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

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S1. Materials

The following reagents were used for the synthesis and modification (Sigma-Aldrich, Germany): triphosgene (98%), α-pinene (99%), ε-carboxybenzyl-L-lysine (Lys(Z)) (≥99%), *p*-nitrophenol (≥99%), *N*,*N'*-diisopropylcarbodiimide (DIC) (≥98), γ-aminobutyric acid (GABA, ≥99%), HBr solution in acetic acid (33 wt%), trifluoroacetic acid (TFA, ≥98%), sodium periodate (≥99%), sodium borohydride (≥98%), lithium bromide (≥99%). NH_2-PEG_4-N_3 and dibenzocyclooctyne amine (DBCO-NH_2) were purchased from Lumiprobe (Russia). Solvents, namely, dimethyl sulfoxide (DMSO), *N*,*N*-dimethylformamide (DMF), 1,4-dioxane, diethyl ether, ethyl acetate, petroleum ether, chloroform and acetone, as well as salts for buffer preparation, sodium hydroxide and glacial acetic acid were purchased from Vecton (Russia) and purified by standard methods prior to use. Dialysis cellulose membranes (MWCO 1000, 3500, 12000-14000, Orange Scientific) were used for the purification of polymer samples. Millipore syringe filters with MCE and PTFE membranes with pore diameters of 0.22 and 0.45 μm were used for the filtration of samples for chromatographic analysis. Oligothymidine dT (23 nucleotides) and oligoadenine dA (23 nucleotides), labeled oligothymidine dT-TAMRA (23 nucleotides) and labeled oligoadenine dA-TAMRA (23 nucleotides) were purchased from Biobeagle (Russia) and used to prepare duplexes dT-dA (23 bp) and study their binding by polymers.

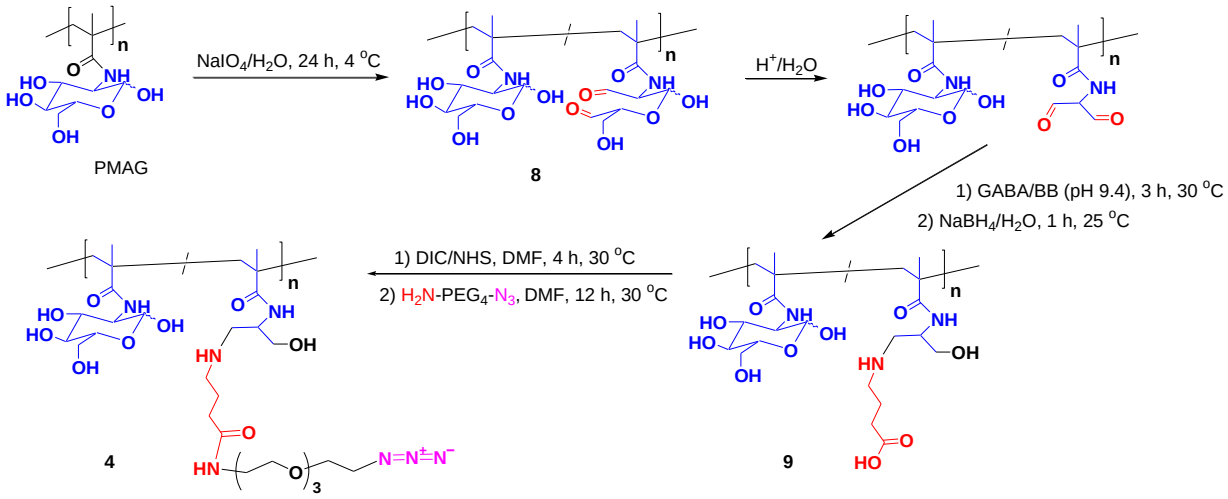
S2 Comments for the synthesis of polymers 6 and 7

Ring opening polymerization of *N*-carboxyanhydride of Cbz-protected lysine **2** was initiated by primary amino group [H. Petersen, P. M. Fechner, A. L. Martin, K. Kunath, S. Stolnik, C. J. Roberts, D. Fischer, M. C. Davies and T. Kissel, *Bioconjug. Chem.*, 2002, **13**, 845, https://doi.org/10.1021/bc025529v] of compound **1** (section S3). The ^{1}H NMR spectrum of **3** contains all signals of chemical shift corresponding to PLys as well as signals of aromatic protons of **1** overlapping with aromatic protons of Cbz-group of **2** (Figure S1). Due to the superposition of lysine and DBCO signals, the correct calculation of the degree of polymerization from ^{1}H NMR spectrum is not feasible.

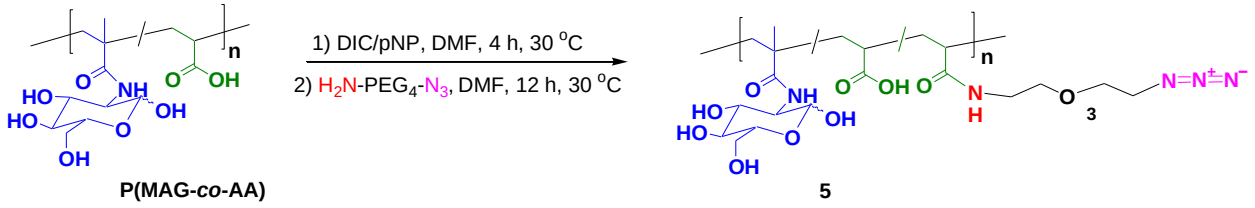
In order to introduce azide functionality into PMAG, it was oxidized to generate aldehyde functionality, then was modified with γ-aminobutyric acid (GABA) and finally with H_2N-PEG-N_3 after activation of carboxylic groups (Scheme S1). Periodate oxidation of a part of glucose units was carried out at [$NaIO_4$]/[MAG] = 0.7 [V. Korzhikov, S. Roeker, E. Vlakh, C. Kasper and T. Tennikova, *Bioconjug. Chem.*, 2008, **19**, 617–625; https://doi.org/10.1021/bc700383w]. After the modification of the oxidized PMAG **8** with GABA (section S3), the content of carboxy groups in

the modified PMAG **9** was determined by the reverse conductometric titration of the polymer solution (Figure S2). The content of carboxylic groups was found to be 1.1 ± 0.1 μmol mg^{-1} that corresponded to 5.5 mol% of the modified units. The structure of **9** was confirmed by ^{1}H NMR spectroscopy. The spectrum of **9** contains all the proton signals of PMAG moiety, as well as methylene protons of aliphatic GABA linker (Figure S3).

In the next step, the carboxy groups of **9** and P(MAG-*co*-AA) were activated and then the functionalization with H_2N-PEG_4-N_3, necessary for further click-reaction with **3**, was performed (sections S4-S5). In the case of P(MAG-*co*-AA), 15 mol% of carboxy groups was activated. For both selected glycopolymers, the reaction between amino groups of NH_2-PEG_4-N_3 with activated ester groups of **9** and P(MAG-*co*-AA) was carried out to obtain **4** and **5**, respectively (Schemes S1, S2). The presence of the azide group was confirmed by ATR-FTIR spectroscopy. The characteristic band corresponding to the asymmetric vibrations of -N_3 group (2115 cm^{-1}) is clearly visible in the obtained FTIR spectra of both **4** and **5** (Figure S4). In addition, activated form of polymer P(MAG-*co*-AA) as well as **5** were analyzed by ^{1}H NMR spectroscopy. The registered spectra contained the main signals of MAG and AA (Figures S5 and S6) as well as signals of aromatic protons of *p*-nitrophenol (7.45 and 8.28 ppm). The latter are strongly decreased in the spectrum after modification.



Scheme S1

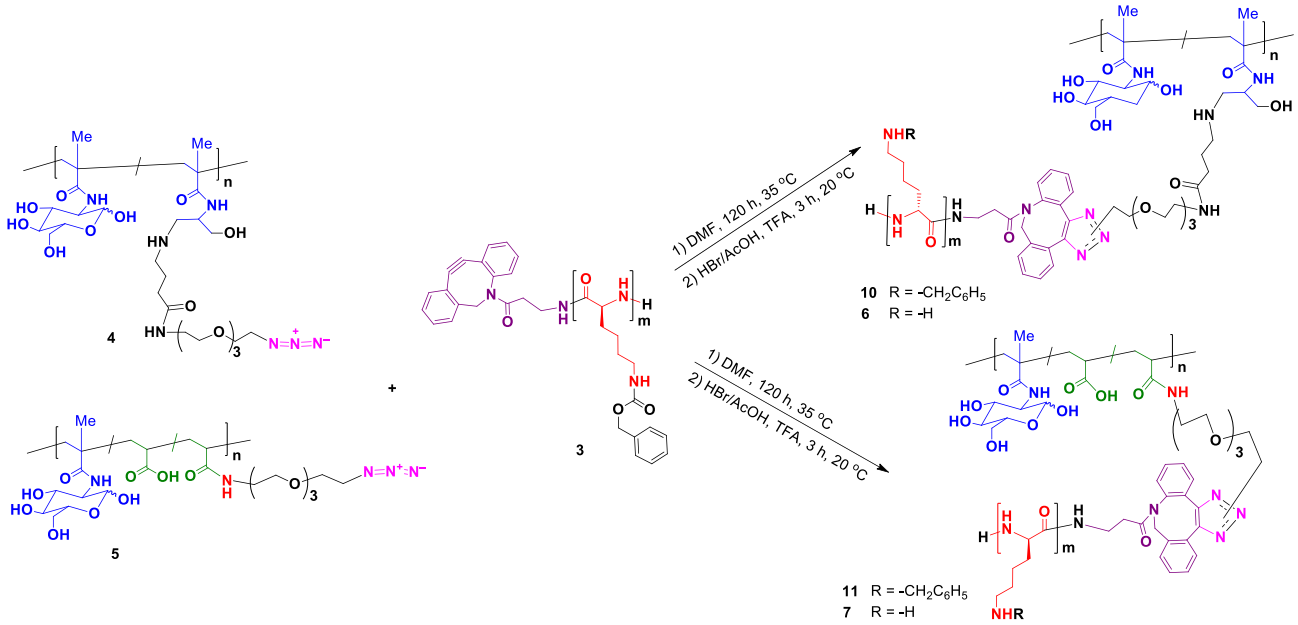


Scheme S2

Finally, the click reactions between **4** or **5** with **3** were performed (Scheme S3, section S6). The resulting copolymers **10** and **11** markedly changed their solubility relative to those of its individual components. Thus, **11** stopped dissolving in water and started dissolving in DMSO, whereas its precursor **5** dissolved well in water and DMF and practically did not dissolve in DMSO, but **3** dissolved well in DMF and DMSO and did not dissolve in water. In turn, the

obtained **10** was not soluble in water and poor soluble DMSO, unlike its precursor **7**, which was well soluble in water and insoluble in DMSO.

The progress in the click reaction was confirmed by ATR-FTIR spectroscopy. The disappearance of the characteristic band corresponding to the valence vibrations of azide group compared to the initial sample (2115 cm^{-1}) was observed in the spectra of both grafted copolymers that indicate the success of reaction (Figure S4). In addition, ^{1}H NMR spectroscopy was used to analyze the product. ^{1}H NMR spectrum of carefully purified **11** contains all signals of both **3** and **5** (Figure S7).



Scheme S3

S3. Synthesis of DBCO-PLys^{Cbz} (3)

The polymerization of N-KA Lys^{Cbz} was carried out in dry *N*,*N*-dimethylformamide (DMF). For this purpose, a 4% solution of the monomer in the solvent was prepared and then a solution of DBCO-NH_2 was added (monomer/initiator = 50). The polymerization mixture was purged with argon and left for polymerization at 30 °C for 72 h. The obtained homopolymers were precipitated with a fourfold excess of diethyl ether. The precipitate was separated by centrifugation. After decantation, the precipitate was washed three times with excess of diethyl ether followed by centrifugation and dried in air at room temperature in dark. The yield of DBCO-PLys^{Cbz} was 85%. The molecular weights and dispersity ($Đ$) values for the homopolymer were determined by size-exclusion chromatography (SEC) in DMF containing 0.1 M LiBr at 40 °C. SEC was performed using a Shimadzu LC-20 Prominence chromatograph with refractometric detection (RID-20A) using a Styragel Column (HMW6E, particle size 15-20 μm, column size 7.8×300 mm (Waters, USA). The obtained experimental data were analyzed and processed using LS Solution Shimadzu software (Tokyo, Japan). The obtained polymer was characterized by ^{1}H NMR spectroscopy using a Bruker Avance III instrument at 400 MHz. ^{1}H NMR (DMSO-d_6), δ (ppm): 1.31-1.86 (6H, 3 -CH_2), 2.97 (2H, -C$\underline{H}_2$-NH), 3.86 (1H, CH), 4.98-5.01 (4H, -C$\underline{H}_2$-N and --C$\underline{H}_2$-C_6H_5), 6-99-7.32 (13H, -$CH_2C_6\underline{H}_5$; 2 -$C_6\underline{H}_4$ of DBCO).

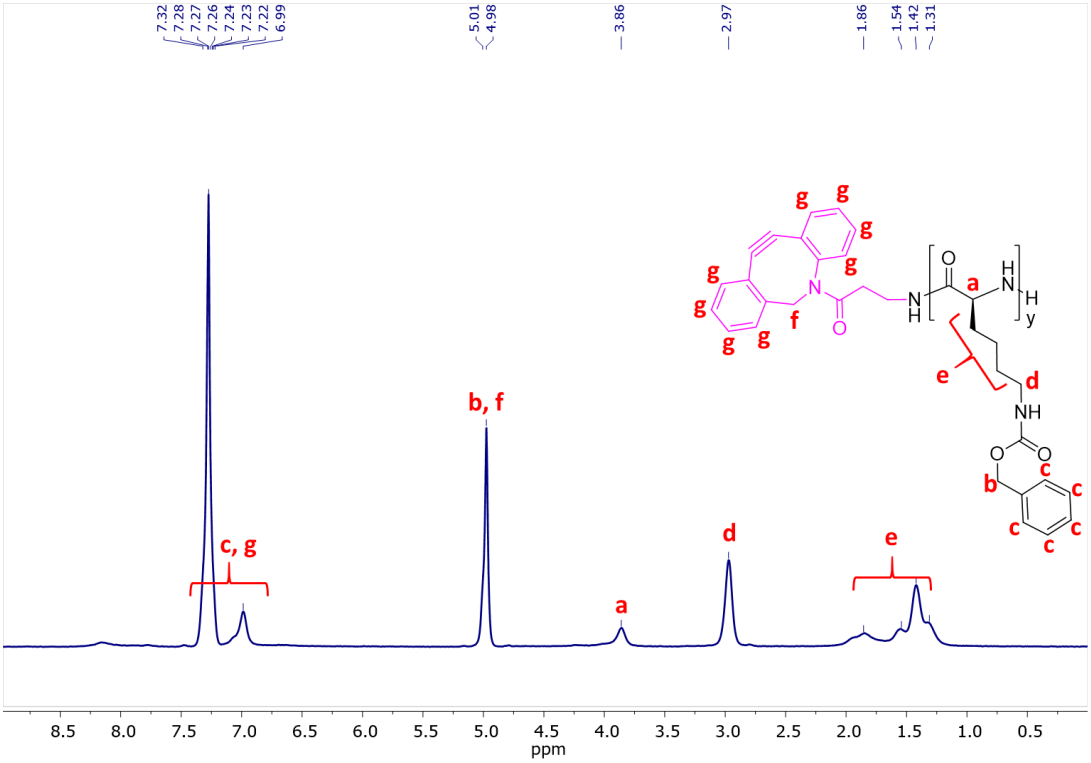


Figure S1. ^{1}H NMR spectrum of polymer **3** (DMSO-d_6, 40 °C).

S4. Oxidation and Modification of PMAG with GABA-linker (synthesis of 9, Scheme S1)

PMAG was modified by oxidation of the part of glucose units followed by condensation of the resulting aldehyde groups with the amine-containing component and reduction of the imine bond by sodium borohydride. For this purpose, a 2% solution of 300 mg PMAG in deionized water was cooled to 4 °C. A solution of 180 mg $NaIO_4$ in 1 mL of water (molar ratio $[NaIO_4]/[MAG] = 0.7$) was added to the solution. The reaction mixture was protected from light and left in the refrigerator (4 °C) with stirring for 24 h. After that, the solution was warmed to room temperature and purified by dialysis against acidified water using a membrane bag with MWCO 1000. After purification, oxidized PMAG was precipitated with a 30-fold excess of acetone and dried in vacuo at 30 °C. After that, oxidized PMAG was dissolved in water (2 mass%) and an aliquot of GABA (3 eq with respect to aldehyde groups) in borate buffer solution (pH 9.4) was added. The reaction mixture was left for 3 h at 30 °C. Finally, an aliquot of $NaBH_4$ (4 eq relative to the amount of amine-containing component) was added to the solution and left for 1 h at room temperature. The polymer was purified by dialysis against water for 24 h using dialysis bag with MWCO 1000. The product was then precipitated with a 30-fold excess of acetone and dried in vacuo at 30 °C. The yield of **9** was 87%.

The content of carboxy groups in polymer **9** was determined by conductometric titration. The latter was performed using a Mettler-Toledo InLab 731-ISM conductivity sensor. A 0.1% solution of the polymer with 1 mL of 0.01 M HCl solution was titrated with 0.01 M NaOH solution. The concentration of carboxyl groups was 1.1 μmol/mg of polymer.

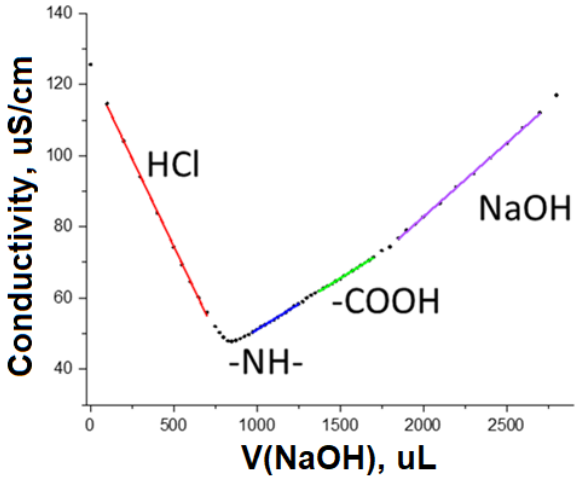


Figure S2. Conductometric titration curve of P(MAG-*co*-MAG(GABA)) solution.

The obtained polymer was characterized by ^{1}H NMR spectroscopy using a Bruker Avance III instrument at 400 MHz. ^{1}H NMR (D_2O), δ (ppm): 1.36 (3H, -CH_3), 2.04 (4H, 2 -CH_2), 2.17 (2H, -C$\underline{H}_2$COOH), 2.54 (2H, -C$\underline{H}_2$NH), 3.51-4.31 (8H, H_2-H_6 of glucose ring), 5.32 (1H$^{\alpha}$).

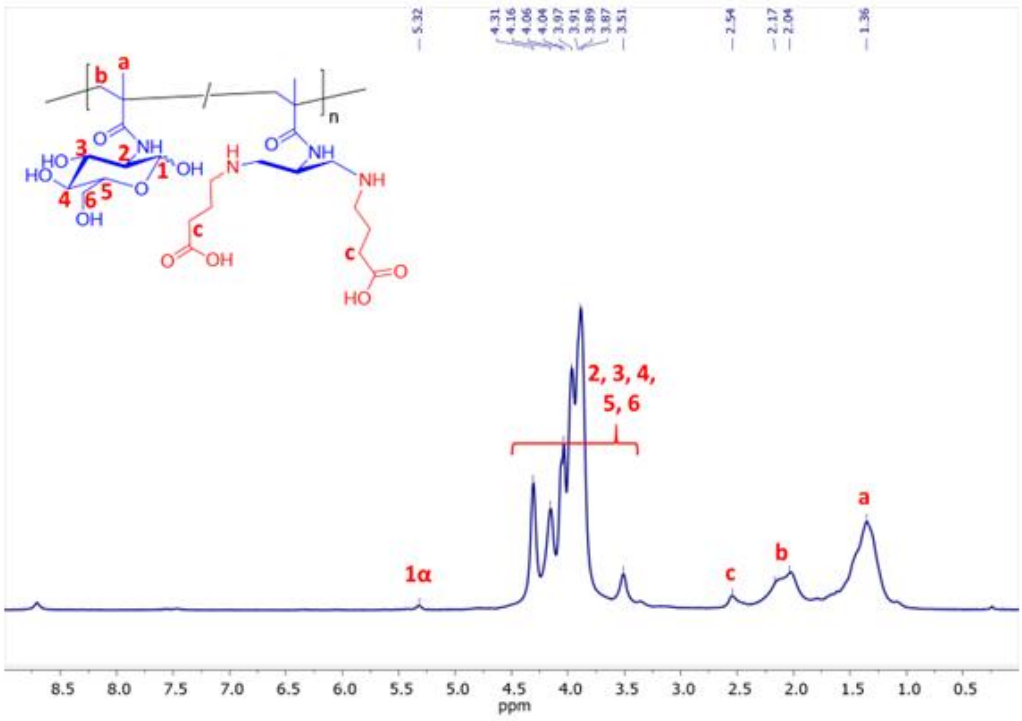


Figure S3. ^{1}H NMR spectrum of polymer **9** (D_2O, 60 °C).

S5. Modification of P[MAG-*co*-MAG(GABA)] with H_2N-PEG-N_3 (synthesis of 4, Scheme S1)

To introduce azide functionality into the polymer chain for further click chemistry, the carboxy group of GABA in P[MAG-*co*-MAG(GABA)] was activated. First, a solution of *N*-hydroxysuccinimide (NHS) in DMF (4 eq relative to the number of carboxyl groups) was added to a solution of 150 mg of polymer in 40 mL of DMF and left for 5 min under stirring at 40 °C. Thereafter, a solution of *N*,*N*'-diisopropylcarbodiimide (DIC) in DMF (2 eq relative to the

number of carboxyl groups) was added and left for 30 min under stirring at 40 °C. A solution of NH_2-PEG_4-N_3 (1.5 eq relative to the number of carboxyl groups) was added to the resulting mixture under stirring and the mixture was left for 12 h at 40 °C. After that, the solution was transferred to a dialysis bag with a MWCO 3500. Dialysis was carried out for 12 h against a 20% solution of DMF in water and then 24 h against water. The polymer product was precipitated by 30-fold excess of acetone and dried in vacuo at 35 °C. The yield of P[MAG-*co*-MAG(GABA-PEG_4-N_3)] was 77%. FTIR spectroscopy was performed using an IRAffinity-1 FTIR spectrometer with the use of ATR console (Shimadzu, Japan). Copolymer was analyzed by FTIR spectroscopy (**Figures S4, a**).

S6. Modification of P(MAG-*co*-AA) with H_2N-PEG-N_3 (synthesis of 5, Scheme S2)

Carboxylic groups of starting copolymer were activated using *p*-nitrophenol (pNP) (2 eq regarding 15 mol% of -COOH) and DIC (1.2 eq regarding 15 mol% of -COOH) in DMF (other conditions as in section S5) and then modified with H_2N-PEG-N_3. In the latter case, a solution of H_2N-PEG-N_3 (1.5 eq relative to the number of activated carboxyl groups) was added to a solution of 150 mg of P[MAG-*co*-AA-*co*-AA(OpNP)] in 30 mL of DMF. The mixture was left for 12 h under stirring at 40 °C. After that, the solution was transferred to a dialysis bag with MWCO 3500. Dialysis was carried out for 12 h against 20% DMF aqueous solution and then 24 h against water. The polymer was precipiateted by 30-fold excess of acetone and dried in vacuo at 35°C. The yield of P[MAG-*co*-AA-*co*-AA(PEG_4-N_3)] **5** was 83%. Copolymer was analyzed by FTIR spectroscopy (**Figures S4, b**).

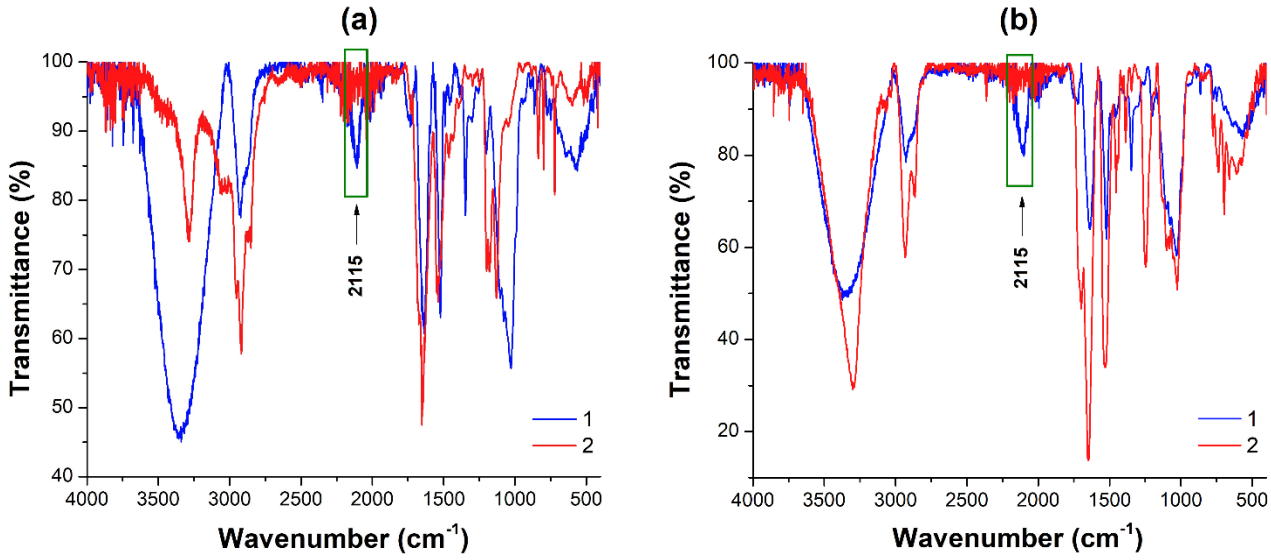


Figure S4. ATR-FTIR spectra of PEG-N_3 modified MAG-based polymers before click-reaction (*1*) and after click-reaction (*2*): (*a*) ***1*** – polymer **4**, ***2*** – graft-copolymer **10**; (*b*) ***1*** – polymer **5**, ***2*** – graft-copolymer **11**.

The obtained copolymers were characterized by ^{1}H NMR spectroscopy using a Bruker Avance III instrument at 400 MHz. ^{1}H NMR (DMSO-d6), δ (ppm): 1.24 (3H, -CH_3), 1.89 (4H, 2 -CH_2), 2.79 (1H, -C<u>H</u>COOH), 3.41-3.88 (8H, H_2-H_6 of glucose ring), 4.78 (1H$^{\beta}$), 5.18 (1H$^{\alpha}$), 7.45 (2H, 2 -CH of pNP aromatic ring), 8.28 (2H, 2 -C<u>H</u>CNO_2) of pNP aromatic ring).

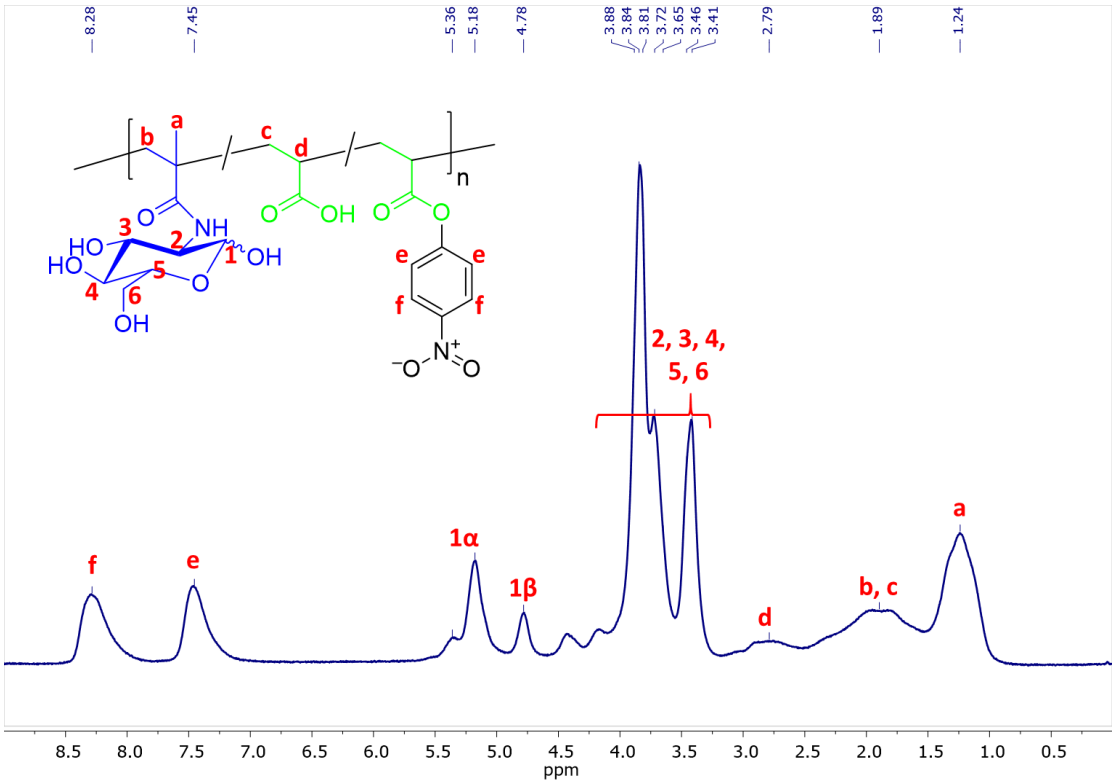


Figure S5. ^{1}H NMR spectrum of P(MAG-*co*-AA) activated with NP (DMSO-d_6, 60 °C).

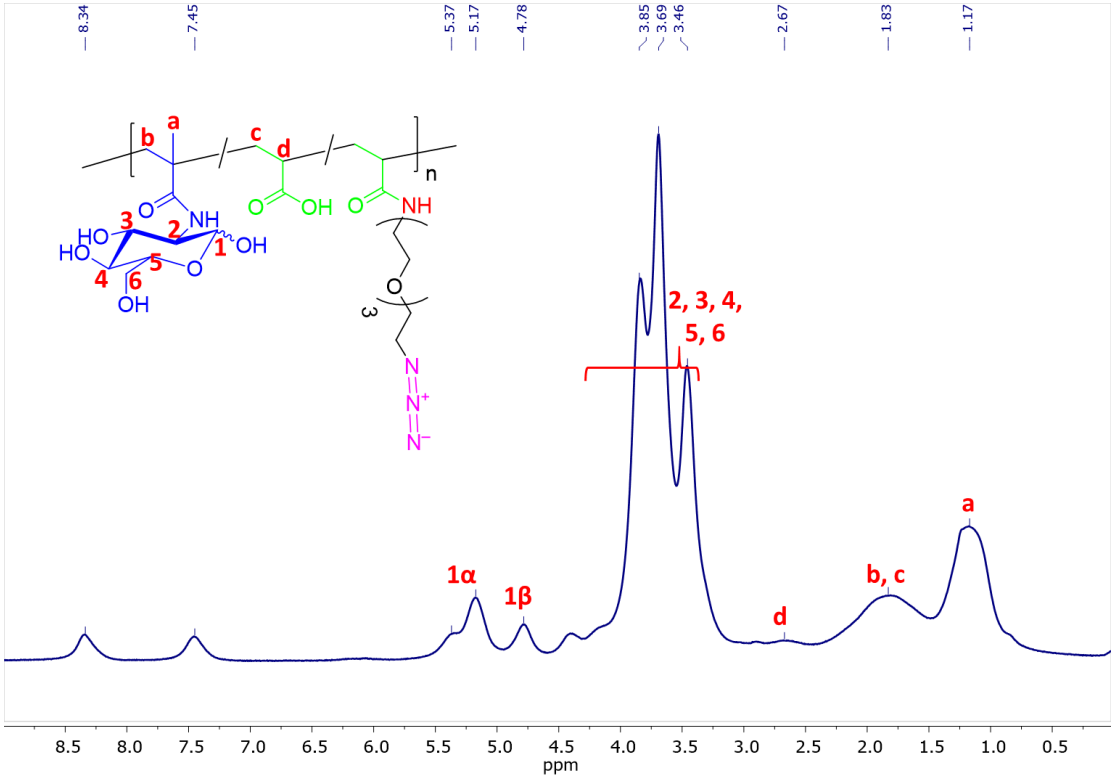


Figure S6. ^{1}H NMR spectrum of **5** (DMSO-d_6, 60 °C).

S7. Synthesis of Graft-copolymers 6,7 by Metal-free Click Chemistry

To synthesize graft-copolymers, a click reaction between a glycopolymer containing azide groups and DBCO-PLys^{Cbz} was carried out. For this purpose, 100 mg of copolymer was

dissolved in 15 mL of DMF. 150 mg of DBCO-PLys^{Cbz} **3** was dissolved in 5 mL of DMF and after complete dissolution, the solution was injected into a vial containing the azide-containing polymer **4** or **5**. The vial with the mixture was closed and stirred at 40 °C for 120 h. At the end of the reaction, the solution was diluted with water and transferred to a dialysis bag with MWCO 12000-14000. Dialysis was carried out for 24 h with 50% DMF solution in water and then 24 h against water. Next, 80-90% of water was evaporated and rest was dried in vacuo at 50 °C. The yield of P(MAG-*co*-(MAG-*g*-PLys(Z))) was 68%, and for P[MAG-*co*-AA-*co*-(AA-*g*-PLys(Z))] it was 84%.

To remove Cbz-protection of the ε-amino group of Lys in the graft-copolymer, 8 mL of TFA was added to 50 mg of copolymer and left to stir while cooling with an ice bath for 30 min. Thereafter, 2 mL of a 33% solution of HBr in acetic acid was added to the resulting suspension. The reaction was carried out under stirring for 4 h, after which the polymer was precipitated with a fivefold excess of diethyl ether. The precipitate was dispersed in DMF and purified by dialysis using a bag with MWCO 12000-14000. Dialysis was carried out for 24 h against deionized water. Next, 80-90% of water was distilled off on a rotary evaporator and dried *in vacuo* at 35 °C.

The obtained copolymers were characterized by ^{1}H NMR spectroscopy using a Bruker Avance III instrument at 400 MHz. ^{1}H NMR spectrum of **11** is shown in Figure S7. ^{1}H NMR (DMSO-d$_6$), δ (ppm): 1.09 (3H, -CH$_3$), 1.29-1.54 (6H, 3 -CH$_2$), 1.78 (1H, -CHCOOH), 2.97 (2H, -CH$_2$-NH), 3.19-3.63 (8H, H$_2$-H$_6$ of glucose ring), 4.24 (6H, 3 -CH$_2$O) 4.79 (1H$^{\beta}$), 5.18 (1H$^{\alpha}$), 4.97 (4H, -CH$_2$C$_6$H$_5$, -CH$_2$N), 6-90-7.49 (13H, -CH$_2$C$_6$H$_5$; 2 -C$_6$H$_4$ of DBCO).

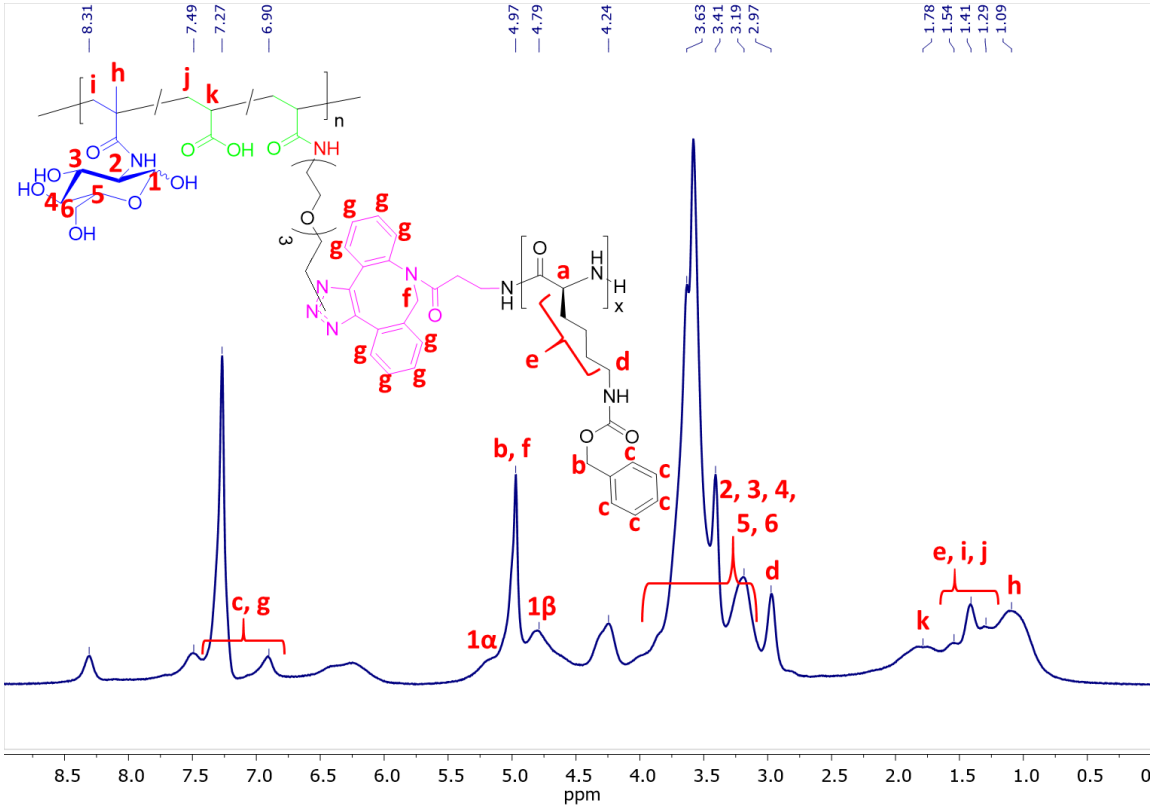


Figure S7. ^{1}H NMR spectrum of graft-copolymer **11** (DMSO-d$_6$, 60 °C).

S8. HPLC analysis

In order to quantify the content of PLys in graft copolymers **6** and **7**, hydrolysis of the copolymers to free lysine was performed. For hydrolysis, 100 mL of 6H HCl with 0.0001% phenol content was prepared. The copolymer sample of 4 mg was put into the ampoule for hydrolysis and hydrolysis solution in the amount of 1 mL/0.5 mg of copolymer was added. Then the ampoule was sealed and incubated at 110 °C for 3 days. After hydrolysis, the solution was evaporated to dryness, washed with water and evaporated again until neutral pH. The precipitate was then dissolved in 0.5 mL of water and the resulting mixture was analyzed by ion-exchange HPLC with mass spectrometric detection. Calibration curve was pre-built for standard solutions of lysine with concentrations in the range of 0.05-0.5 μg/mL. The analysis was performed using a LC-20 Prominence Shimadzu HPLC system; mobile phase – 6 mM H_3PO_4, mobile phase flow rate – 1.0 mL/min, analysis time – 15 min.

S9. Preparation of Polymer Particles

The formation of polymer particles was carried out as follows: 5 mg of deprotected graft-copolymer **6,7** was placed to 5 mL of deionized water or PB and dispersed under ultrasound treatment for 30 s using Bandelin SONOPULS HD 2070.2 ultrasonic probe homogenizer (Germany). The hydrodynamic diameter and zeta potential were measured using a Zetasizer Nano S90 (Malvern Instruments Ltd.) dynamic and electrophoretic light scattering (DLS) instrument (scattering angle 173° and temperature 25°C). The measurements were performed for solutions with the concentration of 0.1 mg/mL.

S10. Formation of Polyplexes

A duplex was pre-prepared by mixing equimolar amounts of dA and dT. Polyplexes with polymers were obtained by adding an aqueous solution of oligo-dT-dA with a given amount of duplex to a polymer dispersion (in water or PB) of a given concentration to reach a ratio of copolymer/oligo-dT-dA equal to 6, 10, 15, 20 and 25. The resulting dispersion was then thoroughly mixed for 2-3 min and incubated at 25 °C for 12 h for complete binding of the polymer to the oligonucleotide duplex and stabilization of the resulting polyplexes. After incubation, the hydrodynamic diameter and ζ-potential were measured for the obtained polyplexes as described in section S8.

S11. Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed using the BlueMarine 200 system (Serva Electrophoresis GmbH). In this case, solution of oligo-dT-dA-TAMRA and dispersions of its polyplexes with copolymers were prepared at a copolymer/oligo-dT-dA ratio 6, 10, 15, 20 and 25. Before analysis, 20 μL of the sample solution was mixed with 5 μL of dye (xylene blue and bromophenol blue, Thermo Scientific, USA) and loaded onto a 1.5% agarose gel in Tris-acetate buffer solution, pH 8.3. Electrophoresis of the samples was performed at an operating voltage of 40 V for 30 min, and then the gel was analyzed using the Gel Doc EZ gel documentation system (Bio-Rad, USA).

S12. Cytotoxicity Assay

The cytotoxicity of polymer particles was examined using HEK 293 cells by MTT assay (MTT reagent: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) based on the

ability of living cells to metabolize MTT reagent into insoluble formazan. The amount of formazan is proportional to the number of viable cells. For the study, 5000 cells in 100 μL of DMEM culture medium containing 10% fetal calf serum and 1% of antibiotics (penicillin and streptomycin) were seeded per well of 96-well plates. The next day, the medium was replaced by dispersions of the analyzed particles in the culture medium at different concentrations (4-500 μg/mL). After 72 h, the solution of medium containing polymeric particles was removed and a solution of 0.5% MTT in basal medium was added to the cells, incubated for 2-3 h, then the MTT solution was removed and the formazan crystals formed at the bottom of the plate were dissolved in 100 μL of DMSO. The optical density of solutions in wells was measured spectrophotometrically at 555 nm a Fluoroscan Ascent plate reader (ThermoFisher Scientific, USA). Wells in which cells were incubated in medium containing no polymeric particles were used as a negative control. The optical density values of the tested samples were normalized as a percentage relative to the control. Each concentration was analyzed in 5-fold repetition within one experiment.

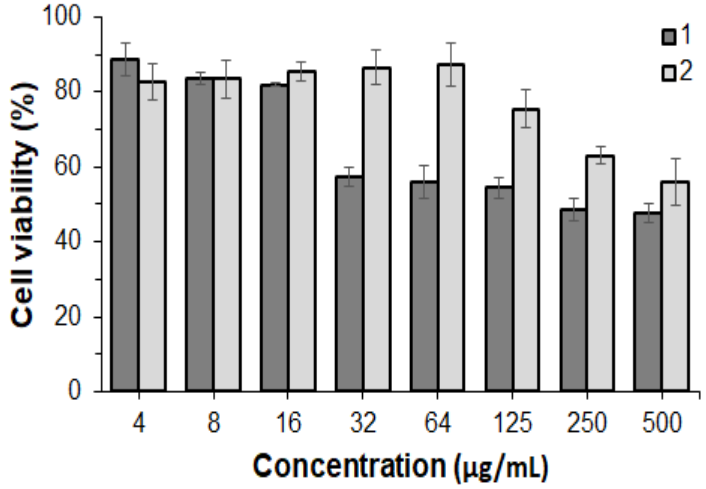


Figure S8. Cytotoxicity of **6** (***1***) and complex of **6** with oligo-dT-dA (copolymer/oligo-dT-dA = 10 (wt/wt) equal to N/P = 9) (***2***) in HEK 293 cells (MTT, 72 h). The X axis indicates the concentration of copolymer.