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# APPLICATION OF POLYMERIC DIMETHYLAMINOETHYL METHACRYLATE-BASED CARRIERS OF PLASMID DNA FOR GENETIC TRANSFORMATION OF *CERATODON PURPUREUS* MOSS

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**Introduction.** Genetic engineering in plants is of great importance for agriculture, biotechnology and medicine, and nanomaterials are widely used for genetic engineering. The **aim** of the present study was to evaluate the potential of poly(2-dimethylamino) ethyl methacrylate (DMAEMA)-based comb-like polymers as gene delivery systems in moss *Ceratodon purpureus* (Hedw.) Brid. protoplasts and determine the level of phytotoxicity of these polymers.

**Materials and Methods**. In order to confirm the formation of a complex of poly-DMAEMA carrier with plasmid DNA pSF3, gel retardation assay was used. The PEG-mediated transformation protocol was adapted to transform the protoplasts of *C. purpureus* moss with poly-DMAEMA carriers. Light microscopy was used to study the toxicity of polymers for moss protoplasts. The level of the polymers toxicity was estimated as $IC_{50}$ value.

**Results and Discussion**. The formation of pDNA complex with DMAEMA-based carriers took place at 0.03 % concentration of the polymers BGA-21, BGA-22(2ph), BG-24, BG-25, BG-26 or 0.1 % concentration of the BGA-22 polymer. Poly-DMAEMA carriers were able to deliver plasmid DNA pSF3 into protoplasts of *C. purpureus* moss. Three stable transformants of *C. purpureus* were obtained using BGA-22 polymer, 2 clones – using BGA-21 carrier, and 1 clone – using BGA-22(2ph), BG-24, BG-25,

BG-26 polymers. Poly-DMAEMA carriers at the working dose of 0.0025 % were relatively non-toxic for protoplasts of *C. purpureus* moss. 83.1–88.4% of viable protoplasts of *C. purpureus* moss were detected after treatment with studied carriers at a 0.0025 % dose. A survival ratio of protoplasts reached 66.7–72.9% under the effect of these polymers at a 0.025 % dose, which is 10 times higher than their working concentration. The $IC_{50}$ value of poly-DMAEMA carriers was in the range of 0.113–0.164 %, which was approximately 10 times higher than that of the PEG-6000 used for gene delivery in plants.

**Conclusion**. Novel synthetic poly-DMAEMA carriers delivered the gene of interest into moss *C. purpureus* protoplasts and possessed a low level of phytotoxicity. Thus, these carriers can be useful for gene delivery into plant cells.

***Keywords***: dimethylaminoethyl-methacrylate, polymeric carrier, protoplasts transformation, moss *Ceratodon purpureus,* phytotoxicity

## INTRODUCTION

Genetic engineering of plants is of great significance for agriculture, biotechnology and medicine. *Agrobacterium*-mediated and biolistic gene delivery methods are widely used tools for genetic transformation of plants. However, the efficiency of transformation remains a challenge because of the limitation of DNA transportation through the plant cell wall, damage of cells/tissues and a disruption of the transported gene [5].

Thus, the development of safe and efficient gene carriers becomes one of the prerequisites for the successful targeted gene transfer. Nanocomposites are widely used in biotechnology and medicine, namely for DNA transfer into the target cells. Cationic polymers have been developed for various biotechnological and biomedical applications. As drug carrier systems, they are widely used for delivery of proteins and peptides, as well as non-viral DNA and RNA vectors [16, 28]. Recently, such polycationic carries as poly(L-lysine) (PLL), poly(amidoamine) (PAMAM), poly(ethylenimine) (PEI), polymethacrylates (e.g. poly(2-dimethylamino)ethyl methacrylate (DMAEMA)) have been developed for gene delivery [2, 16, 20]. Poly-DMAEMA-based polymers were found to be efficient gene transfer carriers into mammalian [1, 6, 19, 22], yeast [7], and plant [8, 9] cells.

The toxic action of polymeric carriers could be a problem in gene therapy and biotechnology [1, 2, 12, 13, 26]. The evaluation of toxicity of polymeric carriers is an important step towards assessing the potential risks of their application. Low cytotoxicity of DMAEMA-based polymers toward mammalian cells was reported [2, 17, 18, 23]. However, the literature does not contain enough information about the phytotoxicity and mutagenicity of DMAEMA-based polymers [8, 10].

The present study was focused on studying the potential of DMAEMA-based block polymers as gene delivery systems for moss *Ceratodon purpureus* protoplasts and determining their biocompatibility.

## MATERIALS AND METHODS

**DMAEMA-based block polymers.** *Materials used for synthesis of the polymers*: 2-(dimethylamino)ethyl methacrylate (DMAEMA), 2-aminoethyl methacrylate hydrochloride (AEM), vinyl acetate (VA), maleic anhydride (MA), poly(ethylene glycol) methyl ether methacrylate (Mn ~470 and 1000 g $mol^{-1}$ (Da)) (PEEM470 and PEEM1000), isopropylbenzene (IPB) were from Aldrich (Milwaukee, WI, USA). N,N-Dimethylformamide (DMF), 1.4-dioxane, acetone and n-hexane obtained from Merck (Darmstadt, Germany).

*Synthesis of polymers*: Novel DMAEMA-containing comb-like polymers were synthesized at the Department of Organic Chemistry of Lviv Polytechnic National University (Lviv, Ukraine) in two stages as described previously [28]: 1) the synthesis of polyfunctional macroinitiators (oligoperoxide metal complex (OMC) with side peroxide groups or telechelic oligoperoxides – polyalcyl acrylate with terminal peroxide groups), and 2) the synthesis by grafting radical polymerization of polycationic polymer chains. The structures and compositions of polymers are shown in **Fig. 1**, and **Table 1**.

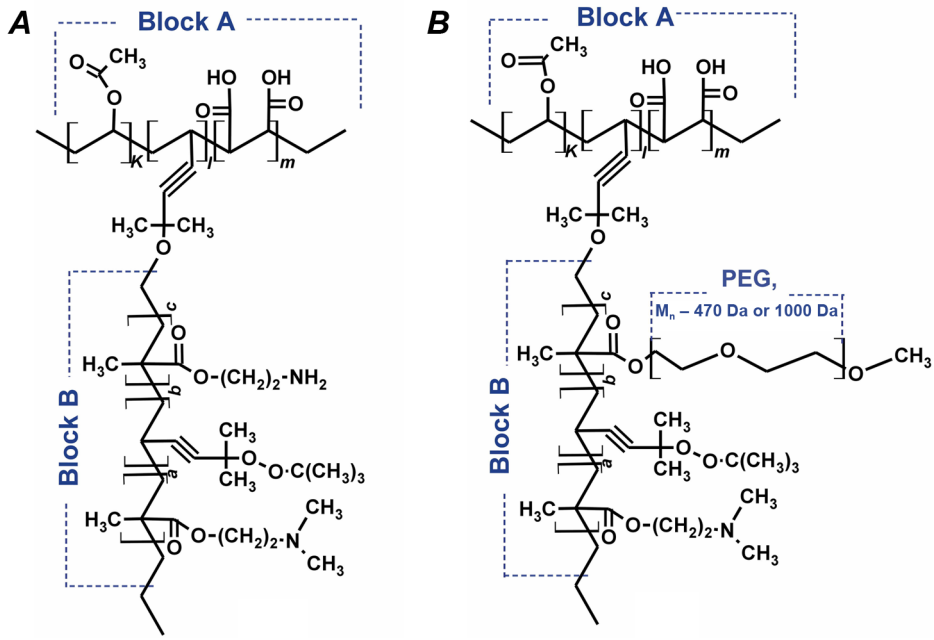


**Fig. 1** Schematic structure of the polymeric carriers: ***A*** – BGA-21, BGA-22, BGA-22(2ph); ***B*** – BG-24, BG-25, BG-26

**Рис. 1.** Схематична структура полімерних носіїв: ***A*** – BGA-21, BGA-22, BGA-22(2ph); ***B*** – BG-24, BG-25, BG-26

*Table 1.* **Comb-like polymers composition and molecular-mass characteristics**

*Таблиця 1.* **Склад гребінчастих полімерів, їхні молекулярно-масові характеристики**

| Polymers | Backbone – Block A (OMC with $M_n$ = 2.0 kDa) % mol | Grafted chain – Block B | | | | | $M_n$, kDa |
|---|---|---|---|---|---|---|---|
| | | Composition, % mol | | | | | |
| | | DMAEMA | VEP | AEM | PEEM470 | PEEM 1000 | |
| BGA21 | 1.2 | 85.3 | 7.5 | 6.0 | – | – | 8.4 |
| BGA22 | 1.2 | 86.3 | 8.7 | 3.8 | – | – | 8.9 |
| BG24 | 1.2 | 90.4 | 7.7 | – | 0.7 | – | 16.5 |
| BG25 | 1.2 | 92.7 | 4.3 | – | 1.8 | – | 17.9 |
| BG26 | 0.9 | 90.4 | 8.3 | – | – | 0.4 | 26.1 |

To obtain mixed micelles (sample BGA-22(2ph)), 0.5 mL of 20% solution of L-α-phosphatidylcholine (Ph) (Sigma-Aldrich, USA) in chloroform (Sigma-Aldrich, USA) was added to 5 mL of the solution of BGA-22 (1 mg/mL, pH 7.2) and sonicated for 25 s.

Then, chloroform was evaporated at 60 °C and stirred for 2 h, and the sample was vacuumized for 1 h.

**Formation of polymer/pDNA complex**. The polymer/pDNA complexes were prepared as described previously [8] by adding the polymer (1 μL) at increasing concentrations (0.003–0.1%) to the plasmid DNA (1 μg) pEGFPc-1 (Clontech, USA) on 6 μL of 20 мМ Tris-HCl, pH 7.4. The complexes were incubated for 20 min at room temperature. Then, the polymer/pDNA complexes were loaded onto 1% agarose gel (Lachema, Czech Republic) and run with 1x Tris-acetate buffer containing 1 μg/mL of Ethydium Bromide (Sigma-Aldrich, USA). Electrophoresis was performed for approximately 1 h at a constant voltage of 90 V. Plasmid DNA bands were visualized with an UV transilluminator (MacroVue UV-20, Hoeffer, USA).

**Transformation of moss protoplasts**. Moss *Ceratodon purpureus* (the Collection of the Institute of Ecology of the Carpathians, National Academy of Sciences of Ukraine, Lviv, Ukraine) was cultured at 24–26 °C in a solid Knop medium [4] with light intensity of 5–20 W/m$^2$ with an alternate 16/8 h day/night cycle. The culture of *C. purpureus* was initiated from the spores. Protoplasts of *C. purpureus* were isolated by digestion of cell walls of 100 mg of protonemal tissue with 1% Driselase (Sigma-Aldrich, USA) for 1 h in darkness at room temperature with slight continuous shaking. The digested moss tissue was transferred to the wet with a D-mannitol (Sigma-Aldrich, USA) nylon filter. The tube with the filtrated protoplasts was centrifuged at 200×g for 5 min. The pellet was gently resuspended in 10 mL of 9% D-mannitol. The number of protoplasts was counted using Neubauer chamber [15]. Protoplasts were recentrifuged at 200×g for 5 min. The pellet was gently resuspended in the MMM solution (9% D-mannitol, 0.015 M $MgCl_2$, 0.1% MES-KOH, pH 5.6) to obtain the protoplasts suspension at the concentration of $1.6\times10^6$/mL [4].

Plasmid DNA pSF3 [8, 9] that contained a gene of green fluorescent protein and hygromycin B resistance gene was used to transform the protoplasts of *C. purpureus* moss. The modified method of PEG-mediated transformation, developed for moss *Physcomitrella patens,* was used to transform the protoplasts of *C. purpureus* [4, 11]. The pDNA complexes with the poly-DMAEMA carrier (1 μL of pDNA and 2.5 μL of 0.1% of polymer) or with 2.5 μL of 40% PEG-6000 (LobaChemie, Austria) were used for moss protoplasts transformation. Complexes were added to 0.1 mL of protoplasts in the MMM solution (the final concentration of polymeric carriers was 0.0025%). Tubes with transformed protoplasts were transfered in a light-tight cardboard box and incubated for 24 h at 24–26 °C. The transformed protoplasts were mixed with 2 mL of PRMT medium (approximately 37–42 °C) and placed on Petri dishes (9 sm) with a solid PRMB medium containing 0.5% glucose. The regenerants were transferred on a selective medium containing hygromycin B (50 μg/mL, Sigma-Aldrich, USA) after 14 days at 24–26 °C with light intensity of 5–20 W/m$^2$ with alternate 16/8 h day/night cycle. The regenerants were sub-cultivated for next 14 days on the medium without or with hygromycin B antibiotic for approximately 8 weeks in order to obtain stable transformants [8].

**Toxicity of polymers for moss protoplasts.** The effects of poly-DMAEMA carriers and PEG-6000 on *C. purpureus* protoplasts were studied in the following range of the final carriers' concentrations of 0.0025 (working concentration), 0.025 and 0.25%. Polymers were added to 0.1 mL aliquots of protoplasts suspension ($1.6\times10^6$/mL) in 8% of D-mannitol and gently mixed by pipetting. Protoplasts were co-cultivated with the carriers for 24 h at 24–26 °C with light intensity of 5–20 W/m$^2$ with an alternate 16/8 h day/night cycle. The cytotoxicity of polymers was examined by calculating the amount of normal and damaged protoplasts using Neubauer chamber [15]. The level of polymer

toxicity was evaluated using $IC_{50}$ value (concentration of polymer that reduced protoplasts viability by 50%).

**Data analysis**. All data are presented as a Mean (M) ± Standard deviation (SD) from three replications. Statistical analysis was performed using one-way ANOVA test at GraphPad Prism 6 software (RRID:SCR_002798). P value of < 0.05 was considered as statistically significant.

## RESULTS AND DISCUSSION

**pDNA binding properties of polymers.** The electrostatic interactions between positively charged groups of the carrier and negatively charged phosphate groups of DNA are important for DNA binding and efficient delivery [24].

A gel retardation assay was used to confirm the formation of poly-DMAEMA carrier and plasmid DNA pEGFPc-1 complex. Plasmid DNA migrated during agarose gel electrophoresis (lane 5 of **Fig. 2**). The electrophoretic mobility of pDNA slowed down with an increase of the polymer concentration. The retardation of pDNA migration was found when it was mixed with 0.03% of BGA-21 (lane 2 of **Fig. 2*A***) point to the polymer/pDNA complex formation. The formation of a pDNA complex with BGA-22(2ph), BG-24, BG-25, BG-26 took place at 0.03% concentration of the polymer (**Fig. 2*C–F***). BGA-22 at 0.01% form complex with pDNA (lane 3 of **Fig. 2*B***).

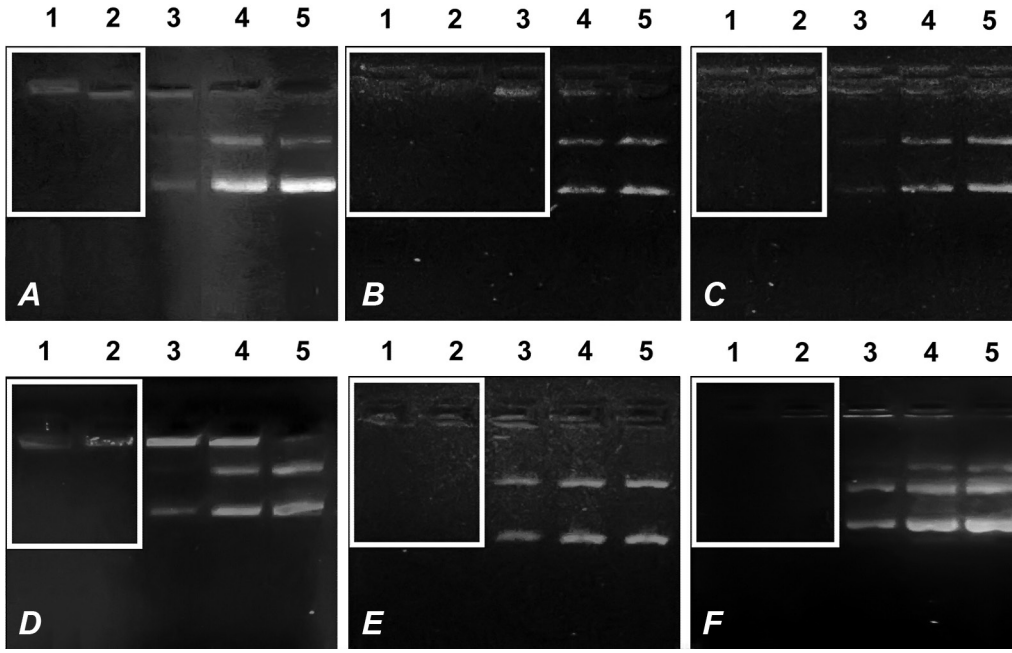


**Fig. 2.** Electrophoregram of gel retardation assay of the polymeric carriers BGA-21 (***A***), BGA-22 (***B***), BGA-22(2ph) (***C***), BG-24 (***D***), BG-25 (***E***), BG-26 (***F***) and plasmid pEGFPc-1 complexes in 1% agarose gel. Lines **1** – pDNA + 0.1% of polymer; **2** – pDNA + 0.03% of polymer; **3** – pDNA + 0.01% of polymer; **4** – pDNA + 0.003% of polymer; **5** – intact pDNA. The white rectangle points to the polymer/pDNA complex

**Рис. 2.** Електрофореграма ДНК плазміди pEGFPc-1 і полімерних носіїв BGA-21 (***A***), BGA-22 (***B***), BGA-22(2ph) (***C***), BG-24 (***D***), BG-25 (***E***), BG-26 (***F***) в 1% гелі агарози. Лінія **1** – пДНК + 0,1% полімер; **2** – пДНК + 0,03% полімер; **3** – пДНК + 0,01% полімер; **4** – пДНК + 0,003% полімер; **5** – інтактна пДНК. Білий прямокутник вказує на комплекс полімер/пДНК

All studied poly-DMAEMA carriers at concentration of 0.03–0.1% were able to form complexes with plasmid DNA.

**Transformation of *Ceratodon purpureus* moss.** We adapted the protocol of PEG-mediated transformation of moss protoplasts to transform the *C. purpureus* moss protoplasts using poly-DMAEMA carriers. We were not able to obtain moss transformants by application of the conventional PEG-based transformation method [4] (**Fig. 3*G***). Three stable transformants of *C. purpureus* were obtained using BGA-22 polymer, 2 clones – when using BGA-21 carrier, and 1 clone – when using BGA-22(2ph), BG-24, BG-25, BG-26 polymers (**Fig. 3, Table 2**).

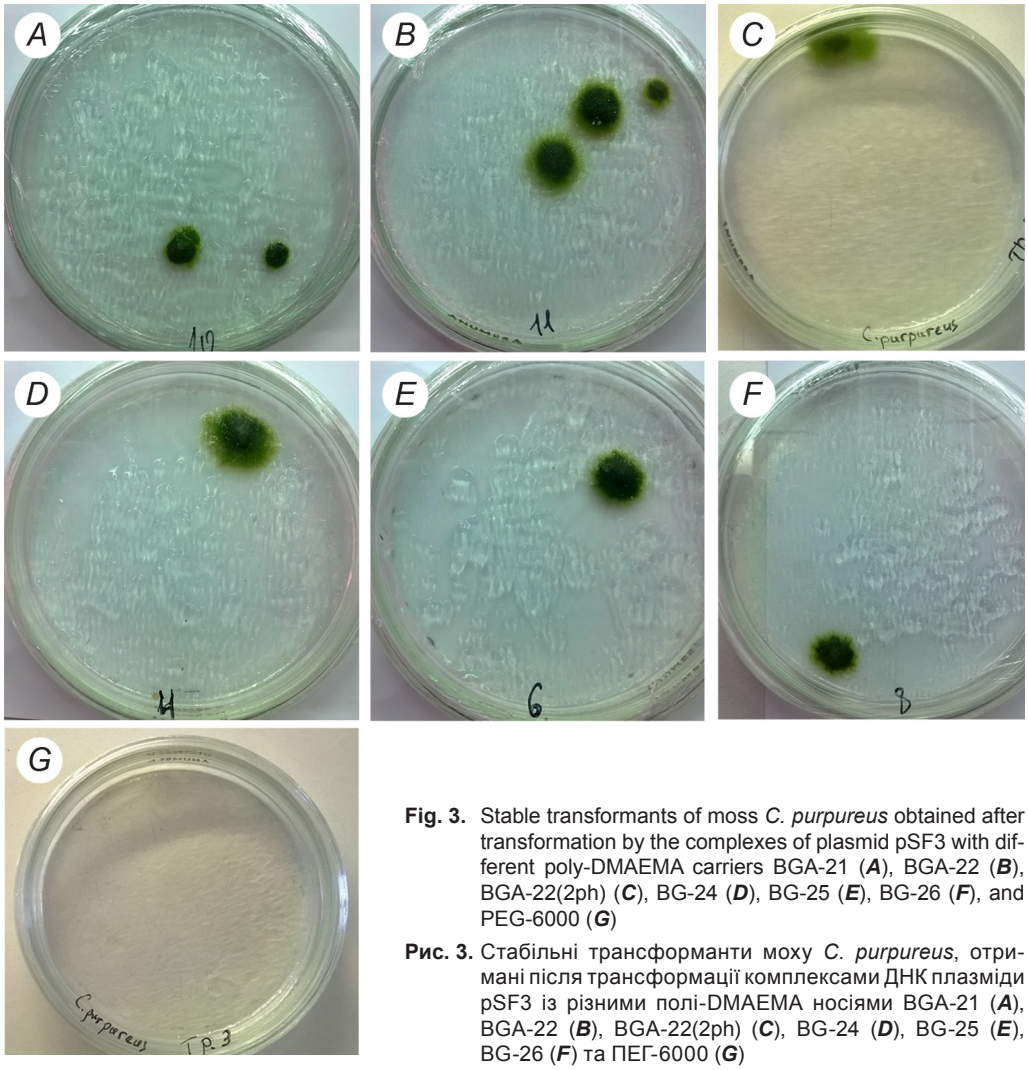


**Fig. 3.** Stable transformants of moss *C. purpureus* obtained after transformation by the complexes of plasmid pSF3 with different poly-DMAEMA carriers BGA-21 (***A***), BGA-22 (***B***), BGA-22(2ph) (***C***), BG-24 (***D***), BG-25 (***E***), BG-26 (***F***), and PEG-6000 (***G***)

**Рис. 3.** Стабільні трансформанти моху *C. purpureus*, отримані після трансформації комплексами ДНК плазміди pSF3 із різними полі-DMAEMA носіями BGA-21 (***A***), BGA-22 (***B***), BGA-22(2ph) (***C***), BG-24 (***D***), BG-25 (***E***), BG-26 (***F***) та ПЕГ-6000 (***G***)

Thus, poly-DMAEMA carriers BGA-21, BGA-22, BGA-22(2ph), BG-24, BG-25, and BG-26 were able to deliver plasmid DNA pSF3 into protoplasts of *C. purpureus*.

*Table 2.* **The effectiveness of the transformation of *C. purpureus* moss protoplasts using poly-DMAEMA and PEG-6000 as pDNA carriers**

*Таблиця 2.* **Ефективність трансформації протопластів моху *C. purpureus* із використанням полі-ДМАЕМА і ПЕГ-6000 як носіїв пДНК**

| Polymer | The number of transformants |
|---|---|
| BGA-22 | 3 |
| BGA-21 | 2 |
| BGA-22(2ph) | 1 |
| BGA-24 | 1 |
| BGA-25 | 1 |
| BGA-26 | 1 |
| PEG-6000 | 0 |

**Toxicity of polymers towards protoplasts of *C. purpureus.*** All poly-DMAEMA carriers demonstrated a slight decrease in the level of viability of *C. purpureus* protoplasts (**Fig. 4**).

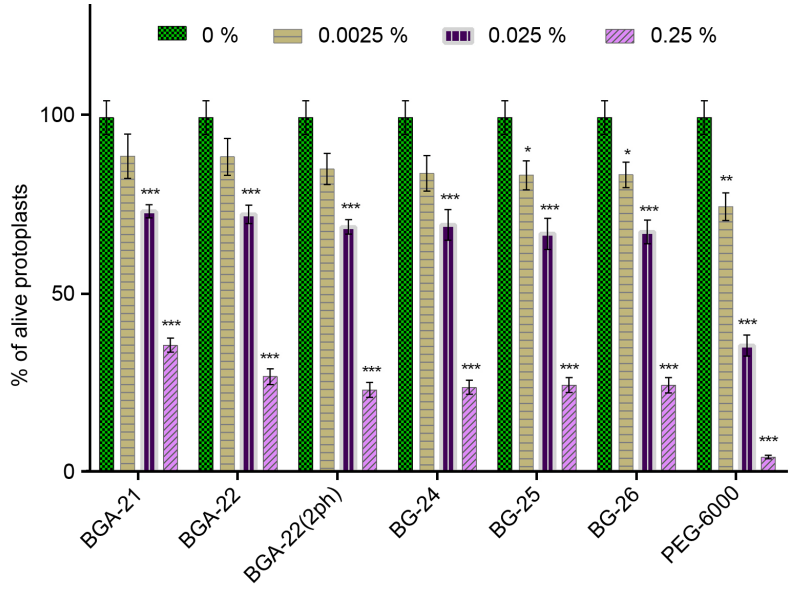


**Fig. 4.** The effect of poly-DMAEMA carriers and PEG-6000 at different concentrations on viability of *C. purpureus* protoplasts

**Рис. 4.** Вплив полі-ДМАЕМА носіїв і ПЕГ-6000 у різних концентраціях на життєздатність протопластів *C. purpureus*

The levels of viable protoplasts after treatment with BGA-21, BGA-22 and BGA-22(2ph), BG-24, BG-25 and BG-26 at the concentration of 0.0025%, that was used for transformation of *C. purpureus* protoplasts, was of 88.4, 88.2, 84.8, 83.5, 83.1 and 83.1%, respectively (**Fig. 4**). A survival ratio of protoplasts reached 66.7–72.9% under the effect of these polymers at 0.025% (10 times higher than working concentration).

The polymers at 0.25% (100 times higher than working concentration) decreased the viability of protoplasts to 23.1–35.6%. The most pronounced cytotoxic effects were found for PEG-6000. PEG-6000 used at 0.0025, 0.025 and 0.25% decreased moss *C. purpureus* protoplasts viability to 74.3, 35.5 and 4.3%, respectively (**Fig. 4**). The $IC_{50}$ value of BGA21 was 0.164%; 0.135 – for BGA-22; 0.119 – for BG-24; 0.117 – for BG-26 and BGA-22(2ph); 0.113 – for BG-25. The $IC_{50}$ value of PEG-6000 was 0.016%.

Different studies reported that modification of polymers with PEGylation substantially reduce the toxicity of carriers against mammalian cells [14, 16, 21, 22, 25]. Here, we found that the efficiency of PEG-containing DMAEMA-based polymers as gene delivery vectors for transformation of moss protoplasts was relatively low. The transformation efficiency of PEG-containing poly-DMAEMA carriers was not associated with the increased cytotoxicity of these carries. Previously, we found that poly-DMAEMA carriers did not demonstrate a genotoxic action in the ana-telophase test in *A. cepa,* and in the Ames test (with and without metabolic activation) [10]. One can speculate that PEG-containing poly-DMAEMA carriers form highly stable complexes with pDNA that reduced the dissociation of the polyplex. Previously, we demonstrated that linear DMAEMA-based polymers were more effective for plant transformation. 25 stable transformants of *C. purpureus* were obtained using TN 83/6 and 26 stable transformants – using TN 84/5. At the same time, a PEG-containing carrier DLM-9-DM was considerably less effective in *C. purpureus* transformation [8].

## CONCLUSION

The studied poly-DMAEMA carriers form complexes with plasmid DNA. Poly-DMAEMA carriers are capable of delivering plasmid DNA pSF3 into protoplasts of *C. purpureus* moss. The poly-DMAEMA carriers at working concentration were relatively non-toxic for protoplasts of *C. purpureus* moss. The poly-DMAEMA carriers at 0.113–0.164% (approximately 45 times higher concentration than that used for moss transformation) reached their $IC_{50}$ value. Thus, novel synthetic poly-DMAEMA carriers demonstrate their prospects as a system for the delivery of plasmid DNA into plant cells.

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## COMPLIANCE WITH ETHICAL STANDARDS

**Conflict of Interests:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Human Rights**: This article does not contain any studies with human subjects performed by any of the authors.

**Animal Studies**: This article does not contain any studies with animal subjects performed by the any of the authors.

## AUTHOR CONTRIBUTIONS

Conceptualization, [RSS, ASZ, NSF]; methodology, [RSS, ASZ, OVL, NSF, NEM]; polymers synthesis and characterization, [NEM, ASZ]; moss culture, [OVL, NSF]; DNA

electrophoresis, moss transformation, toxicity study, [NSF]; data analysis, [NSF, OVL, NEM, ASZ, RSS]; writing – original draft preparation, [NSF]; writing – review and editing, [RSS, NSF, OVL, NEM, ASZ]; visualization, [NSF]; supervision, [RSS, ASZ]; funding acquisition, [RSS, NSF].


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## ВИКОРИСТАННЯ ПОЛІ(ДИМЕТИЛАМІНО) ЕТИЛМЕТАКРИЛАТ-ВМІСНИХ НОСІЇВ ДНК ПЛАЗМІДИ ДЛЯ ТРАНСФОРМАЦІЇ МОХУ *CERATODON PURPUREUS*

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**Обґрунтування**. Генна інженерія рослин є перспективною галуззю в сільському господарстві, біотехнології, медицині. На сьогодні наноматеріали є привабливими системами для генетичної інженерії рослин. Метою представленої роботи було визначити потенціал полімерів гребінчастої будови на основі полі(2-диметиламіно) етилметакрилату (ДМАЕМА) як носіїв для введення генів у протопласти моху *Ceratodon purpureus* (Hedw.) Brid. та оцінити рівень фітотоксичності цих полімерів.

**Матеріали та методи.** Електрофорез ДНК проводили для вивчення комплексоутворення полі-ДМАЕМА носіїв і плазмідної ДНК pSF3. Адаптований протокол ПЕГ-трансформації використано для трансформації протопластів моху *C. purpureus* за допомогою полі-DMAEMA носіїв. Для оцінювання токсичності полімерів щодо протопластів моху застосовано світлову мікроскопію. Загальну токсичність полімерів оцінювали за показником $IC_{50}$.

**Результати і обговорення.** Формування комплексу ДНК плазміди pSF3 з носіями полі-ДМАЕМА виявлено за дії полімерів BGA-21, BGA-22(2ph), BG-24, BG-25, BG-26 у дозі 0,03 %, а полімера BGA-22 – у дозі 0,1 %. Полі-ДМАЕМА носії здатні доставити плазмідну ДНК pSF3 у протопласти моху *C. purpureus*. Три стабільних

трансформанти моху *C. purpureus* отримано з використанням полімера BGA-22, два клони – за використання носія BGA-21 і один клон – за використання полімерів BGA-22(2ph), BG-24, BG-25, BG-26. Полі-ДМАЕМА носії за робочої концентрації (0,0025 %) проявили низьку токсичність для протопластів моху *C. purpureus*. Виявлено 83,1–88,4 % життєздатних протопластів моху *C. purpureus* за впливу досліджених носіїв. Показник виживання протопластів сягав 66,7–72,9 % за дії полімерів у концентрації 0,025%, яка в 10 разів перевищувала робочу концентрацію, використану для трансформації протопластів моху. $IC_{50}$ полі-ДМАЕМА носіїв сягав 0,113–0,164 % за концентрації, яка у 10 разів перевищувала $IC_{50}$ традиційного носія – ПЕГ-6000.

**Висновок.** Нові синтетичні полі-DMAEMA носії здатні доставляти ген інтересу у протопласти моху *C. purpureus* і водночас мають фітотоксичність. Отже, полі-ДМАЕМА носії є перспективними для доставки генів у клітини рослин.

***Ключові слова:*** полі(2-диметиламіно)етилметакрилат, полімерний носій, трансформація протопластів, мох *Ceratodon purpureus,* фітотоксичність

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