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Transformation of the moss (*Ceratodon purpureus*) with plasmid DNA delivered by novel block-copolymers of the dimethylaminoethyl methacrylate

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Aim. To investigate the potential of poly(2-dimethylamino)ethyl methacrylate (DMAEMA)-based block-like polymers to serve as gene delivery carriers in moss *Ceratodon purpureus* (Hedw.) Brid. protoplasts, and to evaluate the level of their phytotoxicity. **Methods.** Organic synthesis; DNA gel retardation assay; adapted PEG-mediated transformation protocol; PCR; light microscopy. **Results.** The formation of pDNA complex with DMAEMA-based carriers took place at 0.01-0.1 % concentrations of the polymer. The poly-DMAEMA carriers F8-DM1, F8-DM2 (fluorine-containing), LAcr-DM1, LAcr-DM2 (lauryl acrylate-containing), BAcr-DM1, and BAcr-DM2 (butyl acrylate-containing) were effective as carriers of plasmid DNA pSF3 at *C. purpureus* transformation. PCR analysis confirmed that the transformants of *C. purpureus* moss contain *GFP* as a gene of interest after the protoplast transformation by polymers LAcr-DM2, LAcr-DM1, BAcr-DM2, BAcr-DM1 and F8-DM2. The poly-DMAEMA carriers at working concentration (0.0025 %) were relatively non-toxic for protoplasts of *C. purpureus* moss. 83.1-93.9 % of viable protoplasts of *C. purpureus* moss were found after the treatment with studied carriers at that dose. However, at 0.25 % i.e. 100 times higher concentration than that used for moss transformation, the poly-DMAEMA carriers reached their IC_{50} level. **Conclusion.** The novel block-like poly-DMAEMA carriers were effective in transformation of *C. purpureus* moss protoplasts and demonstrated low toxicity.

K e y w o r d s: polymeric carrier, poly(2-dimethylamino)ethyl methacrylate, moss *Ceratodon purpureus,* protoplasts transformation, toxicity.

Introduction

Genetic engineering of plants is crucial at crop engineering to achieve higher yields and resistance to different environmental stresses and diseases [1, 2], as well as to meet the energy demands [3] and to provide pharmaceutical manufacturing of recombinant proteins and small-molecular drugs [4, 5]. The *Agrobacterium*-mediated delivery is the most commonly used tool for gene delivery into plants [6]. The biolistic particle delivery with "gene gun", PEG-mediated, chemical-based methods are also used for plant transformation [7–9]. The plant viral vectors (e.g. tobacco mosaic virus-based) could be applied for the plant transformation [5].

The plant cell wall is a main barrier for exogenous gene delivery to plants. Current delivery methods are limited by low transformation efficiencies, cell/tissue damage or unavoidable DNA integration into the host genome [5, 10]. The viral vectors are effective only for selected plant species and limited by the content and size of the expression cassette [5, 11].

Nanomaterials are intensively studied as the carriers for gene delivery in animal cells [12–15]. However, their potential for plants remains understudied [1, 5]. Thus, mesoporous silica nanoparticles (MSNs) [16], carbon (CNTs) [17], silicon carbide whiskers (SCWs) [5], gold nanoclusters [18] and nanosheets [19] have been reported for gene delivery to plant cells. Noteworthy, the application of the reported carriers could be limited due to their reduced bio-compatibility [1, 20]. Polymethacrylates are widely used for gene delivery into mammalian cells [21–23]. The poly-DMAEMA-based polymers were found to be efficient gene transfer carriers into the mammalian [24–26], yeasts [27], and plants [28].

Genetic engineering of mosses might serve multiple purposes, such as identifying molecular mechanisms of environmental adaptation of mosses and formation of biologically active products. *Ceratodon purpureus* is a moss used as a model system in genetic and developmental studies [29, 30].

Present study was aimed at investigation of the potential of DMAEMA-based block polymers to serve as gene delivery carriers in the protoplasts of the model species *Ceratodon purpureus* moss and at evaluation of cytotoxicity.

Materials and Methods

DMAEMA-based block polymers

Materials used for synthesis of polymers: 2-(dimethylamino)ethyl methacrylate (DMAEMA), 2-aminoethyl methacrylate hydrochloride (AEM), vinyl acetate (VA), maleic anhydride (MA), butyl acrylate (BA), lauryl acrylate (LA), isopropylbenzene (IPB) were from Aldrich (Milwaukee, WI, USA). Fluorine-containing monomer (octafluoropentyl methacrylate (F8MA)) was synthesized at the Institute of Organic Chemistry of NAS of Ukraine and used without further purification. N,N-Dimethylformamide (DMF), 1,4-dioxane, acetone and n-hexane were obtained from Merck (Darmstadt, Germany).

Novel DMAEMA-containing block-copolymers were synthesized at the Department of Organic chemistry of Lviv Polytechnic

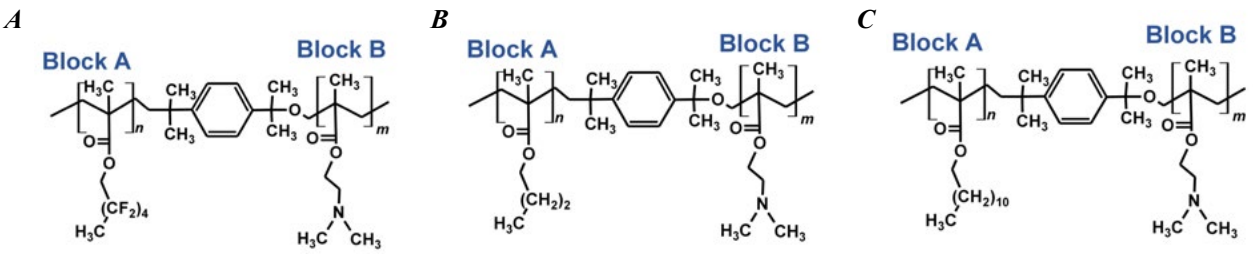

Fig. 1. Schematic structure of the polymeric carriers F8-DM1, F8-DM2 (**A**), BAcr-DM1, BAcr-DM2 (**B**), LAcr-DM1, LAcr-DM2 (**C**).

National University (Lviv, Ukraine). The block-copolymers were synthesized in two stages: 1) synthesis of polyfunctional macroinitiators (telechelic oligoperoxides — polyalcyl acrylate or fluorinated poly(meth)acrylate with terminal peroxide groups), and 2) synthesis by grafting radical polymerization of the polycationic polymer chains [24, 31]. The structures and compositions of polymers are shown in Figure 1 and Table 1.

Complex polymer/pDNA formation. The polymer/plasmid DNA complexes were prepared as described previously [28] by adding the polymer (1 μL) at increasing concentrations (0.003–0.1 %) to the plasmid DNA (pDNA, 1 μg) pEGFPc-1 (Clontech, USA) in 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. The bands of plasmid DNA were visualized with UV transilluminator (MacroVue UV-20, Hoeffer, USA).

Transformation of moss protoplasts. Moss *Ceratodon purpureus* (obtained from the collection at the Institute of Ecology of the Carpathians, National Academy of Sciences of Ukraine, Lviv, Ukraine) was cultured at 24–26 °C in solid Knop medium with light intensity of 5–20 W/m^2 with alternate 16/8 h day and night cycle. The culture of *C. purpureus* moss was initiated from the spores. Protoplasts of *C. purpureus* were isolated by a digestion of cell wall of 100 mg of protonemal tissue

Table 1. **Composition of block-copolymers and their molecular-mass characteristics**

Polymers	Block A					Block B	M_n, kDa
	F8MA	BA	LA	MP_{rest}	$M_{n(A)}$, kDa	DMAEM	
F8-DM1	37.3	-	-	0.96	4.7	61.7	12.6
F8-DM2	26.7	-	-	0.69	4.7	72.6	17.6
BAcr-DM1	-	49.3	-	0.5	6.5	50.0	13.0
BAcr-DM2	-	37.4	-	0.4	6.5	62.1	17.2
LAcr-DM1	-	-	26.0	0.6	6.0	73.2	18.0
LArc-DM2	-	-	12.2	0.4	6.0	87.3	34.0

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 8 % D-mannitol (Sigma-Aldrich, USA) nylon filter. The tube with filtrated protoplasts was centrifuged at 200×g for 5 min. The pellet was gently resuspended in 10 mL of 8 % D-mannitol. The number of protoplasts was counted using Neubauer chamber [9]. The 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 1.6 $\times 10^6$/mL concentration.

Plasmid DNA pSF3 [28] that contained the gene of green fluorescent protein and the gene of Hygromycin B resistance, was used to transform the protonema of *C. purpureus* moss. The modified PEG-mediated transformation method, developed for moss *P. patens,* was used to transform the protoplasts of *C. purpureus* [32]. The pDNA complexes with poly-DMAEMA carrier (1 μL of pDNA and 2.5 μL of 0.1 % polymer) or with PEG-6000 (1 μL of pDNA and 2.5 μL of 40 % PEG-6000, LobaChemie, Austria) were used. The complexes were added to 0.1 mL of protoplasts suspension (1.6 $\times 10^6$/mL) in 8 % of D-mannitol (Sigma-Aldrich, USA) (the final concentration of polymeric carriers was 0.0025 %). The tubes with transformed protoplasts were transferred 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 containing 0.6 % agar (approximately 37–42 °C) and placed on Petri dishes with solid PRMB medium containing 0.5 % glucose. The regenerants were transferred on a selective medium containing Hygromycin B (50 μg/mL, Sigma-Aldrich, USA) for 9 days at 24–26 °C with light intensity of 5–20 W/m^2 with alternate 16/8 h day and night cycle. The regenerants were cultivated for the next 14 days on the medium without Hygromycin B antibiotic. After that, they were sub-cultivated for next 8 weeks on the medium with or without Hygromycin B antibiotic in order to obtain stable transformants of *C. purpureus* moss [28].

Molecular-genetic analysis of transformants of C. purpureus moss with PCR. To verify the presence or absence of the transferred gene of interest in the obtained transformants, PCR analysis was performed. DNA from transgenic plants was isolated after 8 weeks of cultivation according to the described method [32]. The primers VN10 (forward) and VN11 (reverse) (Genomed, Poland) were used to detect the transgenesis of the *GFP-PTS1* chimeric gene (Table 2).

Table 2. **The nucleotide sequence of the primers used in this work**

Primer	5'-3' oligonucleotide sequence of primers
VN10	AAGAATTCATGGTGAGCAAGGGCGAG
VN11	AAAGCGGCCGCTGCAGATCTGAGTACTTGT

PCR analysis was performed under the following conditions: 1 cycle at 94 ºC for 5 min; 30 cycles at 94 ºC for 30 s, 57 ºC for 30 s, 72 ºC for 80 s; 1 cycle at 72 ºC for 1 min. PCR products were analyzed by electrophoresis in 1 % agarose gel [33].

Toxicity of polymers for moss protoplasts. Protoplasts of *C. purpureus* moss were isolated, as described previously. The effects of polymeric carriers and PEG-6000 on *C. purpureus*

protoplasts were studied in the following range of the final concentrations of carriers: 0.0025 (effective concentration), 0.025 and 0.25 %. Polymers were added to 0.1 mL aliquots of protoplasts suspension (1.6 $\times 10^6$/mL) in 8 % of D-mannitol and gently mixed. Protoplasts were co-cultivated with carriers for 24 h at 24–26 °C with light intensity of 5–20 W/m^2 with alternate 16/8 h day and night cycle. The cytotoxicity of polymers was examined via calculating the amount of normal and damaged protoplasts using Neubauer chamber. The level of polymer toxicity was evaluated using IC_{50} value (the concentration of polymer that reduced protoplasts viability by 50 %).

The obtained data are presented as Mean (M) ± Standard deviation (SD) from three replications. Statistical analysis was performed using one-way ANOVA test at GraphPad Prism 6 software. P value < 0.05 was considered as statistically significant.

Results and Discussion

pDNA binding properties of studied polymers

The electrostatic interactions between positively charged groups of the carrier and negatively charged phosphate groups of nucleic acid are important for the DNA (RNA or oligonucleotides) condensation and efficient gene delivery [21–23, 34].

The gel retardation assay was used to confirm the formation of poly-DMAEMA carrier

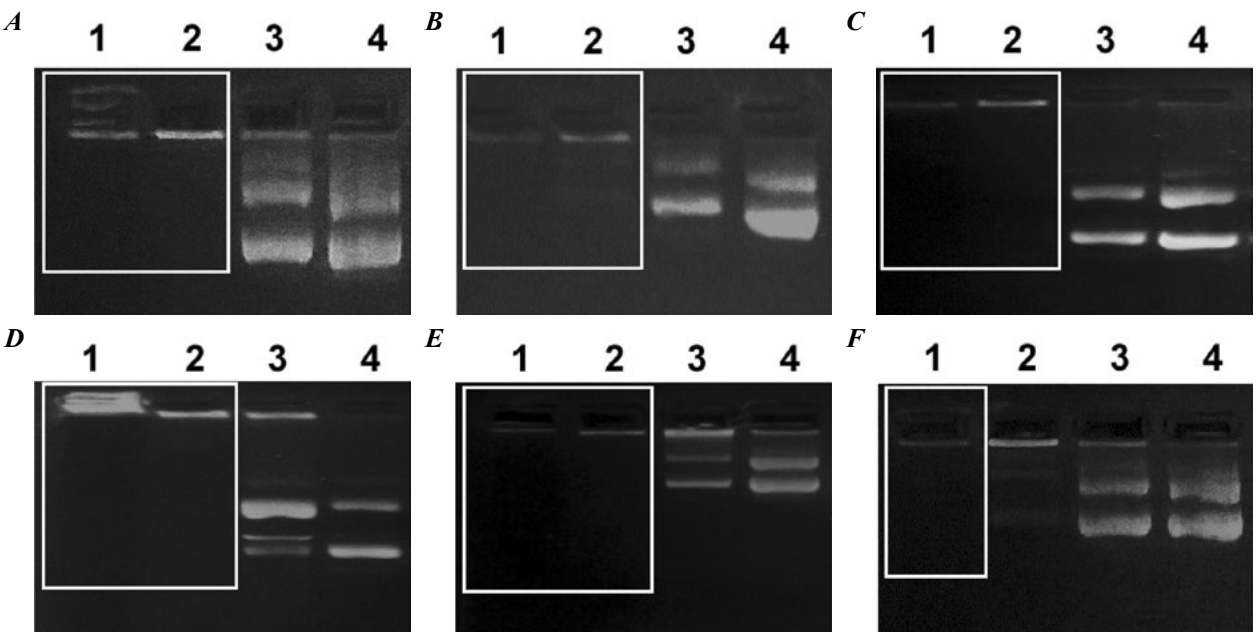

Fig. 2. Gel retardation assay of the polymeric carriers: F8MA-DM1 (**A**), F8MA-DM2 (**B**), LA-DM1 (**C**), LA-DM2 (**D**), BA-DM1 (**E**), BA-DM2 (**F**) and plasmid DNA pEGFPc-1 complexes in 1 % agarose gel. Line 1 — pDNA mixed with polymer at 0.1 %; 2 — pDNA + polymer at 0.01 %; 3 — pDNA polymer + polymer at 0.001 %; 4 — intact pDNA. The white rectangles indicate the plasmid DNA that was bound with polymeric carrier.

and plasmid DNA pEGFPc-1 complex. Plasmid DNA pEGFPc-1 migrated during electrophoresis in the agarose gel (lane 4 of **Fig. 2**). The electrophoretic mobility of pDNA was hold back with an increase of the poly-DMAEMA carrier concentration.

Poly-DMAEMA carriers F8MA-DM1, F8MA-DM2, LA-DM1, LA-DM2, BA-DM1 at 0.01 % retarded the electrophoretic mobility of pDNA. We assumed that the formation of pDNA complex with polymers F8MA-DM1, F8MA-DM2, LA-DM1, LA-DM2, BA-DM1

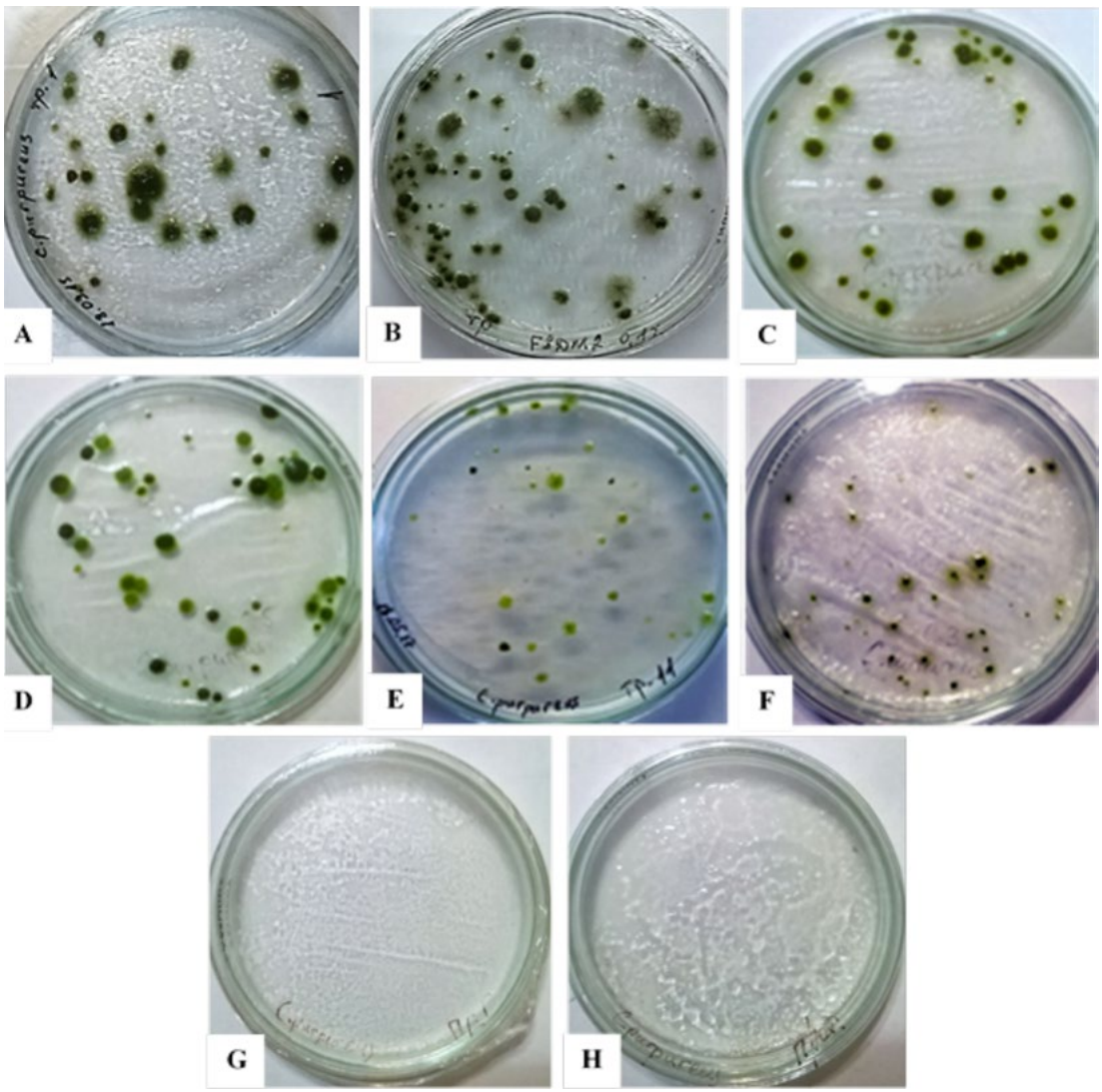

Fig. 3. Stable transformants of moss *C. purpureus* obtained after treatment of protoplasts with plasmid DNA pSF3 in complex with different poly-DMAEMA carriers: F8MA-DM1 (**A**), F8MA-DM2 (**B**), LA-DM1 (**C**), LA-DM2 (**D**), BA-DM1 (**E**), BA-DM2 (**F**), PEG-6000 (**G**), and DNA-minus sample (**H**).

took place at 0.01 % concentration of the polymer (**Fig. 2A-E**). Polymer BA-DM2 at 0.1 % concentration fully retarded the electrophoretic mobility of pDNA (lane 1 of **Fig. 2F**), and this carrier at 0.01 % partly induced retardation of electrophoretic mobility of pDNA (lane 2 of **Fig. 2F**). BA-DM2 at 0.1 % forms the complex with pDNA.

Thus, the studied poly-DMAEMA carriers are capable of forming the complexes with plasmid DNA.

Moss (Ceratodon purpureus) transformation

We have adapted the protocol of PEG-mediated transformation of moss (*C. purpureus)* protoplasts using poly-DMAEMA carriers. The same number of protoplasts (1.6 $\times 10^6$/mL) was taken for transformation with each carrier. No moss transformants were received when using conventional PEG-based transformation protocol (**Fig. 3G**). However, we obtained 75 stable transformants of *C. purpureus* moss using F8MA-DM2 carrier, 26 transformants — using F8MA-DM1, 41 — using LAcr-DM2, 38 — using LAcr-DM1, 33 — using BAcr-DM2, 28 — using BAcr-DM1 (**Fig. 3, Table 3**).

Table 3. **The effectiveness of transformation of moss** *C. purpureus* **using different poly-DMAEMA carriers and PEG-6000**

Polymer	The amount of stable transformants
F8-DM-2	75
LAcr-DM2	41
LAcr-DM1	38
BAcr-DM2	33
BAcr-DM1	28
F8-DM1	26
PEG-6000	0

The PCR analysis confirmed the presence of the gene of interest — *GFP* — in transformants of *C. purpureus* moss at using LAcr-DM2, LAcr-DM1, BAcr-DM2, BAcr-DM1, F8-DM1, and F8-DM2 carriers of plasmid DNA (**Fig. 4**).

Thus, the poly-DMAEMA carriers F8-DM1, F8-DM2, LAcr-DM1, LAcr-DM2, BAcr-DM1, and BAcr-DM2 were effective carriers of plasmid DNA at transformation of protoplasts of *C. purpureus* moss. The PCR-analysis confirmed that transformants of *C. purpureus* moss contained a gene of interest *GFP* after protoplast transformation with the studied poly-DMAEMA carriers. Thus, poly-DMAEMA carriers possess the structure providing a possibility of surface adjustment for excellent self-assembly [35, 36].

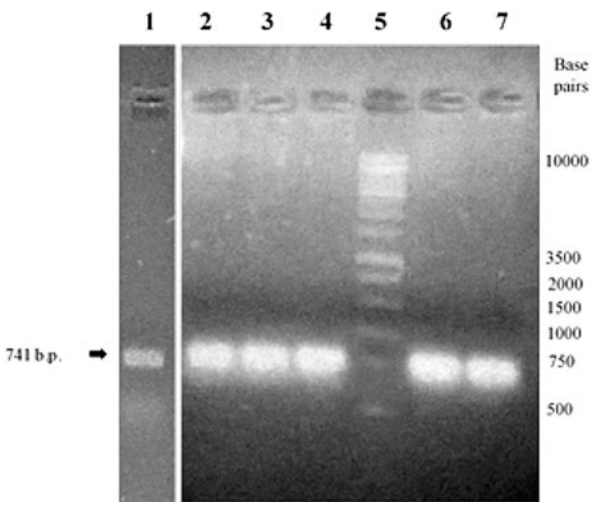

Fig. 4. Electrophoregram of the products of PCR analysis of *C. purpureus* moss transformants: **1** — delivery of pDNA with LAcr-DM2 carrier; **2** — LAcr-DM1; **3** — BAcr-DM2; **4** — BAcr-DM1; **5** — markers of molecular weights; **6** — delivery of pDNA with F8-DM2; **7** — F8-DM1 carrier.

It was found that poly-DMAEMA carriers with higher amount of DMAEMA blocks (marked as DM2) were more effective for transformation of moss protoplasts. Synatschke *et al.* reported that the polymers with a branched architecture and an intermediate molecular weight were more prominent for the gene delivery, since they combined low cytotoxicity with acceptable transfection efficiency [37]. Besides, the fluorinated polymers are characterized as hydrophobic and lipophobic carriers with high chemical stability, bio-inertness, and low surface energy [38, 39].

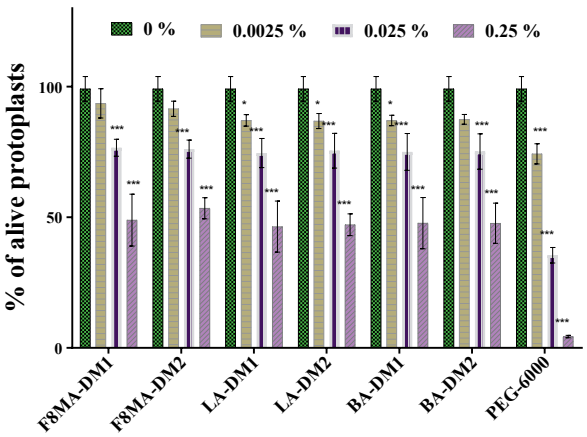

Fig. 5. Effect of polymeric carriers used at different concentrations on the viability of protoplasts of *C. purpureus* moss.

Toxicity of polymers towards protoplasts of C. purpureus moss

The poly-DMAEMA carriers under study demonstrated moderate toxicity for the protoplasts of *C. purpureus* moss (**Fig. 5**).

The poly-DMAEMA carriers F8MA-DM2, F8MA-DM1, LAcr-DM2, LAcr-DM1, BAcr-DM2, and BAcr-DM1 used in 0.0025 % working concentration were relatively non-toxic for protoplasts of *C. purpureus*. 87.1–93.9 % of viable protoplasts of *C. purpureus* moss were detected after treatment. The poly-DMAEMA carriers used at 0.025 % (10 times higher concentration than that used for moss transformation) caused a reduction of alive protoplasts amount to 74.7–76.6 %. At the same time the poly-DMAEMA carriers used at 0.25 % dose reached their IC_{50} value and only 46.5–53.5 % of alive protoplasts of *C. purpureus* moss were found (**Fig. 5**). The most pronounced cytotoxic effects were demonstrated by the PEG-6000. When used at 0.0025, 0.025 and 0.25 % doses, a decrease in the viability of protoplasts of *C. purpureus* moss to 74.3, 35.5 and 4.3 %, respectively, was detected (**Fig. 5**). Several researchers reported that fluorine-containing polymers possessed a reduced toxicity toward targeted cells [40, 41]. We did not find a significant difference in the toxicity of the butyl acrylate-, lauryl acrylate-, and fluorine-containing poly-DMAEMA carriers for the protoplasts of *C. purpureus* moss.

Conclusion

The poly-DMAEMA carriers caused an efficient condensation of plasmid DNA into stable complexes. We found that such carriers noted as F8-DM1, F8-DM2, LAcr-DM1, LAcr-DM2, BAcr-DM1, and BAcr-DM2 were effective in transformation of *C. purpureus* moss with plasmid DNA. PCR-analysis confirmed that transformants of *C. purpureus* moss contained the gene of interest, namely *GFP,* after protoplast transformation with the synthetic polymers LAcr-DM2, LAcr-DM1, BAcr-DM2, BAcr-DM1, F8-DM2, and F8-DM1. The poly-DMAEMA carriers at the effective concentration were relatively non-toxic for protoplasts

of *C. purpureus* moss. However, the poly-DMAEMA carriers used at 0.25 % dose, that is 100 times higher concentration than that used for moss transformation, reached their IC_{50} value. Thus, the novel synthetic poly-DMAEMA carriers demonstrated their potential as a promising system for delivering plasmid DNA into plant cells, and the applied approach seems to be beneficial in plant biotechnology.

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Трансформація моху *Ceratodon purpureus* плазмідною ДНК, доставленою новими блок-кополімерами диметиламіноетил метакрилату

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Мета. Вивчити здатність полімерів блочної будови на основі полі(2-диметиламіно) етилметакрилату (ДМАЕМА) слугувати носіями генів для трансформації протопластів моху *Ceratodon purpureus* (Hedw.) Brid. та оцінити їхню токсичність. **Методи.** Органічний синтез; електрофорез ДНК; адаптований протокол ПЕГ-трансформації; ПЛР-аналіз; світлова мікроскопія. **Результати.** Формування комплексу ДНК плазміди з полі-ДМАЕМА носіями виявлено за 0,01–0,1 % концентрації полімерів. Полі-DMAEMA носії F8-DM1, F8-DM2 (містять фтор у структурі), LAcr-DM1, LAcr-DM2 (містять лаурилакрилат), BAcr-DM1 та BAcr-DM2 (містять бутилакрилат) були ефективними носіями плазмідної ДНК pSF3 для трансформації моху *C. purpureus*. За допомогою ПЛР-аналізу підтверджено, що трансформанти цього моху містять ген інтересу *GFP* після трансформації протопластів комплексом полі-DMAEMA з ДНК плазміди pSF3. Носії полі-ДМАЕМА за ефективної концентрації (0,0025 %) проявили низьку токсичність для протопластів моху *C. purpureus*. Виявлено 83,1–93,9 % життєздатних протопластів цього моху за дії досліджуваних носіїв. Проте, за концентрації 0,25 %, яка у 100 разів перевищувала концентрацію, використану для трансформації моху, досліджувані полі-ДМАЕМА носії досягали IC_{50}. **Висновки.** Нові блок-подібні полі-DMAEMA носії були ефективними для трансформації протопластів моху *C. purpureus* і при цьому демонстрували низьку токсичність.

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

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