Encapsulation of genistein in glycidylated G3 polyamidoamine dendrimers enables diffusion of genistein through biological membranes and anti-nematode activity of the encapsulate

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ABSTRACT

Introduction and aim. Poorly soluble isoflavonoid genistein is known as an anti-nematode agent and also it decreases the risk of certain types of cancer. The biological activity of genistein is limited mostly by its low solubility. Therefore many attempts to increase genistein solubility in water were reported. We applied a polyamidoamine dendrimer, modified its surface by glycidylation, and used this macromolecule as a guest for genistein.

Material and methods. Polyamidoamine dendrimer 3rd generation was substituted with 64 glycidol residues to obtain a macromolecule host for genistein. The stoichiometry of this host-guest complex was determined. The complex was tested for skin model permeability, toxicity on fibroblast (BJ) and keratinocyte (HaCaT) cell lines in vitro and anthelmintic activity on the Caenorhabditis elegans nematode.

Results. The partition coefficient of genistein between octanol and water was determined (K_{O/W}). The 1:1 host-guest complex was isolated and used as drug delivery system for genistein delivery. PAMAM G3 glycidyled dendrimer containing genistein indicated an anthelmintic activity at 50 µM concentration.

Conclusion. The solubility of genistein in water increases 640 times in presence of an equimolar concentration of the dendrimer. One molecule of host dendrimer encapsulates 3 molecules of genistein. The encapsulate is an efficient anti-nematode formulation.

Keywords. C. elegans, fibroblast BJ toxicity, genistein, keratinocyte HaCaT toxicity, PAMAM dendrimer
protect against many chronic diseases (including coronary heart diseases, subclinical atherosclerosis, type 2 diabetes), and decrease the risk of certain types of cancer such as breast and prostate cancers.

Fig 1. Formula of GEN with atom numbering

On the other hand, IFLs including GEN are anti-nematode agents. IFLs were demonstrated to impede soybean cyst nematode (SCN) by influencing sex ratios and the number of female eggs. GEN was shown to strongly influence the physiological index of SCN in a dosage and time dependent way.2 When SCNs were exposed to 0.37 mM GEN (100 µg/mL) aqueous solution (with 1% DMSO), a 50% reduction of SCN mobility (measured by reversal frequency assay), ca 35% reduction of oxygen consumption, and severe body leaking after 24 hours treatment were observed.

Another nematode model organism, a transgenic Aβ-expression Caenorhabditis elegans strain CL4176 was used to determine the ability of IFLs to alleviate Aβ expression-induced paralysis in C. elegans, which correlated with a reduced level of hydrogen peroxide and β amyloid.3 This relationship was observed only for gly-

cer such as breast and prostate cancers.

Aim

We have designed the DDS for GEN delivery based on a modified polyamidoamine (PAMAM) dendrimer generation 3. The native dendrimer was used before to encapsulate riboflavin and 8-methoxypsoralene for transdermal delivery.6,9 However, recently, we modified the G3 dendrimer by glycidylation in order to eradicate primary amine groups of native G3.10 Those G3-functionalized dendrimers were very well soluble in water and efficiently internalized into normal human fibroblasts and two cancer lines within 1 hour.

Material and methods

Materials

Genistein (>98% purity) was purchased from Merck KGaA (Darmstadt, Germany). Third generation poly-
amidoamine dendrimer (G3) was synthesized according to Tomalia’s protocol and then modified by conversion of primary amine groups into bis-2,3-dihydroxypropylamine groups as was described before to yield G3-functionalized dendrimers were very well soluble in water and efficient-
ym at 0.7 hour for GEN-M in comparison with 1.0 hour T_max for GEN alone. Many other DDSs for GEN delivery were discovered, which slightly improved solubility and permeability of GEN, and the subject was recently reviewed.7

methods

The 1-D 1H and 13C NMR spectra and 2-D 1H-1H correlations spectroscopy (COSY), 1H-13C heteronuclear
single quantum correlation (HSQC), and heteronuclear multiple bond correlation (HMBC) spectra were recorded in DMSO-d$_6$ using Bruker 300 MHz instrument (Rheinstetten, Germany) at College of Natural Sciences, University of Rzeszow.

UV-Vis spectra were recorded with Hitachi U-1900 spectrometer (Tokyo, Japan).

The permeation GEN and GEN@G3$^{30}$ was studied using Franz diffusion assembly (Thermo Scientific (UK) model DC 600) equipped with 6 cm$^3$ acceptor compartments. Polyvinylidifluoride (PVDF, 0.125 mm thickness) or prepared rabbit skin (RS, 0.55 mm thickness) model membranes were used for permeation studies.

Partition of GEN between octanol and water containing host G3$^{30}$

Analytical protocols used in partition experiments and transdermal diffusion experiments (vide infra) required determination of extinction coefficients in various solvents. The UV-vis spectra were taken in water, ethanol, octanol, and in 70% aqueous phosphate buffered pH 7.4 and 30% ethanol (receiving solution in diffusion tests) are shown at Figure 2.

Solubility of GEN in octanol was determined by suspending 5.1 mg GEN in 5 mL octanol, stirring the mixture at 25 °C for 12 hours, filtration off the undissolved octanol, and determination of GEN concentration using previously determined extinction coefficient at 336 nm, $e_{336}=3.52\times10^4$ mol$^{-1}$×dm$^3$×cm$^{-1}$. Finally, the 3.77 M concentration of GEN in octanol saturated solution was elucidated.

In o/w partition experiments, 60 mM solutions of GEN in octanol was prepared. Several partition experiments were performed by equilibration of 10 mL GEN in octanol against 10 mL of water, 0.21 mM G3$^{30}$ (aq), 0.63 mM G3$^{30}$ (aq), 0.63 mM G3$^{30}$ (aq), 1.91 mM G3$^{30}$ (aq), and 5.70 mM G3$^{30}$ (aq) with vigorous magnetic stirring at 25°C for 48 hours. Then, the aqueous and octanol phases were separated and concentration of GEN in octanol ($\lambda$=336 nm, $e_{336}=3.52\times10^4$ mol$^{-1}$×dm$^3$×cm$^{-1}$) and in water ($\lambda$=255 nm, $e_{255}=3.82\times10^4$ mol$^{-1}$×dm$^3$×cm$^{-1}$) were determined spectrophotometrically. Thus the partition coefficients log$K_{ow}=\log(c_o/c_w)$ were obtained in function of concentration of G3$^{30}$. The results are presented graphically in the Figure 3.

Transdermal permeation of GEN and GEN@G3$^{30}$ encapsulate

To analyze the ability of GEN to diffuse through skin models, synthetic polyvinylidifluoride (PVDF) and prepared rabbit skin (RS) were studied using hydroxyethylcellulose-based hydrogel as delivery form and a mixture of phosphate-buffered water pH 7.4 (70 %) and ethanol (30 %) as receiving solutions. The ability of GEN (guest) to release from the dendrimer (host) and from hydrogel followed by permeation of skin model was monitored spectrophotometrically at 266 nm ($\lambda_{max}$, $e_{266}=4.0\times10^4$ mol$^{-1}$×dm$^3$×cm$^{-1}$). The delivery gels were prepared as follows: 1:1 G3$^{30}$ : GEN solution was prepared by dissolving 0.7386 g G3$^{30}$ in 4.0 mL water, 17.1 mg GEN in 1.5 mL ethanol and mixing the solutions. The mixture was stirred at 50°C for 2 hours and then cooled to room temperature. The final concentrations of GEN and G3$^{30}$ were both 11.5 mM. In the meantime, the hydrogel was prepared by addition of 0.75 g hydroxyethylcellulose to phosphate-buffered water (25 mL). After homogenization, 0.5 mL of 11.5 mM GEN or G3$^{30}$ : GEN solution was added to freshly prepared gel and homogenized rapidly to obtain the delivery system. Typically 0.32 g of delivery sample was mounted topically on membrane of a Franz chamber filled with 6 mL receiving solution below the membrane (PVDF or RS). The initial amount of GEN in the mounted sample was between 16 and 20 µg (ca 75 nmoles) and equimolar amounts of G3$^{30}$. The receiving solution (6 mL) was removed in 2 hr time intervals and replaced with new portions. The concentration of GEN in receiving solution was quantified by measurements of absorption at 266 nm. The results are presented as cumulative percentage of released GEN in time for PVDF and RS vs time (vide infra).
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**Cell cultures**
BJ human normal skin fibroblasts, doubling time 1.9 days, and HaCaT human immortalized keratinocytes, doubling time 24 hours, were propagated in a DMEM medium supplemented with 10% FBS, and 100 U/ml penicillin, 100 µg/ml streptomycin. Cells were cultured at 37°C in 5% CO₂ atmosphere and 95% humidity, with growth medium changed every 2–3 days and passaged at 60-80% confluence, using 0.25% trypsin-0.03% ethylenediaminetetraacetic acid in PBS (calcium- and magnesium-free). Cell morphology was checked under the Nikon Eclipse TE2000 inverted microscope (Tokyo, Japan) equipped with phase contrast. The number and viability of cells were estimated by the trypan blue exclusion test, with Automatic Cell Counter TC10™ (Bio-Rad Laboratories, Hercules, CA, USA).

**Cytotoxicity of GEN, G3gl and GEN@G3gl encapsulates**
Alamar Blue assay
BJ or HaCaT cells were seeded in flat-bottom 96-well culture plates at a density of 1×10⁴ cells/well (200 µL cell suspension/well) and allowed to attach for 24 hours. After culture medium removal, GEN, G3gl and GEN@G3gl in the concentration range 1–100 µM were added (100 µL/well) and incubated for 24 hours. Then, tested drugs solutions (GEN, G3gl and GEN@G3gl) were aspirated and 60 µM resazurin solution was added (100 µL/well). The plates GEN@G3gl were incubated at 37°C for 2 hours. After that, the fluorescence was measured at λ=570 nm with a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The control sample was cells (BJ and HaCaT separately) grown in DMEM medium without addition of tested samples, for which viability was assumed to be 100%. An experiment was performed in three independent assays in triplicate.

**Toxicity against Caenorhabditis elegans**
The C. elegans nematode wild-type culture (strain N2, variety Bristol) was used to estimate the in vivo activity of the G3gl and GEN@G3gl as described earlier. After the transfer of nematodes to a 96-well plate (about 20 individuals in 50 µL), the solutions of the studied compounds in a complete medium were added (50 µL/well). The maximal DMSO final concentration was equal to 0.2% and had no significant influence on nematode viability. Five replicates were made for each concentration. The plate was incubated at 21°C for seven days. During this time, live and dead worms were counted under an inverted microscope (Delta Optical IB-100).

**Statistical analysis**
To estimate the differences between treated samples and non-treated controls for the cell culture assays, a nonparametric Kruskal–Wallis test was performed because the data did not show signs of a normal distribution. To analyze differences in nematode viability between the control and the nematodes incubated with dendrimer and encapsulate, the Kaplan-Meier estimator was used. Statistically significant differences against the control were determined with Gehan’s Wilcoxon test. The p<0.05 was considered statistically significant. Analyses were performed with Statistica 13.3 software (StatSoft, Tulsa, OK, USA).

**Results and discussion**
**Stoichiometry of host-guest encapsulates**
Genistein (GEN) was characterized by the 1-D ¹H, ¹³C, and heteronuclear HSQC, and HMBC NMR spectroscopy. 2-D NMR measurements enabled us to unambiguously assign the ¹H and ¹³C resonances (Fig. 4).

Then the G3⁰ dendrimer in D₂O was titrated with GEN in order to determine the ability of the host to encapsulate the guest. The starting spectrum of G3⁰ in D₂O...
(9 mM) is presented at Figure 5A. To this, a solution GEN in DMSO-d6 (125 mM) was added stepwise until no precipitate was formed. The 1H NMR spectra of the solutions are presented at Figure 5, traces B-D. We found that the maximum concentration of GEN in the mixture was 31 mM (Fig. 5D); above this, the GEN (or GEN@G3gl encapsulate) precipitated upon addition to the aqueous solution. Thus, we concluded that solubility of GEN in water increased from 4.8 µM to 31×10³ µM concentration in presence of G3gl host, i.e. four orders of magnitude. Furthermore, the molar stoichiometry of the GEN@G3gl encapsulate was finally at least 3.5:1 GEN:G3gl (Fig. 5D).

Skin permeation
GEN is known to be highly hydrophobic. We measured the K_{O/W} parameter using standard procedure of partition between octanol and water. We obtained logK_{O/W}=3.34, which was close to the value of 3.04 determined before. Then we determined the logK_{O/W} in presence of various concentrations of the G3gl host in the water phase (Fig. 3). We found that K_{O/W} decreased almost two orders of magnitude in presence of 5.7 mM G3gl in water phase, namely from 2157 to 33.7 (corresponding to logK_{O/W} 3.34. and 1.53, respectively). We have assumed that the highly hydrophobic G3gl host was absent in the octanol phase, which is reasonable considering that logK_{O/W}=2.24 in case of PAMAM G3 dendrimer, indicating that solubility of that hydrophilic dendrimer in water is 5754 times higher than in octanol. From partition experiments, we conclude that GEN@G3gl encapsulate loses the guest GEN molecule, which is promising in terms of biological conditions

Fig. 5. 1H NMR titration of G3gl (in D₂O) with GEN (in DMSO-d₆). A – spectrum of G3gl in D₂O (ca 9 mM concentration); B – D: spectra of solution containing G3gl and 1.1, 1.6, and 3.4 equivalents of GEN; E – spectrum of GEN in DMSO-d₆. The residual resonances of HDO, DMSO-d₆, and methanol are labeled with asterisks. The [128H] integral intensity signal of 2,3-dihydroxypropyl substituents (64 residues) centered at 3.5 ppm were used as an internal reference
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which might be met when GEN@G3<sup>δ</sup> in water is delivered into tissue.

Considering the determined stoichiometry achievable in aqueous environment (at least 3 GEN per 1 G3<sup>δ</sup> host) we obtained the encapsulate 1:1 GEN-G3<sup>δ</sup> and tested this as DDS in skin permeation experiments, as well as toxicity against human cell lines and C. elegans.

Skin permeation of GEN was tested using hydroxyethylcellulose hydrogel as a donor, polyvinylidene fluoride (PVDF) and freshly prepared rabbit skin (RS) membranes as skin models, and phosphate buffered water/ethanol receiving solutions. The amount of transferred GEN from donor into receiver was determined spectrophotometrically within 48 hours (Fig. 6 and 7).

The cumulative amount of GEN permeated through PVDF indicated 3-fold enhancement of GEN release from encapsulate GEN@G3<sup>δ</sup> donor in comparison with GEN alone in donor. The time of 10% GEN transfer, \( t_{0.1} \) was used as comparative parameter. Thus the estimated \( t_{0.1} \) was 3.2 ± 0.2 hour in case of GEN@G3<sup>δ</sup>, while 6.50 ± 0.2 hour in case of GEN alone (Fig. 6). The values of \( t_{0.1} \) for GEN@G3<sup>δ</sup> were shorter than those obtained in similar delivery system for 8-methoxypsoralen in PAMAM G3 and G4 dendrimers, which were 6.5 and 9.0 hours,<sup>7</sup> respectively as well as riboflavin in PAMAM G3,5 dendrimer, which was determined as 6.5 hours.<sup>8</sup> The release of GEN from both hydrogel and from GEN@G3<sup>δ</sup> in hydrogel was relatively fast; the control experiment after 24 hours indicated that permeation was completed within the first 12 hours.

**Fig. 6.** The permeation of GEN from hydrogel containing GEN and GEN@G3<sup>δ</sup> encapsulate through PVDF. Standard deviation for 5 times repeated experiments was not higher than 1.5%

In case of permeation through rabbit skin, the release of GEN from hydrogel and GEN@G3<sup>δ</sup> was stopped after 32 hours of experiments (Fig. 7). No statistically relevant enhancement of GEN release from GEN@G3<sup>δ</sup> versus GEN alone in the hydrogel donor was found, although the release of GEN from encapsulate was ca 1% faster than from GEN in hydrogel within first 30 hours. Because no 10% of transfer was achieved in either case, the time of 3% transfer was chosen for comparison with other results, \( t_{0.03} \). Thus the \( t_{0.03} = 7.5 ± 0.2 \) hour was determined for GEN release from GEN@G3<sup>δ</sup>, while it was 12.0 ± 0.2 hour for GEN alone. For comparison \( t_{0.03} = 1.5 ± 0.1 \) hours was determined for riboflavin release from riboflavin – PAMAM G3 encapsulate in o/w emulsion through porcine skin membrane and 7.4 ± 0.1 hour for 8-methoxypsoralen in PAMAM G3 in an analogous experiment.<sup>1, 5</sup>

**Fig. 7.** The permeation of GEN from hydrogel containing GEN and GEN@G3<sup>δ</sup> encapsulate through rabbit skin. Standard deviation for 5 times repeated experiments was not higher than 0.3%

Kitagawa et al. used w/o microemulsions prepared from isopropyl myristate (oil phase), 150 mM NaCl(aq), ethanol and Tween 80 surfactant to enhance the solubility of GEN and other isoflavones.<sup>13</sup> Using various microemulsions, they were able to enhance the GEN dispersion 1300–2000 times. The microemulsions were then used as GEN delivery system to accumulate GEN in guinea pig dorsal skin and Yukatan micropig skin. The amount of accumulated GEN increased 25-59 times in comparison with GEN in a NaCl(aq) delivery experiment. Accumulated GEN in skin significantly inhibited lipid peroxidation in vitro dose-dependently. Furthermore, pretreatment of guinea pig dorsal skin with GEN containing microemulsions prevented UV irradiation-induced erythema formation. As described here, solubilization effects for GEN using G3<sup>δ</sup> is more efficient than the aforementioned microemulsion dispersion.

**In vitro toxicity of GEN, G3<sup>δ</sup> and GEN@G3<sup>δ</sup>**

In order to estimate the biological properties of GEN, G3<sup>δ</sup> and GEN@G3<sup>δ</sup> encapsulates, we performed the toxicity Alamar Blue assay on BJ normal human fibro-
blasts and immortalized human keratinocytes (HaCaT cell line). We determined that neither GEN, nor G3gl or GEN@G3gl exerted any toxic effect in range of 1–100 µM concentration (Fig. 8).

Moreover, a slight increase of cell viability over the entire range of used concentrations (1–100 µM) was noted, which could be related to the trophic effect of the substances used. Obtained results were consistent with others, where GEN did not influence on HaCaT cell viability up to 100 µM concentration after 24 h incubation (MTT assay or Sulforhodamine B (SRB) assay).16,17 Also Pawlicka et al. indicated that genistein was not toxic up to 150 µM concentration against BJ fibroblasts, and observed that genistein at lower concentrations (10–100 µM) stimulated fibroblast growth (MTT assay, 24 h incubation).18

It is worth mentioning that increases of cell viability under the influence of GEN@G3gl was probably an effect of presence of GEN in encapsulate, since viability after incubation with GEN and GEN@G3gl was always higher than G3gl alone at appropriate concentrations (Fig. 8).

**In vivo toxicity against C. elegans**

Due to previously reported anti-nematode activity of GEN, we have tested the GEN@G3gl biological effect on *C. elegans* in comparison with G3gl.2,3 We found that after 7 days incubation, glycidylated G3 PAMAM dendrimer did not influence on *C. elegans* lifetime (Fig. 9).

A similar pattern was observed for GEN@G3gl up to 25 µM concentration. At 50 µM concentration, viability of nematodes decreased significantly, achieving a value of 63% after 7 days incubation. It was interesting that 100 µM concentration of both G3gl and GEN@G3gl had a slighter effect than 50 µM concentration. In summary, at a 50 µM concentration, the genistein delivered as GEN@G3gl encapsulate induced an anthelmintic effect.

**Conclusion**

Third generation polyamidoamine dendrimers modified by exhausting glycidylation, act as a solubilizer for the highly hydrophobic isoflavonoid genistein. The host dendrimer macromolecule is able to encapsulate more than 3 molecules of guest genistein. Water solubility of genistein increased about 27 times in presence of the host. The 1:1 genistein : dendrimer encapsulate promotes skin permeation of genistein. It was non-toxic against normal BJ fibroblast and immortalized HaCaT keratinocyte human cells. The 1:1 genistein : dendrimer

![Fig. 8. Biological activity of GEN, G3gl, and GEN@G3gl against normal human BJ fibroblasts and immortalized HaCaT keratinocytes after 24 hours incubation. Results are presented as medians (percentage of non-treated control). Whiskers indicate the lower (25%) and upper (75%) quartile ranges. * p≤0.05, Kruskal–Wallis test (against non-treated control).](image)

![Fig. 9. The Kaplan–Meier survival curves of *C. elegans* after 7 days of incubation with G3gl and GEN@G3gl. Results are presented as cumulative proportion surviving. Statistically significant differences against DMSO-treated control (0.2%) obtained by Gehan’s Wilcoxon test are marked with asterisks * (p≤0.05) in the colors corresponding to the tested concentrations.](image)
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encapsulate indicated anthelmintic action at 50 μM concentration against C. elegans.

Declarations

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Author contributions
Conceptualization, A.F-R. and S.W.; Methodology, S.W. and Ł.U.; Software, Ł.U.; Validation, S.W., Ł.U. and A.F-P.; Formal Analysis, S.W.; Investigation, J.D., A.F-R., M.Z-D, and S.W.; Resources, Ł.U.; Data Curation, Ł.U.; Writing – Original Draft Preparation, Ł.U., S.W., and Ł.U.; Writing – Review & Editing, S.W.; Visualization, S.W. and Ł.U.; Supervision, S.W.; Project Administration, S.W.; Funding Acquisition, S.W.

Conflicts of interest
Authors declare no conflict of interest.

Data availability
Raw data are available from corresponding author on request demand.

Ethics approval
None.

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