CHEMICAL DEPARTMENT

DEPARTMENT OF PHYSICAL CHEMISTRY AND TECHNOLOGY OF POLYMERS

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SUMMARY OF DOCTORAL DISSERTATION

Designing of grafted poly(ionic liquids) as potential drug delivery systems for antibacterial therapy

A guide to a monothematic series of publications

Promoter: prof. dr hab. inż. Dorota Neugebauer

Gliwice, 2024

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THE AIM AND SCOPE OF THE RESEARCH

The aim of the research presented in this doctoral thesis was to develop new polymer systems as drug nanocarriers based on graft polymers that contained units of choline ionic liquid (IL), i.e. [2-(methacryloyloxy)ethyl]trimethylammonium chloride (TMAMA). Therefore, using controlled atom transfer polymerization (ATRP), well-defined copolymers were obtained, which differed with the number of side chains, i.e. the degree of grafting, the degree of polymerization, and the TMAMA content. For comparison purpose, analogous linear copolymers were also synthesized. The presence of chloride anions, both in graft and linear copolymers, was used in the ion exchange reaction to introduce drugs in ionic form (pharmaceutical anions). According to this strategy, chloride polymers could serve as universal templates for obtaining ionic polymer-drug conjugates.

The second important research topic was the use of the amphiphilic nature of the obtained copolymers as micellar carriers in the encapsulation of drugs in non-ionic form. As a result of combining both strategies, i.e. anion exchange and encapsulation, with the use of graft polymers, micellar ionic conjugates were obtained as dual systems for combined therapy transporting two drugs with a synergistic effect which are variably linked to the polymer matrix (ionic bond vs. physical impact). The tested systems were designed for transporting drugs used in the treatment of lower respiratory tract diseases, including tuberculosis. In addition to the basic physicochemical characteristics of the polymers, the influence of the structural parameters of the carrier on the drug release rate was examined, and the cytotoxicity of the systems was assessed.

Due to the diverse nature of polymer matrices and selected model drugs, the tested systems were grouped as follows:

• Polymer conjugates with pharmaceutical anions: *p*-aminosalicylate (PAS^-), clavulanate (CLV^-), piperacillin (PIP^-), fusidate (FUS^-),

• Micelles based on chloride polymers with encapsulated drug in non-ionic form: isoniazid (ISO), tazobactam (TAZ), rifampicin (RIF),

• Micellar ionic conjugate systems for two drugs tramsporting (ionic/non-ionic): PAS^-/ISO, PIP^-/TAZ, FUS^-/RIF.

LIST OF SCIENTIFIC PUBLICATIONS CONSTITUTING A MONOTIVE CYCLE

This dissertation is a monothematic series of seven scientific articles published in 2020-2024 in journals registered in the Journal Citation Records (JCR) database:

P.1. Synthesis and Characterization of Ionic Graft Copolymers: Introduction and In Vitro Release of Antibacterial Drug by Anion Exchange.

K. Niesyto, D. Neugebauer

Polymers. 2020, 12, 2159. (IF2020= 4.329; MEiN=100 pkt)

P.2. Linear Copolymers Based on Choline Ionic Liquid Carrying Anti-Tuberculosis Drugs: Influence of Anion Type on Physicochemical Properties and Drug Release.

K. Niesyto, D. Neugebauer

International Journal of Molecular Sciences 2021, 22, 284 (IF2021= 6.208; MEiN=140 pkt)

P.3. Dual-Drug Delivery via the Self-Assembled Conjugates of Choline-Functionalized Graft Copolymers.

K. Niesyto, A. Mazur, D. Neugebauer

Materials 2022, 15, 4457 (IF2022= 3.4; MEiN=140 pkt)

P.4. Ionic Liquid-based Polymer Matrices for Single and Dual Drug Delivery: Impact of Structural Topology on Characteristics and In Vitro Delivery Efficiency.

K. Niesyto, S. Keihankhadiv, A. Mazur, Mielańczyk, A., D. Neugebauer

International Journal of Molecular Sciences 2024, 25, 1292 (IF2022=5.6; MEiN=140 pkt)

P.5. Piperacillin/Tazobactam co-delivery by micellar ionic conjugate systems carrying pharmaceutical anions and encapsulated drug

K. Niesyto, A. Mazur, D. Neugebauer

Pharmaceutics 2024, 16, 198 (IF2022= 5.4; MEiN=100 pkt)

P.6. Biological in vitro evaluation of PIL graft conjugates: cytotoxicity characteristics.

K. Niesyto, W. Łyżniak, M. Skonieczna, D. Neugebauer

International Journal of Molecular Sciences 2021, 22, 7741 (IF2021= 6.208; MEiN=140 pkt)

P.7. Toxicity evaluation of choline ionic liquid-based nanocarriers of pharmaceutical agents for lung treatment.

K. Niesyto, M. Skonieczna, M. Adamiec-Organiściok, D. Neugebauer

Journal of Biomedical Materials Research Part B - Applied Biomaterials 2023, 111(7), 1374-1385 (IF2022= 3.4; MEiN=140 pkt)

SUMMARY OF THE DOCTORAL STUDENT'S OWN CONTRIBUTION

Participation in the development of the concept and research plan; conducting the synthesis of macroinitiator precursors, macroinitiators and linear and graft polymers; obtaining conjugates, micelles and micellar conjugates as drug carriers containing one or two types of drugs; conducting physicochemical characterization of the obtained polymers and carriers; conducting drug release studies; conducting biological research; development, analysis and interpretation of results; preparation of original drafts of publication manuscripts; Scholarship holder - research contractor under the OPUS program (grant no. 2017/27/B/ST5/00960; 2019-2022).

Declarations of the co-authors of the publication detailing their individual authorial contribution are included in the appendices to this dissertation.

DESCRIPTION OF THE SUBJECT OF THE RESEARCH, RESULTS AND CONCLUSIONS

Synthesis of copolymers using the ATRP method (P.1.; P.2.)

During studies, innovative polymer systems were developed as drug carriers for antibacterial therapy of lower respiratory tract diseases, using grafted copolymers containing units of choline ionic liquid, i.e. [2-(methacryloyloxy)ethyl]trimethylammonium chloride (TMAMA), which is known from biological activity. For this purpose, controlled Atom Transfer Radical Polymerization (ATRP) was used to obtain well-defined copolymers. The ionic structure of TMAMA enabled the ion exchange of the chloride anion for pharmaceutical anions, increasing the biological activity of the systems. At the same time, the use of the hydrophilic TMAMA comonomer in various initial proportions (TMAMA/MMA = 25/75, 50/50, 75/25) allowed to achieve a different hydrophilic-hydrophobic balance in the tested polymer systems. Comparatively, a series of tests were carried out using analog linear polymers. Carrier studies included well-defined copolymers with diverse topologies, i.e. grafted G1-G8 vs. linear L1-L3, which were synthesized using different initiator systems (initiator vs. macroinitiator). Grafted copolymers G1-G8 were obtained as a result of a two-stage controlled atom transfer radical polymerization (ATRP) reaction (Fig. 1, Table 1). In turn, linear copolymers L1-L3 were obtained by a one-step ATRP reaction (Fig. 2, Table 2).

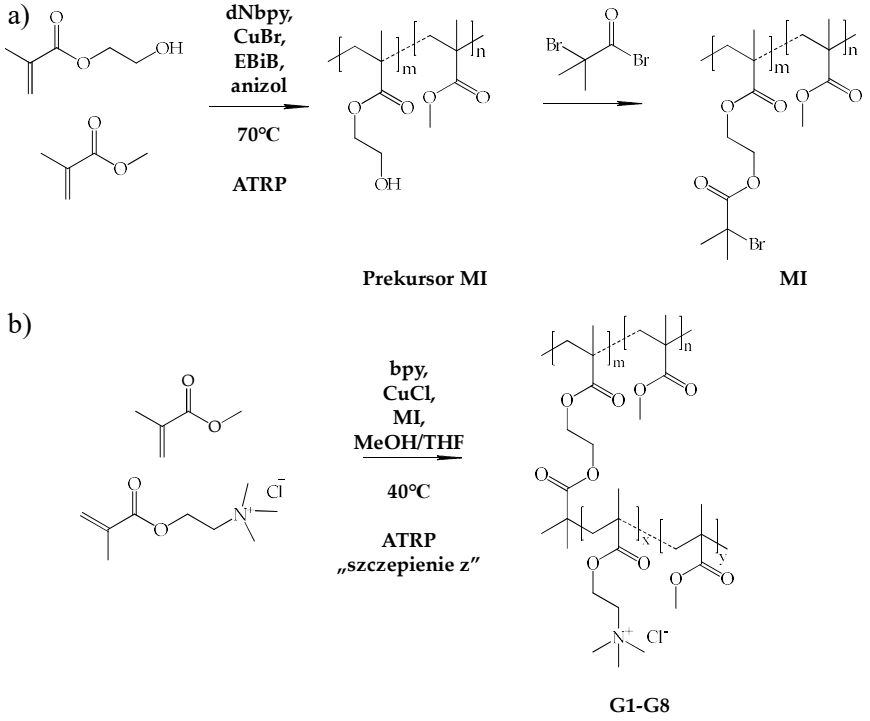

Figure 1. Reaction schemes for preparing a) P(MMA-*co*-BIEM) as macroinitiator, and b) graft copolymer, where EBiB is the initiator.

Table 1. Characteristics of graft copolymers.

	TMAMA/ MMA	DP_n	n_{sc}	DG (mol.%)	DP_{SC}	F_{TMAMA} (mol.%)	Mn × 10^{-3} (g/mol)	Đ
G1	25/75	186	48	26	16	13	115	1,68
G2	25/75				24	21	169	1,90
G3	50/50				31	42	244	1,31
G4	50/50				35	43	273	1,15
G5	25/75				29	28	554	1,24
G6	25/75	292	133	46	65	18	1 091	1,11
G7	50/50				28	39	584	1,03
G8	50/50				48	46	1 007	-

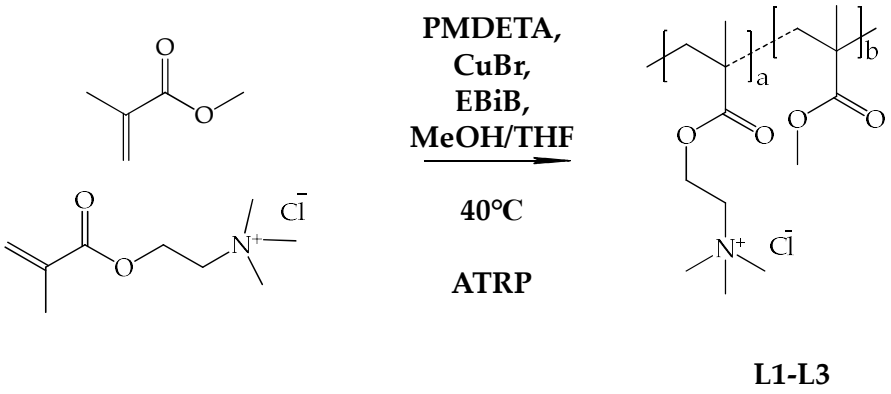

Figure 2. Reaction scheme for obtaining a linear copolymer.

Table 1. Characteristics of linear copolymers.

	TMAMA/MMA	DP_n	F_{TMAMA} (mol.%)	M_n × 10^{-3} (g/mol)	Đ
L1	25/75	370	24	47	1,74
L2	50/50	477	49	73	1,36
L3	75/25	526	75	96	1,27

Most copolymers were characterized by a low dispersity determined by GPC (for graft copolymers Đ = 1.03-1.90, for linear copolymers Đ = 1.27-1.74). In most cases, this analysis confirmed the controlled course of the reaction and the initial ratios of TMAMA/MMA comonomers (25/75, 50/50 for graft copolymers and 25/75, 50/50 or 75/25 for linear copolymers).

The ion exchange of chloride anions for pharmaceutical one – ionic conjugates obtaining (P.1.; P.2.; P.3.; P.5.)

PIL-drug ion conjugates were obtained as a result of the ion exchange reaction. For this purpose sodium or potassium salts containing the following anions were selected: *p*-aminosalicylate (PAS^-), clavulanate (CLV^-), fusidate (FUS^-) and piperacillin (PIP^-). The exchange reaction and the amount of drug introduced into the polymer matrix were indirectly analyzed by the drug content (DC), which was determined with UV-Vis spectra. Both the topology and structure of the copolymer, as well as the structure of the drug, significantly influenced the DC values, as showed by the differences for systems with similar ionic fraction content (Fig. 3). Considering the structure of the copolymer, the content of the hydrophilic fraction and the degree of grafting had a big impact on the exchange reaction effectiveness. It was noticed that the higher ionic fraction, with loosely grafted side chains, affected better DC results were achieved for CLV^-, PAS^- and FUS^-, which was especially observed for the G4 copolymer. An inverse relationship was noted for systems with PIP^-, in which a higher density of side chains and lower F_{TMAMA} led to higher DC values, as in the case of the G6 copolymer, while for the remaining copolymers G4 and G7, the DC values were twice lower than for CLV^-.

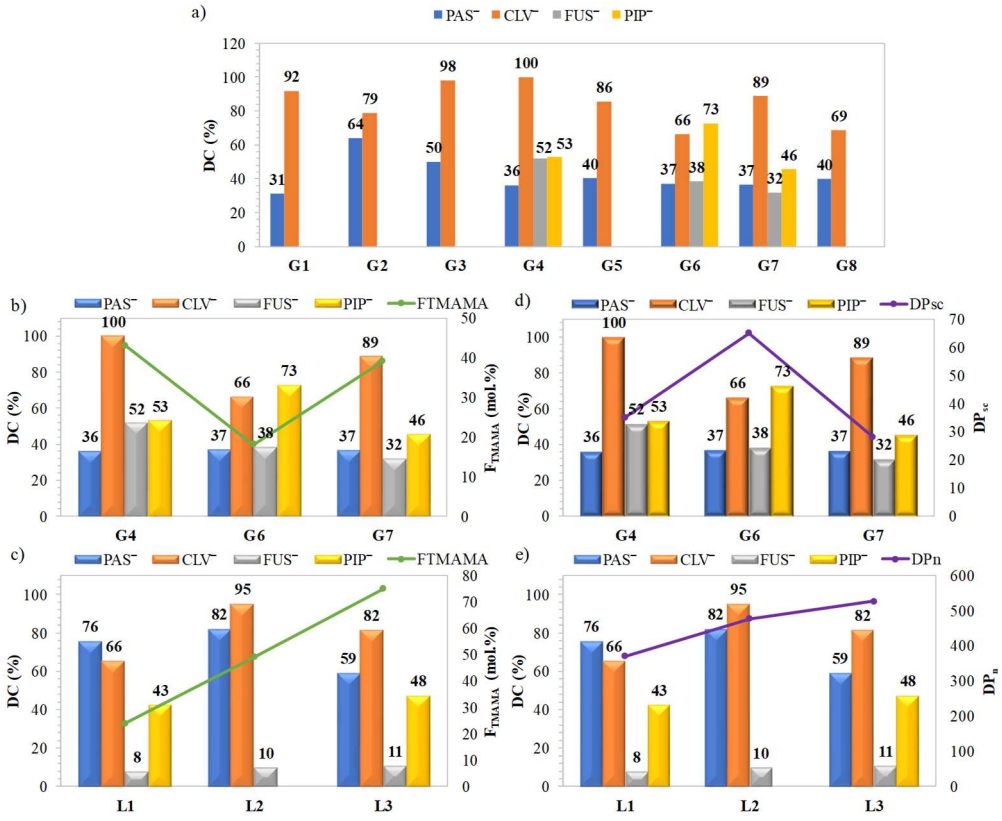

Figure 3. Summary of drug content (DC) for a) all tested conjugates of graft copolymers, b-c) DC in correlation with the content of the ionic fraction FTMAMA and d-e) the length of the chain containing TMAMA units.

In the case of linear copolymers (Fig. 3c, e), DC CLV^- and PAS^- were the highest for the system with the medium F_{TMAMA} value and chain length (L2). A higher content of hydrophobic units limited drug loading into the matrix, resulting in lower DC values. It was noticed that the higher content of hydrophobic units in the linear copolymers, and thus their looser distribution in the chain, the higher DC was achieved, suggesting better accessibility to ionic moieties. Analogous to grafted systems, DC PIP^- was higher for less hydrophilic systems with longer chain lengths.

Behavior of polymers and their conjugates in an aqueous environment (P.1.; P.2.; P.3.; P.5.)

The obtained conjugates based on linear copolymers in aqueous solution formed nanoparticles with sizes of 9–306 nm. In turn, copolymers grafted with a chloride counterion formed structures reaching hydrodynamic diameters (Dh) of 18-368 nm. Their conjugates with PAS^- and CLV^- had similar sizes, 23,354 nm, 18-357 nm, respectively. FUS^- conjugates formed slightly smaller particles in the range of 26-208 nm, and exchange with PIP^- resulted in an increase in Dh values, reaching sizes between 20-451 nm.

The ability of the obtained graft and linear copolymers to form nanoparticles was confirmed by the critical micellization concentration (CMC), which was determined both for copolymers with a chloride counterion, as well as for selected drug conjugates (Fig. 4). CMC values were determined by the measured interfacial tension (IFT). The highest CMC values were recorded for densely grafted copolymers G7 and G8 characterized by a high content of the TMAMA fraction in the side chains (DG = 46 mol%; F_{TMAMA} = 39 and 46 mol% for G7 and G8, respectively) and the L3 copolymer with the longest chain and the highest ionic fraction content (DPn = 396; F_{TMAMA} = 75 mol%). After ionic exchange with PAS^-, CLV^- and PIP^-, an increase in CMC was observed in the grafted polymers. In the case of FUS^-, for the copolymer with lower DG, drug replacement resulted in an increase in CMC values (0.013 vs. 0.025 mg/mL at DG = 26 mol%), while CMC values were not changed for copolymers with a higher grafting degree (DG = 46 % mol.), due to the more hydrophobic nature of the drug, as well as the DC value in the FUS conjugate, which was almost twice as high for G4 compared to G6 and G7.

The water contact angle (WCA) of the polymer layer surface was also determined using a goniometer. The WCA may change due to the structure of the polymer matrix and the nature of the introduced drug (Fig. 5). It was noticed that as the degree of grafting and F_{TMAMA} increased, the WCA values decreased, indicating the increasing hydrophilicity of the systems. Similarly, in the case of linear copolymers, wettability increased with the TMAMA fraction content. Moreover, the layers of linear copolymers showed greater hydrophilicity as compared to the graft copolymers, which can be caused by the predominance of hydrophobic units and much longer side chains in the graft copolymers. Ion exchange for pharmaceutical anions in graft copolymers resulted in a decrease in the WCA value, which means that in this case the conjugated drugs increased the solubilization of the systems. The inverse relationship after the introduction of drugs in ionic form into matrices based on linear copolymers resulted from the lack of phase separation effect, which occurs in graft copolymers due to the hydrophobic main chain. These observations confirmed that the topology, structural parameters, chain length and at the same time the chemical nature of the drug had a significant impact on the wettability of the copolymer layers.

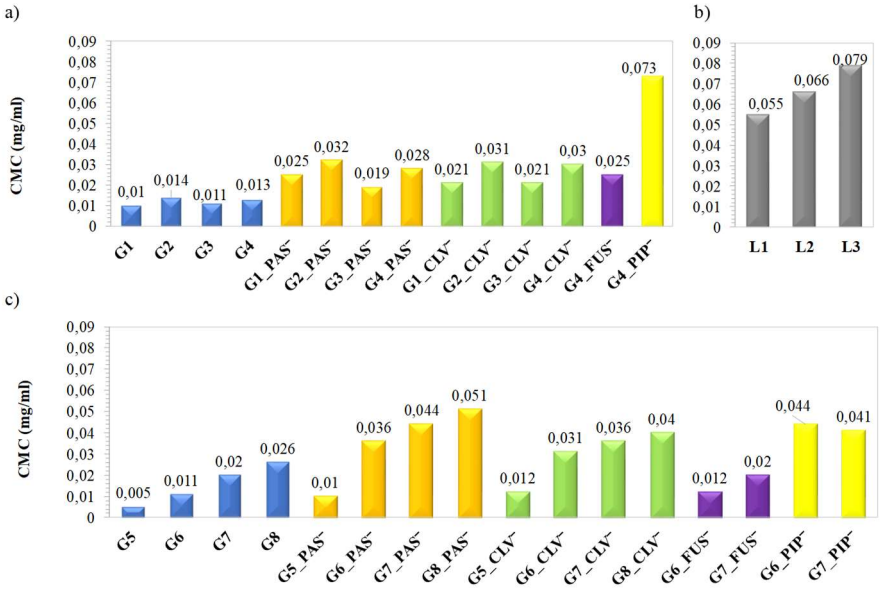

Figure 4. Critical micellization concentration (CMC) values of a) G1-G4 graft copolymers and their drug conjugates, b) L1-L3 linear copolymers, and c) G5-G8 graft copolymers and their drug conjugates.

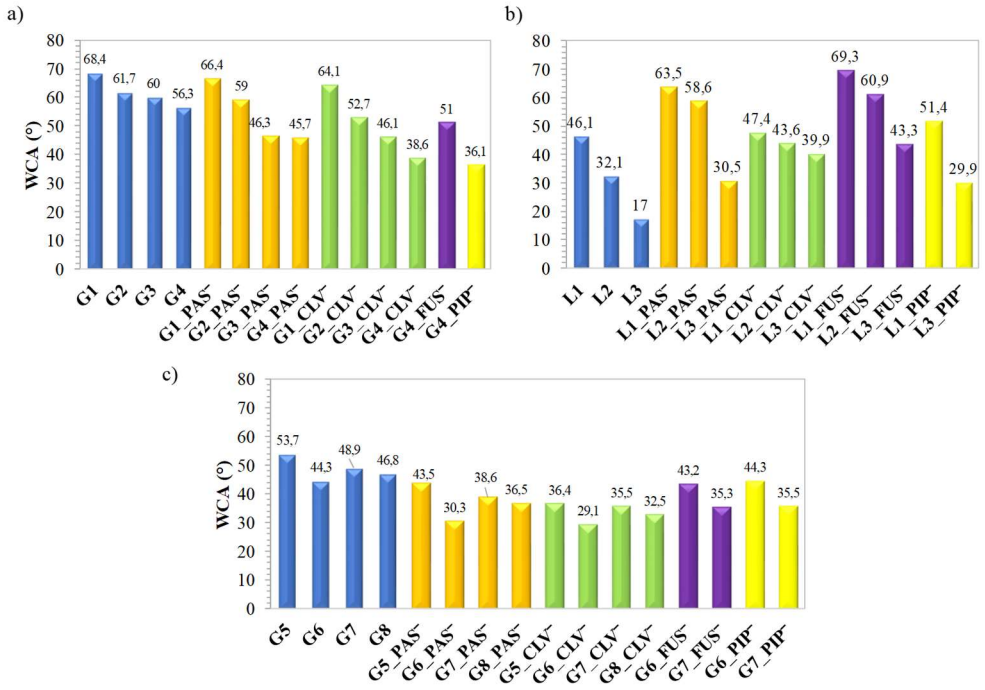

Figure 5. Water contact angles (WCA) for copolymers a) G1-G4 with a lower degree of grafting and their conjugates, b) L1-L3 and their conjugates and c) G5-G8 with a higher degree of grafting and their conjugates determined by the goniometric method.

Release of the conjugated drug in the form of a pharmaceutical anion (P.1.; P.2.; P.3.; P.5.)

The in vitro release process of ionically conjugated drugs was carried out in phosphate buffer saline (PBS, pH = 7.4, 37°C). The release was carried out for 72 hours, however, an effective process could be observed for up to 4 hours, followed by a slower release lasting up

to 24-48 hours. The drug release strongly depended on the structure of the polymer, including its topology and the number of ionic groups. In the case of graft copolymers, the degree of grafting also played a significant role. Moreover, a relationship was noticed between the type of conjugated pharmaceutical anion and the drug release rate.

Due to the greater steric hindrance of the FUS^- and PIP^- anions distributed in the side chains, the release of these drugs occurred at a significantly lower rate (Fig. 6). Among the tested systems, these drugs were released in the largest amounts for the G6 sample, which had densely spaced side chains, at the same time with the lowest content of the hydrophilic fraction. In turn, a lower grafting degree was more beneficial for the release of drugs creating less steric hindrance, i.e. PAS^- and CLV^-, which resulted in faster drug diffusion, where after 4 hours most of the active substance was released.

The release process from linear copolymers as systems for comparing the efficiency of ionic drug delivery was the most favorable for PAS^- in terms of the percentage of drug released as well as the initial drug content. Similarly, FUS^- proved to be a convenient drug for release from linear polymers, however, a low DC value generated a low concentration of released drug. In turn, the release of CLV^- and PIP^- from these copolymers was much less efficient, suggesting stronger interactions of these pharmaceutical anions with the polymer matrix, creating relatively stable ion pairs.

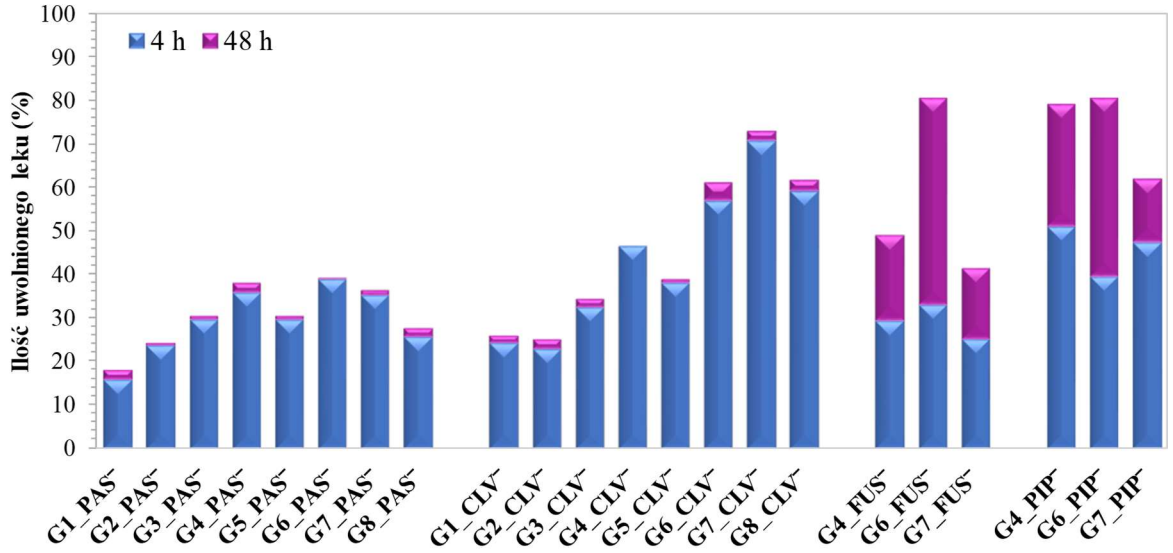

Figure 6. Amounts of released ionic drugs for graft systems.

Encapsulation of drugs - systems based on graft copolymers and their conjugates (P.3.; P.4.; P.5.)

Due to the demonstrated ability to self-organize in aqueous solutions of graft copolymers, they were used for non-ionic drug encapsulation that physically interacts with the polymer matrix. In the case of copolymers with a chloride counterion, drug loading led to obtaining single systems transporting one type of drug. A special approach was the encapsulation of drugs in self-assembled conjugates with a pharmaceutical counterion, which resulted in doubly active systems with a pair of interacting drugs, i.e. an ionic one connected by a chemical bond and a non-ionic one physically interacting with the polymer matrix.

For this purpose, three model drugs were selected, i.e. isoniazid (ISO), rifampicin (RIF) and tazobactam (TAZ). The efficiency of the encapsulation process during self-assembly of selected graft copolymers and their conjugates was assessed based on the Drug-Loading Content (DLC) (Fig. 7). A positive effect of the pharmaceutical anion presence on the encapsulation efficiency was noted. Moreover, a relationship between the grafting degree and the nature of the drug on the encapsulation efficiency was observed. The hydrophilic ISO drug was significantly better encapsulated by systems with a higher DG, i.e. G6 and G7, compared to G4. In turn, encapsulation of drugs that are sparingly soluble in water, i.e. RIF and TAZ, turned out to be more effective in G4 systems with a lower DG. The obtained results confirm that the type of non-ionic drug in correlation with the structural parameters of the polymer matrix affects the DLC values.

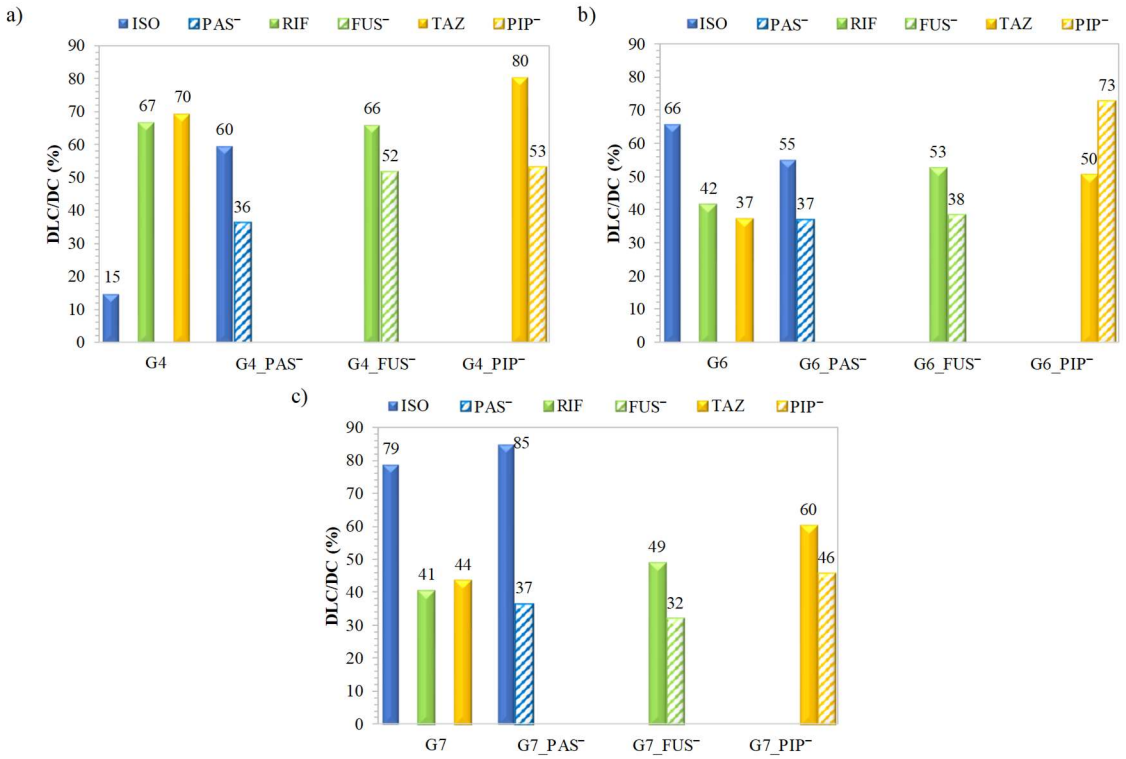

Figure 7. Contents of non-ionic drugs in single systems and contents of non-ionic drugs in dual systems vs. pharmaceutical anion content.

Studies on the size of nanoparticles of dual systems in aqueous solution showed that, as compared to single systems, they formed smaller structures (Fig. 8), i.e. 30-175 nm for PAS^-/ISO systems and 31-184 nm for FUS^-/RIF systems. A similar effect was obtained for the PIP^-/TAZ systems, in which the particle sizes reached 24-192 nm. Although they showed a greater tendency to aggregate, as indicated by the presence of a significant fraction >500 nm (46%), while in the case of the remaining systems, the aggregates occurred only in small amounts (<10%). The presented commentary refers only to the prevailing factions.

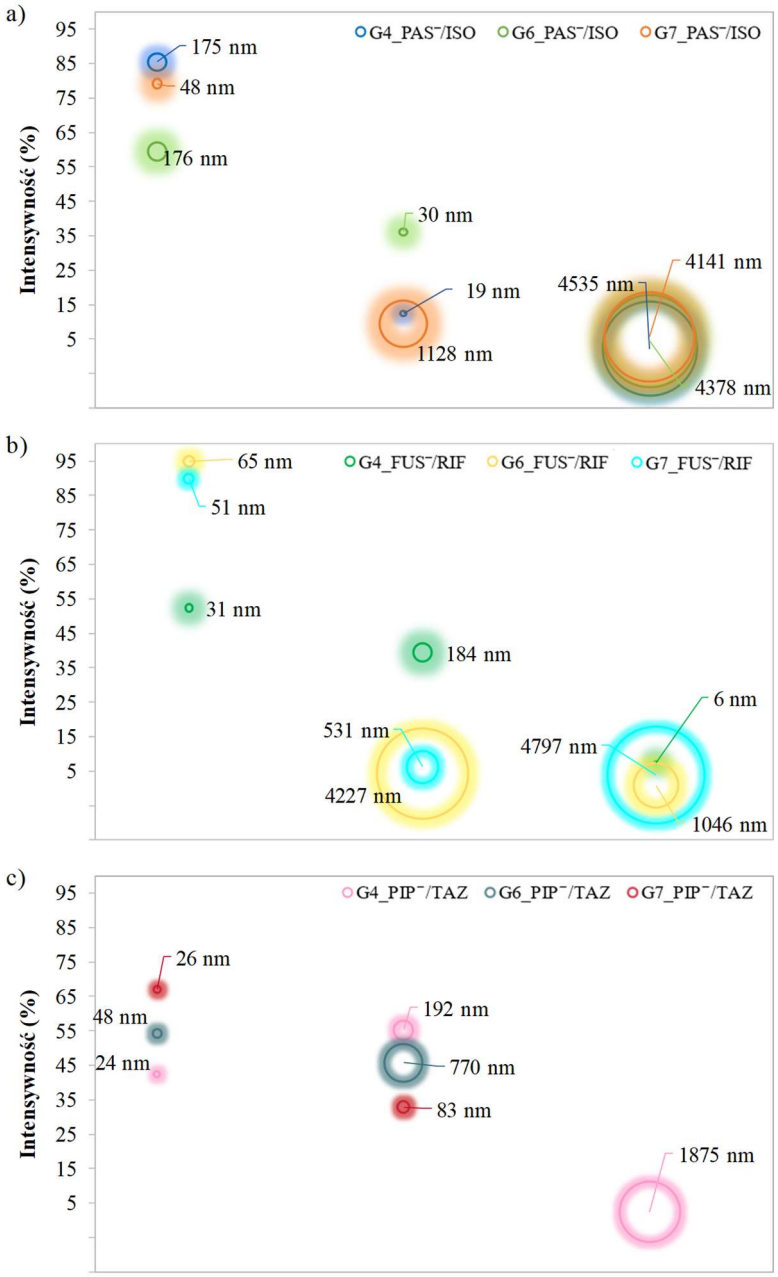

Figure 8. Hydrodynamic diameters (Dh) of polymer nanoparticles determined by DLS for dual systems.

Release of non-ionic drugs and co-release of a pair of drugs with a synergistic effect (P.3.; P.4.; P.5.)

The in vitro release process, in similarity to the conjugates with a pharmaceutical anion, was carried out in conditions imitating the environment of body fluids, i.e. PBS solution (pH = 7.4, 37°C). The obtained results showed that the presence of the encapsulated drug reduced the amount of the released ionic drug, as compared to single systems carrying pharmaceutical anions (Fig. 9a). The largest differences were noted in the case of PIP^- release, where after drug encapsulation during co-release, the pharmaceutical anion was released in a much smaller amount.

As in the case of ionic drugs, the release of non-ionic drugs depended strongly on both the polymer matrix and the nature of the drug (Fig. 9b). The most favourable matrix in terms of

the amount of released ISO was the G4 copolymer with the shortest side chains, lower degree of grafting and the highest ionic fraction content among the tested systems. In turn, drugs that were sparingly soluble in water, i.e. RIF and TAZ, were released in the highest rate from the G6-based system with the longest side chains, a higher degree of grafting, and the smallest TMAMA fraction.

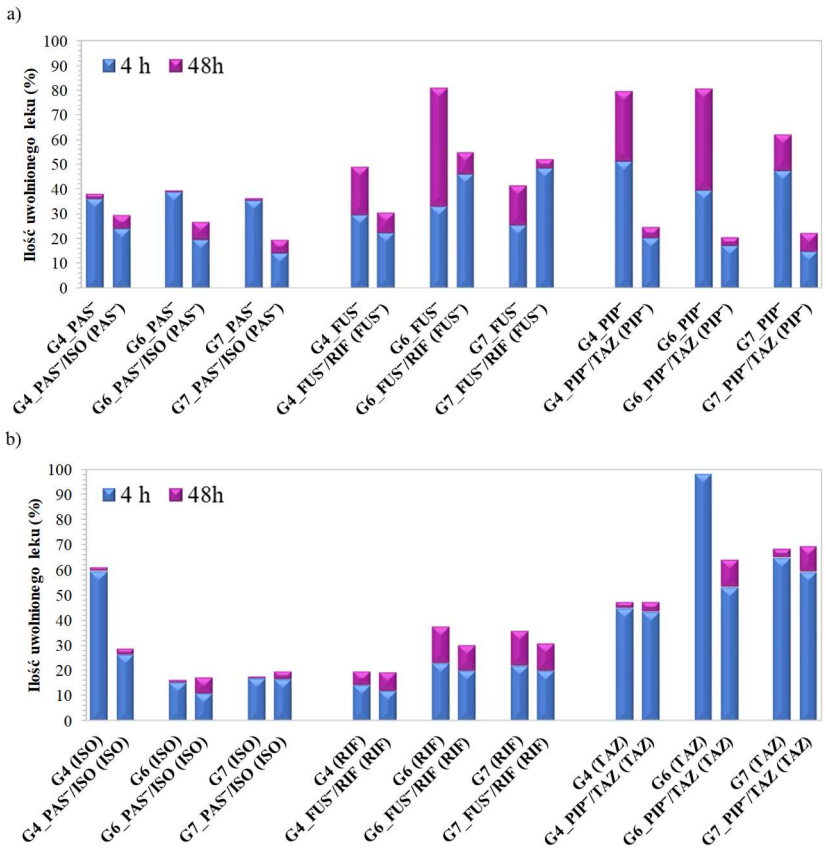

Figure 9. The amounts of released drugs in single vs. dual systems for a) ionic drugs b) non-ionic drugs after 4 and 48 h.

Biological assessment of drug delivery systems - cytotoxicity tests (P.6.; P.7.)

An evaluation of the cytotoxicity of drug delivery systems was carried out, based on colorimetric tests using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and flow cytometric analysis, including an apoptosis test and cell cycle analyses. Since the obtained carriers were tested for the transport of drugs used in the treatment of the lower respiratory tract, including tuberculosis, the model of human bronchial epithelial cells (BEAS-2B) and cancer lines, i.e. human basal alveolar epithelial cell adenoma (A549) and non-small cell lung cancer (H1299), were used to test cytotoxicity. Additionally, gene expression measurements for interleukins IL6 and IL8 were performed for linear copolymer systems.

In vitro cytotoxicity tests performed with the obtained polymer carriers confirmed the lack of cytotoxic effect on the normal BEAS-2B cell line, while conjugates with PAS^{-}, CLV^{-}, FUS^{-} and PIP^{-} showed a negligible effect on cell viability. In turn, the tested systems caused the proliferation of A549 and H1299 cancer cells. The demonstrated selectivity of action of most of the tested systems resulted in no significant changes for normal cell lines and a negative effect on cancer cells.

Summary and conclusions

The use of graft copolymer systems allowed for a slower and more controlled release process due to the greater stability of their micellar structure, compared to systems based on tangled chains of linear copolymers.

An appropriately large amount of drug introduced into the chains and its release in a satisfactory percentage ultimately ensured a relatively high concentration of drug released from the graft copolymers, which may guarantee the effectiveness of the therapy.

The efficiency of drug conjugation and encapsulation, as well as the (ionic and non-ionic) drug release rate can be regulated by the structure of the polymer carrier, where the density of the side chains in the polymer plays a special role, but at the same time the structure and nature of the drugs and their interaction in the matrix are important.

In vitro cytotoxicity studies of the obtained polymer carriers showed a negligible effect on normal BEAS-2B cell lines and cancer A549 cell proliferation. Considering that an immunologically weakened body during disease is susceptible to the cancer, the effect of selective action of drug delivery systems is extremely desirable. This indicated a high application potential as new alternative systems in the treatment of respiratory diseases due to the possibility of their wide use against pathogens or cancer cells.

The presence of choline units in the polymer matrix and the introduced antibacterial drugs make these systems have potential applications in the treatment of respiratory diseases, including combination therapy transporting a pair of synergistic drugs.

In the context of anti-tuberculosis treatment, their rapid action, completing a full cycle of effectiveness within four hours ensure an effective course of treatment. However, their use requires further tests, including detailed *in vivo* biological studies, which will fully confirm the possibility of using the obtained polymer drug delivery systems in the human body.

To sum up, in this research, graft copolymers with pharmaceutical counterions were designed, which seem to be promising drug carriers from the physicochemical point of view and in terms of cytotoxicity.