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# Glycopolymer-*graft*-polypeptide copolymers as potential carriers for nucleic acids

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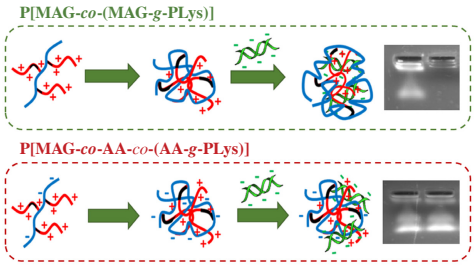
**Bioinspired copolymers consisting of a glycopolymer main chain grafted with polylysine were synthesized using metal-free click chemistry based on a 1,3-dipolar cycloaddition reaction involving strained cyclic alkyne. Poly(2-deoxy-2-methacrylamido-D-glucose)-*graft*-polylysine was efficiently complexed with a double-stranded oligonucleotide duplex. The cytotoxicity of the polyplex was lower than that of the free copolymer.**

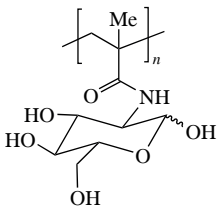



*Keywords*: bioinspired copolymers, synthetic glycopolymers, polypeptides, graft copolymers, metal-free click chemistry, delivery systems, nucleic acids.

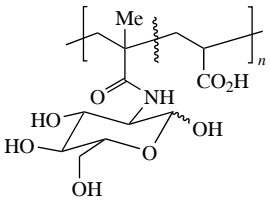
Gene therapy opens a prospect to treat cancer, inherited genetic diseases, diseases associated with hyperexpression of certain genes, *etc*.[1] Since free therapeutic RNA/DNA macromolecules are unstable and cannot independently penetrate into target cells, the development of carriers for gene delivery is one of the urgent tasks of modern biomedicine.[2] Synthetic and natural cationic polymers are widely studied as non-viral delivery systems since they are able to form interpolyelectrolyte complexes (IPECs) with negatively charged nucleic acid.[3–8] Among the bioinspired polymers for nucleic acid delivery, synthetic cationic polypeptides are of particular interest.[9] To enhance cell entry, drug delivery systems can be further modified with specialized peptides or small vectors.[10,11]

A significant disadvantage of any cationic polymers is their excessive cytotoxicity.[11] The latter is determined by a number of factors such as the p$K_a$ of monomer units, the degree of polymerization, and the architecture of macromolecule (linear, branched, *etc*.).[12,13] Graft copolymers can provide the same high transfection efficiency as hyperbranched copolymers such as b-PEI, but have less cytotoxicity. Typically, graft copolymers used for gene delivery contain rather short grafted cationic chains, which by themselves have low transfection efficiency and low cytotoxicity.[14,15] When these short chains are grafted onto the polymer backbone, the delivery characteristics are improved by increasing the charge density.[15]

To shield the surface positive charge, copolymerization of polycations with neutral biocompatible polymers is a common approach.[16] Similar to PEG,[17] neutral glycopolymers can ensure the stability of IPECs in body environment (blood plasma) and protect the polyplex from the entrapment by macrophages (stealth effect).[18] Considering this, we proposed the synthesis of graft copolymer consisting of a neutral glycopolymer, namely, poly(2-deoxy-2-methacrylamido-D-glucose) (PMAG) as a main chain playing a shielding role, and grafted cationic polylysine responsible for the binding with nucleic acids. PMAG is water-soluble and biocompatible polymer.[19] The unique properties of glycopolymers are attributed to the facilitation of carrier penetration into the cell due to the affinity of sugars to membrane lectins.[18] In particular, glucose has an affinity for cellular GLUT-1 receptors, which performs many physiological functions in the body. Specifically, it is known that GLUT-1 receptor is overexpressed in glioma cells.[20] To date, there are the results on the synthesis of various linear copolymers of MAG with cationic methacrylate monomers.[16] In addition, the synthesis of block-copolymers consisting of PMAG and poly(lysine-*co*-phenylalanine) and its evaluation as delivery system for hydrophobic drugs has been recently reported.[21] However, there are no data on the synthesis and evaluation of PMAG-*g*-polypeptide copolymers.




PMAG




P(MAG-*co*-AA)

Click reactions based on azide–alkyne 1,3-dipolar cycloaddition have been widely used for the preparation of graft copolymers.[22] However, the employment of reliable copper(I)-catalyzed (CuAAC) reaction requires the use of metal catalysts, which becomes a problem for further applications of synthesized polymers in biomedical fields. In this way, metal-free click reactions look more attractive.[23]

In this study, a 'grafting to' strategy using metal-free click chemistry was selected to synthesize PMAG-*g*-PLys. The

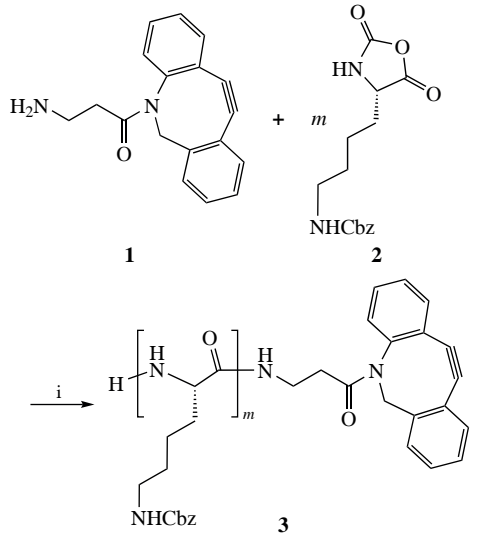
synthetic route included synthesis of PLys$^{Cbz}$ bearing a terminal substituent incorporating strained cyclic triple bond, covalent modification of homo- and copolymer MAG with a linker containing an azide group, and conjugation of PLys to PMAG by metal-free click-chemistry. PMAG used herein was obtained by free radical polymerization of MAG as described previously.[24] The used sample had the following characteristics: $M_w$ = 50 000, $M_n$ = 26 300, and $Đ$ = 1.9. Recently, it has been shown that introduction of anionic groups into a cationic polypeptide chain facilitated the release of nucleic acid from a polyplex that resulted in the higher transfection efficacy.[25] Considering this, P(MAG-*co*-AA)[26] being a copolymer of MAG with acrylic acid (AA) as a source of negative charge was also used in the study. Substance P(MAG-*co*-AA) had the following characteristics: $M_w$ = 36 000, $M_n$ = 16 200, $Đ$ = 2.2, 40 mol% of AA units. This copolymer is characterized by a heterogeneity in distribution of AA units since MAG units have much higher reactivity ratio than that for AA units[26] (for details, see Online Supplementary Materials, section S1).

As shown in Scheme 1, ring opening polymerization of *N*-carboxyanhydride of Cbz-protected lysine **2** was initiated by primary amino group[27] of compound **1**. Due to the superposition of signals of the reactant moieties, the correct calculation of the degree of polymerization from $^1$H NMR spectrum is not feasible. The weight-average molecular weight ($M_w$) and dispersity ($Đ$) of product **3** were determined by size-exclusion chromatography (SEC) regarding poly(methyl methacrylate) standards: $M_w$ = 26 000, $M_n$ = 23 000 and $Đ$ = 1.13. The calculated $M_n$ for deblocked PLys, which is presented as grafted chains in the final copolymer, is ~11 300 (degree of polymerization is ~86).

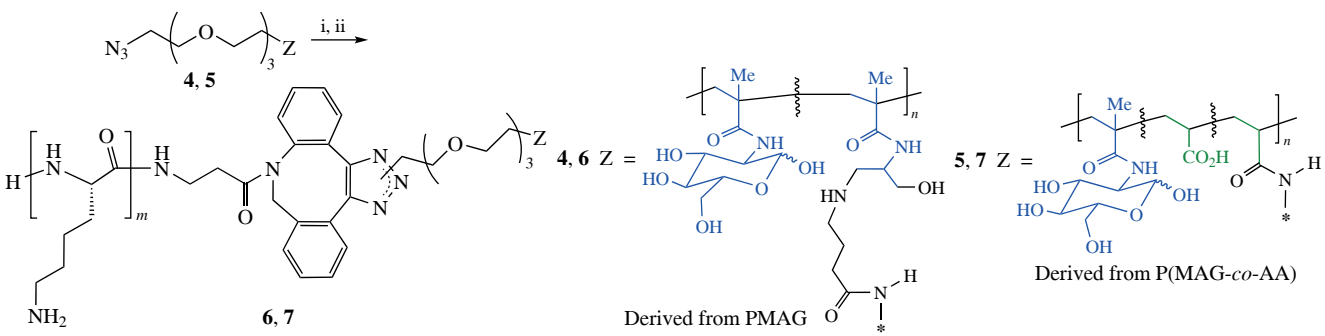
In order to introduce azide functionality into PMAG and its copolymer P(MAG-*co*-AA), they were subjected to special multistep transformations affording two key polymers **5** and **6**, respectively (Scheme 2, for synthetic details and comments see Online Supplementary Materials). These transformations involved the coupling of carboxy functionalities in the polymers with azido amine $H_2N(CH_2CH_2O)_3(CH_2)_2N_3$ to produce the corresponding azido amides. The click reactions between **4** or **5** with **3** afforded the corresponding copolymers containing Cbz protection at the lysine moiety. Their solubility in various solvents may be of note (see Online Supplementary Materials). After Lys deprotection, the solubility of the target grafted copolymer **7** in organic solvents decreased, the polymer became limitedly soluble in water with improvement after acidification (pH 2), which indicated the presence of a large number of deprotected ε-amino groups of PLys. Similarly, substance **6** became limitedly soluble in DMF, DMSO and water. As in previous case, water acidification (pH 2) improved its solubility. In order to determine the PLys content in these copolymers, hydrolysis of the samples was carried out until complete destruction of the polypeptide chains, and the hydrolysates thus obtained were quantitatively analyzed by HPLC for Lys content (see Online Supplementary Materials, section S8). The following content of Lys (equal to content of PLys) was found in the copolymers: 38 wt% (51 mol%) for **6** and 41 wt% (44 mol%) for **7**.

Direct dissolution of the polymers in water or 0.01 M sodium phosphate buffer (pH 7.4) followed by short-term ultrasonication (30 s by ultrasound probe) led to the formation of homogeneous dispersions. Analysis of the dispersions by dynamic and electrophoretic light scattering (DLS/ELS) revealed the formation of nanoparticles (Table 1). In the case of **7**, the formation of particles is driven by electrostatic interactions between negatively charged AA and positively charged Lys units. The molar ratio of Lys to AA in this graft copolymer is 1.6. Thus, at least half of the Lys units may be involved in complexation with AA units. This is the reason for the low surface zeta potential of such nanoparticles. The formation of particles from **6** may occur due to the formation of hydrogen bonds between PMAG and PLys, which promotes the association of the copolymer chains. Furthermore, the presence of hydrophobic dibenzoazocine moieties may additionally contribute to the hydrophobic interactions between the copolymer chains in an aqueous environment. The absence of oppositely charged units resulted in a higher zeta-potential value of nanoparticles formed from **6** (see Table 1).

To evaluate the ability of the obtained copolymers to form IPECs with nucleic acid, the physicochemical model of a short double-stranded RNA (siRNA) was used to obtain IPECs with copolymers. In particular, duplex of oligothymidine and oligoadenine (oligo-dT-dA) consisting of 23 base pairs was selected as a physicochemical siRNA model. The characteristics of the IPECs obtained at copolymer/oligo-dT-dA mass ratios in the range of 6–25 are presented in Table 1. The used range of mass ratios corresponded to the range of N/P ratios of 5.5–22.5 for **6** and 7–24 for **7**, respectively. In the case of **6**, IPECs with




**Scheme 1** *Reagents and conditions*: i, DMF, 30 °C, 72 h.




**Scheme 2** *Reagents and conditions*: i, alkyne **3**, DMF, 35 °C, 120 h; ii, HBr/AcOH, $CF_3CO_2H$, 20 °C, 3 h.

**Table 1** Characteristics of empty nanoparticles based on polymers **6** and **7** as well as their IPECs with oligo-dT-dA (DLS and ELS analysis, 25 °C).

| Copolymer/oligo-dT-dA (wt/wt) | $D_h$/nm | | ζ-potential/mV | |
|---|---|---|---|---|
| | in water | in phosphate buffer | in water | in phosphate buffer |
| | Graft copolymer **6** | | | |
| Only copolymer | 230 ± 20 | 240 ± 40 | 38 ± 5 | 22 ± 5 |
| 20 | – | 260 ± 70 | 1 ± 3 | 1 ± 3 |
| 15 | – | 150 ± 30 | 1 ± 3 | 2 ± 3 |
| 10 | 160 ± 20 | 150 ± 30 | 0 ± 3 | 0 ± 3 |
| 6 | – | 120 ± 40 | 0 ± 3 | 0 ± 3 |
| | Graft copolymer **7** | | | |
| Only copolymer | 650 ± 50 | 430 ± 30 | 11 ± 2 | 8 ± 2 |
| 25 | 300 ± 30 | 290 ± 50 | –15 ± 3 | –17 ± 3 |
| 20 | – | 250 ± 40 | –16 ± 3 | –18 ± 3 |
| 15 | – | 150 ± 20 | –18 ± 3 | –20 ± 3 |
| 6 | 140 ± 20 | 120 ± 20 | –21 ± 3 | –23 ± 3 |

oligo-dT-dA were formed at any of the selected copolymer/oligo-dT-dA ratios (section S10). Increasing the content of oligo-dT-dA led to compactization of the formed IPECs. In the case of **6**, ζ-potential values of all IPECs were close to or equal to zero. Since ζ-potential of empty nanoparticles was quite high positive, such a decrease after complexation with nucleic acid may indicate the presence of PLys complexed with oligo-dT-dA inside the particles, while uncharged PMAG is on the surface and well shields the charges of the polyplex core. In turn, all **7**-based IPECs had negative surface zeta-potential at all N/P ratios. This may indicate that nucleic acid is located on the surface of the IPEC or the units of acrylic acid are located on the surface when the nucleic acid is inside the complex.

Agarose gel electrophoresis was used to evaluate the ability of graft copolymers to retain the nucleic acid (section S11). As can be seen from Figure 1, oligo-dT-dA was not retained at any of the selected ratios for **7**/oligo-dT-dA IPECs. This fact indicates the low retention of the nucleic acid by copolymer due to insufficient positive charge as it is partly involved in the interaction with AA units. At the same time, the oligo-dT-dA was retained at all ratios of **6**/oligo-dT-dA IPECs due to the absence of competitive interactions.

Finally, the cytotoxicity of the more efficient **6** and its IPEC with oligo-dT-dA was evaluated in normal cells (HEK 293: human embryonic kidney cells) (see Online Supplementary Materials, section S12, Figure S8). The results of the long-term MTT assay confirmed the high cytotoxicity of cationic graft copolymer **6**. This copolymer was not toxic up to 16 μg ml$^{-1}$. At the same time, the cytotoxicity of **6** when it was in complex with nucleic acid was significantly diminished (nontoxic up to 125 μg ml$^{-1}$ regarding copolymer). This result is consistent with the measurement of surface zeta potential which is close to zero for the IPEC.

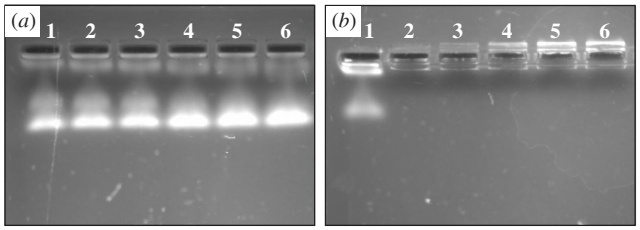



**Figure 1** Images of agarose gel electrophoresis for IPECs based on (*a*) copolymer **7** and (*b*) copolymer **6**. Images 1 stand for oligo-dT-dAs (control); 2 for copolymer/oligo-dT-dA = 6; 3 for copolymer/oligo-dT-dA = = 10; 4 for copolymer/oligo-dT-dA = 15; 5 for copolymer/oligo-dT-dA = 20 and images 6 for copolymer/oligo-dT-dA = 25.

In summary, despite the more complicated procedure of synthesis compared to **7**, graft copolymer **6** can be considered as a more promising carrier for nucleic acids. This copolymer produces complexes with the nucleic acid more efficiently due to the sufficient positive charge of the initial system, and after binding, the surface of the IPECs becomes neutral due to the efficient shielding by the PMAG shell. As a result, the cytotoxicity of such systems is reduced. A similar effect was previously shown for PEI-based PEGylated IPECs, for which the effective shielding of the surface charge also reduced the cytotoxicity of the delivery system.[28]

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*Online Supplementary Materials*

Supplementary data associated with this article can be found in the online version at doi: 10.71267/mencom.7606.

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