **1** absorbed in PAMAM dendrimer was obtained. In this way we have found the stoichiometry of host-guest complexes in methanol, which was: **1**:**G2.5** = 15, **1**:**G3** = 14, **1**:**G3.5** = 30, and **1**:**G4** = 30.

The attempts to dissolve **1** in water in the presence of **G3** and **G4** led to partial decomposition of dendrimers and oxidation of **1** at prolonged heating the mixture of 0.1 M aqueous **G3** and crystalline **1**. Therefore another method of solvent-free samples containing **1** and **Gn** was applied. The neat, oily complexes could be obtained by dissolving **1** and **G3** or **G4** in methanol, followed by evaporation of the solvent. These complexes were soluble in water. No precipitation of **1** in water occurred, when samples of stochiometry 3:1 **1**:**G3** and 7:1 **1**:**G4** were tested. Thus, the maximum weight% load of **1** in dendrimers **G3** and **G4** were: 9.4% and 10.4%, respectively. Furthermore, the dilution of concentrated solutions with water did not cause any precipitation of **1**.

The interaction between **1** and dendrimers is weak. The equilibrium between **1** bound in dendrimers and free **1** is fast on NMR time-scale. The concentration dependence of chemical shift of **1** shows linear instead of sigmoidal shape. The simple UV–Vis experiments performed in tandem cell showed no difference, neither in maxima positions nor extinction coefficients in 1:1–3:1 **1**:**Gn** solutions after mixing of separated solutions of **Gn** and **1**. However, quite remarkable solubilization effect of **G3** and **G4** in methanol, and solubility of oily samples of 3:1 **1**:**G3** and 7:1 **1**:**G4** in water was promising for application of these PAMAM dendrimers as vehicles for **1**.

3.2. Differential scanning calorimetric studies of neat complexes of *1* with dendrimers

Host–guest complexes were obtained by homogenation of **1** with **Gn** dendrimers in methanol followed by extensive removal of the solvent under vacuum (0.2 mm Hg). The oily samples were subjected to DSC measurements in order to obtain glass transition temperature, T_g. DSC runs started from cooling the sample from room temperature to −90 °C and then heating to 120 °C and the cooling–heating loop was repeated, all with rate 10 K/min. The T_g obtained in second loop was compared for the complexes and neat dendrimers. Obtained results showed increase of T_g of complexes in comparison with oily dendrimers until the absorption capacity of dendrimers was exceeded (Table 1). The limiting amount of **1** giving homogeneous oils contained: 7, 3, 6, and 7 molecules per one molecule of **G2.5**, **G3**, **G3.5**, and **G4**, respectively. When these stoichiometries were overcame, the samples lost their homogeneity and the measured T_g returned to their initial values, i.e. to T_g of neat dendrimers. The phenomenon is illustrated at Fig. 3 for **G4** dendrimer and its mixtures with **1**. This method appeared to be useful for the determination of stoichiometry of host–guest complexes and corroborate well with solubility of neat complexes in water. The increase of T_g for **Gn**–**1** complexes was rather expected phenomenon related to incorporation of molecules of **1** into flexible dendrimers, rendering the complexes more rigid. The incorpora-

tion of **1** (m.p. >415.15 K (142 °C)) into **Gn** resulted in increase of T_g, opposite to incorporation of methanol, which resulted in decrease of T_g (when 12 molecules of CH_3OH were absorbed in **G3**, the value of T_g dropped to 201.15 K (−72 °C)). With this experimental procedure we were able to determine the limiting stoichiometry of **Gn**–**1** complexes, i.e. the capacity of dendrimers, which for **G3** and **G4** reached 3 and 7 molecules of **1** for molecule of dendrimer. These water-soluble complexes allow to prepare water solutions of **1** with well controlled concentration. Further we have used them to perform transdermal permeability experiments on skin-model membranes (see below).

3.3. Permeation studies

The permeation experiments of **1** through PVDF and PES membranes using **1** dispersed in o/w emulsion indicated that only traces of **1** reached the receiving solution. The reason was very limited solubility of **1** in water (*ca.* 1×10^{-4} mol dm^{-3}), i.e. typical load of emulsion (*ca.* 250 mg) contained *ca.* 150 mg of water and consequently at most 0.7 mg of **1**, which was beyond the limit of UV detection, when this amount of **1** was received by 100 cm^3 receiver solution composed of buffer pH = 7.4: ethanol 7:3 (v/v). Thus the increase of solubility of **1** was the crucial for successful detection of **1**. This goal was achieved by addition of dendrimers **G3** or **G4** to reach the 3:1 **1**:**Gn** molar ratio, dispersion of the host–guest complex in emulsion, and finally the 250 mg sample containing *ca.* 4 mg of **1** and 40 mg of **G3** or 80 mg of **G4** were mounted on top of receiver, separated from donor by the membrane. The permeation of **1** was monitored spectrophotometrically at two wavelengths (246 and 302 nm) to give the flux (in μmol h^{-1} cm^{-2}) against time of experiment. We have observed that after 0.5 h lag time the breakthrough of membrane led to high initial flux in case of permeation through PVDF and after ca 2 h the flux was stabilized, which was presumably consistent with steady-state conditions. The initial increase can be rationalized in terms of saturation of PVDF membrane with large dendrimeric molecules and **1**. Because the driving force for the diffusion was the gradient of concentration of **1**, the raw data were then converted by multiplying the flux by factor of n_{in}/n_{temp}, where n_{in} was the initial number of micromoles of **1** in releasing emulsion and n_{temp} was the current amount of micromoles of **1**. From the corrected flux data the cumulative curves of **1** in receiving solution were calculated. The flux was also variable when permeation experiments were performed on PES membranes, however in these cases the initial flux was lower before steady-state conditions were achieved. The same corrections were used for experimentally observed flux for experiments performed on PES, as before. In details, for permeation of **1** through PVDF membrane the initial flux, measured within first 4.5 h was above 1 μmol h^{-1} cm^{-2} and after that time it was stabilized at mean 0.8 μmol h^{-1} cm^{-2}. When much thicker PES membrane was used (the thickness was 0.55 mm in comparison with 0.125 mm for PVDF), the initial flux was at the level of 0.3 μmol h^{-1} cm^{-2} and after 5.5 h increased up to mean 0.6 μmol h^{-1} cm^{-2} and was maintained throughout next 29 hours. Thus, in both cases the steady conditions were achieved at almost constant concentration of **G3**–**1** complex in donor (only *ca.* 10% of **1** was transferred within experimental time).

All the cumulative curves (plotted in mg of **1** diffused through membrane) were calculated using corrected flux. The obtained cumulative plots showed slight curvature at the beginning of experiment according to the flux instability and after steady state was reached, the dependence was nicely linear. The linear fit into linear part of plots was calculated by linear regression method; the slopes had the physical sense of permeability. These calculations were performed separately and then the value of time of 10% transfer of **1** ($\tau_{0.1}$) was calculated for every experiment (7 runs) and finally the mean $\tau_{0.1}$ was calculated. The most convenient relative parameter, which can be used for comparison of all the obtained results is the permeation time of 10% of initial amount of **1**, $\tau_{0.1}$. Based upon seven independent experiments for every system the arithmetic mean values of $\tau_{0.1}$ for 3:1 **1**–**Gn** complexes were calculated. The inspection of UV–Vis spectra of receiving solution indicated the presence of PAMAM dendrimers accompanying the **1** by comparison of absorbance at two wavelengths, namely 246 and 302 nm, which correspond to absorption by PAMAM dendrimers and **1**, respectively. The ratio of A_{246}/A_{302} in receiving solution was the same as in solution of 3:1 **1**–**Gn** complexes in receiving solution recorded separately. This was qualitative evidence, that permeating species were indeed the 3:1 **1**–**Gn** complexes.

Table 2
The time of 10% transfer of **1** from emulsions containing dendrimers. The mean value of $\tau_{0.1}$ is based on 7 separate experiments; s.d.: standard deviation.

Formulation	Membrane	$\tau_{0.1}$ [h] (s.d.)
G3–**1** 1:3 in o/w emulsion	PVDF	6.46 (0.32)
	PES	19.50 (0.48)
G4–**1** 1:3 in o/w emulsion	PVDF	9.07 (0.33)
	PES	26.19 (0.76)

The obtained $\tau_{0.1}$ collected in Table 2, it can be seen that release of **1** from emulsion is slowed down by **Gn** dendrimers depending on their molecular weight and size. Control experiment of release of **1** from water through PES (Borowska et al., in preparation) with fluorescence detection showed $\tau_{0.1}$ equal to *ca.* 2 h. From the obtained results it can be concluded that full-generation PAMAM dendrimers: **G3** and **G4** retard the diffusion of 8-methoxypsoralene by factor of 10 and allow to increase considerably the load of 8-methoxypsoralene in providing form in comparison with other dispersed forms like suspensions in mixed solvents or microemulsions.

4. Conclusions

Well-controlled administration of psoralenes, photosensitizers for PUVA therapy is an important issue for treatment of many dermatological diseases. Currently available formulation of 8-MOP: solutions, emulsion and cream do not provide good transdermal permeability of 8-MOP, especially to deeper layers of skin. Thus, there is a need to develop novel systems formulated with non-irritating components that can be applied to more efficiently treat skin disorders like psoriasis, vitiligo, atopic eczema, mycosis fungoides and many others.

The studies on carrier–drug complexes formed between widely used polyamidoamine dendrimers and 8-MOP allowed to elucidate optimum composition of the complexes with full-generation **G3** and **G4** and half-generation **G2.5** and **G3.5** PAMAM dendrimers. Preliminary in vitro transdermal studies indicated that **G3** and **G4** dendrimers can be useful carriers for 8-MOP allowing to increase local concentration of photosensitizer and, on the other hand, to slow down the transdermal diffusion of the complexed drug in comparison with its water solutions (or in the solvent mixture used in medical practice, where ethanol, glycerol, ketone, and water mixture is used). Our preliminary studies (Borowska et al., in preparation) performed on Wistar rats reveal the enhancement of permeability of complexes in comparison with 8-methoxypsoralene. Moreover, the large gradient of concentration of donor form of photosensitizer across skin will probably result in deeper penetration of 8-methoxypsoralene in PUVA therapy.

The concluded stoichiometry of permeable complexes of **G3** or **G4** dendrimers with 8-methoxypsoralene, based on solubilization and DSC experiments is 3:1 and 7:1 8-MOP:Gn, respectively. The molar ratios correspond to mass load of 8-methoxypsoralene equal

to 8.6% and 9.6% of water-soluble formulation, which can be safely diluted with water to desired concentration.

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