FACULTY OF BIOLOGY AND ENVIRONMENTAL PROTECTION
University of Lodz

InterDOC-STARt - Interdisciplinary Doctoral Studies at the Faculty of Biology and Environmental Protection, University of Lodz

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Properties and application of tyrosine-modified and unmodified polyethyleneimine polymers as siRNA carriers

Właściwości i zastosowanie polimerów polietylenoiminowych modyfikowanych i niemodyfikowanych tyrozyną jako nośników siRNA

The doctoral thesis

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Lodz, 2023

Firstly I would like to thank my Supervisors ***Prof. dr hab. Maksim Ionov*** and ***Prof. Dr. Achim Aigner*** for their outstanding guidance, assistance and support. I feel privileged to have had the opportunity to learn so much from them.

I would like to thank all members of Department of General Biophysics, especially ***Prof. dr hab. Maria Bryszewska*** for her substantive assistance and scientific atmosphere in our Department.

Thanks to ***Dr Sylwia Michlewska*** for her enthusiasm and irreplaceable assistance in microscopy analysis.

Thanks to ***Dr Alexander Ewe*** and ***Dr Michael Karimov*** who synthetized all modified polymers used in this study. I am grateful for your patience.

I would like to thank all my friends form the Department of General Biophysics, without you my PhD would never be so enjoyable as it turned to be.

At the end I would like to thank my family, especially to my parents and my fiancé. I could never thank you enough for what you have done for me.

Contents:

Financial sources:

Interdisciplinary doctoral studies InterDoc STARt on Faculty of Biology and Environmental Protection, UL

Project co-financed by the European Union from European Social Fund

Project co-financed from the Operational Program Knowledge Education Development, Priority Axis III Higher education for the economy and development, Measure 3.2 Doctoral studies Competition no. POWR.03.02.00-IP.08-00-DOK/16.

Research work was partially financed from a joint grant from the National Science Centre of Poland, project no: 2018/31/F/NZ5/03454, and the Deutsche Forschungsgemeinschaft (DFG; grant number AI 24/24-1.) within the "BEETHOVEN LIFE 1" program "Novel polymeric nanoparticles for pulmonary nucleic acid therapy – synthesis, toxicological analysis and biological / therapeutic assessment".

Scientific cooperation within the research:

Rudolf Boehm Institute for Pharmacology and Toxicology, Clinical Pharmacology, Medical Faculty, University of Leipzig

Introduction

Gene therapies are promising strategies for the treatment of diseases including cancer and neurodegenerative disorders (Falkenhagen and Joshi, 2018; Fleifel et al., 2018). Solutions based on RNA interference (RNAi) are currently broadly investigated (Kim et al., 2015; Song et al., 2018). RNAi interference may be directed against any gene of interest, offering some advantages over conventional drug-based treatment options (lower toxicity and reduced side effects). Additionally, due to the ability to treat so-called 'undruggable' diseases they may serve as a powerful therapeutic option (Babaei et al., 2017; Günther et al., 2011; Qian et al., 2017). RNA interference relies on the action of small interfering RNAs. They incorporate into the RISC complex and mediate RISC binding to the sequence-complementary mRNA what eventually leads to the cleavage and degradation of targeted mRNA. However, siRNA is unable to cross cellular membranes due to its molecular weight about 13 kDa and its negative charge. Additionally, siRNA must be protected from nucleases and degradation (Benfer and Kissel, 2012; Creusat et al., 2010; Nademi et al., 2020; Thanki et al., 2019). Due to their unique properties, viruses are considered as the most efficient gene vectors. Unfortunately, the usage of viral vectors is limited to some nucleic acids and does not allow the delivery of siRNA. Additionally, safety issues and a risk of immune reactivity are the main drawbacks of viral vectors. Therefore, non-viral platforms should be applied as an alternative for nucleic acid delivery (Dizaj et al., 2014; Kopatz et al., 2004; Li et al., 2017).

Nanoparticles are mainly transported into the cells by endocytosis (Kaksonen and Roux, 2018; Parton and Richards, 2003). For endocytosis, nanoparticles should be ideally around 150 nm in size; however, larger nanoparticles have been found to be efficiently taken up by cells as well (Kaksonen and Roux, 2018). Several types of endocytosis can be recognized and may affect the intracellular fate of nanoparticles. Clathrin-mediated endocytosis is a way found in all mammalian cells and is the best characterized. Nanoparticles are enclosed in 'clathrin-coated vesicles' and transported to the early endosomes. Early endosomes are transformed into late endosomes and eventually into lysosomes. Endosomal/lysosomal escape of nanoparticles may be limited due to the acidic conditions in lysosomes, with the pH value decreasing to pH 5, thus siRNA must be protected from degradation (Kaksonen and Roux, 2018).

Caveolae-mediated endocytosis is another mechanism mediating nanoparticle uptake. After their internalization, nanoparticles are enclosed in caveolin-coated vesicles called caveosomes. They are non-acidic and do not possess any hydrolytic enzymes. Complexes can be translocated directly to the Golgi or the endoplasmic reticulum, avoiding lysosomes (Kiss and Botos, 2009). Caveolae-mediated endocytosis may serve as an attractive alternative for cellular uptake, especially for delivery vectors which are unable to escape from the acidic environment of clathrin-coated endosomes/lysosomes (Parton and Richards, 2003; Yacobi et al., 2010).

Nanoparticles based on lipids, amino acids, dendrimers or polymers have been developed in order to facilitate nucleic acid transport into the cells. For efficient and safe gene delivery, synthetic nanocarriers should mimic the viral mechanisms (genetic material condensation and protection from degradation). Additionally, they should exhibit low toxicity and immunogenicity (Pandey and Sawant, 2016). The therapeutic application of siRNA requires a delivery system which mediates intracellular siRNA trafficking to its site of action (Creusat et al., 2010; Nademi et al., 2020; Thanki et al., 2019).

Cationic polymers are among the most efficient and non-toxic synthetic delivery agents. Their positive charge facilitates interaction with negatively charged nucleic acids and allows for efficient uptake by cells (Wong et al., 2007).

Polyethylenimines (PEI) are a class of synthetic polymers (branched and linear) broadly tested as vehicles for nucleic acid delivery (Lungu et al., 2016; Pandey and Sawant, 2016). Their structure and molecular weight determine their biological activity and efficacy. PEIs have been tested mainly as plasmid DNA nanovectors (Gosselin et al., 2001; Kichler et al., 2001; Sig et al., 2004). It was reported that linear PEIs were able to complex plasmid DNA whereas they poorly bound siRNA. In the contrary, branched PEIs bound effectively plasmid DNA as well as siRNA (Kwok and Hart, 2011; Sadeghpour et al., 2018). The structure of PEIs relies on the nitrogen atoms at every third position, giving a positive charge at physiological pH. Buffering capacity, which is crucial for the PEI mechanism of action, depends on the type and amount of amino groups on their surface. Linear PEIs possess primary and secondary amino groups, whereas branched PEIs have primary, secondary and tertiary amino groups in a 1:2:1 proportion, what improves their complexation properties. PEIs with different structure and molecular weight have been widely investigated. It was demonstrated that low molecular weight PEIs (600 and

1800 Da) did not have significant efficacy as a gene carriers, while PEIs with molecular weights around 25 kDa or higher exhibited a good gene delivery properties (Godbey et al., 1998; Kircheis et al., 2001). The mechanism of PEI/nucleic acid complex interaction with cells has been studied and it was found that syndecan receptors were responsible for the uptake of PEI-based complexes. Complexes could bind to heparan sulfate proteoglycans (HSPGs) found on syndecan. Next, syndecan clustering into rafts and phosphorylation mediated by PKC takes place. Eventually, actin is bound through mediator proteins and actin-mediated phagocytosis occurs (Kopatz et al., 2004).

A proton sponge effect has been described for PEIs (Creusat et al., 2010; Günther et al., 2011; Jin et al., 2018). They are partially protonated under physiological pH, and under acidic conditions in the lysosome they can accept protons due to their buffering capacity, thus protecting their cargo from lysosomal degradation. The influx of chloride ions and water eventually lead to endosome/lysosome disruption and a release of the complexes into the cytoplasm (Kopatz et al., 2004). Despite the advantages of PEIs, their usage is limited due to their cytotoxicity, which increases with molecular weight (Beyerle et al., 2011, 2009; Gholami et al., 2014). PEIs can be easily modified, which may improve their biocompatibility and transfection efficacy. PEI modifications with chitosan, dextran, leucine, histidine, arginine or fatty acids have been described and modified polymers were found to be more efficient and safer than non-modified PEIs (Aldawsari et al., 2011; Ewe et al., 2016). For example, hydrophobic modifications improved polymer interaction with nucleic acids and enhanced cellular uptake. PEGylated polymers exhibited lower toxicity, prolonged circulation half-life in the blood and increased serum tolerance. However, PEGylation disrupted DNA/RNA condensation and inhibited cellular uptake (Beyerle et al., 2010; Merkel et al., 2009; Saqafi and Rahbarizadeh, 2019).

Amino acid residues have been proposed for PEI modification (Chen et al., 2015; Karimov et al., 2021; Yu et al., 2017). It was reported that this approach offered some advantages over other modifications. Wang et al. applied cationic dendrimer modification with twenty amino acids (Wang et al., 2016). Anionic and hydrophilic amino acids were found to be inefficient for nucleic acid transport whereas hydrophobic and cationic amino acids improved complexation efficiency. It was demonstrated that some amino acids (tyrosine, phenylalanine, tryptophan, arginine, lysine or histidine) increased transfection efficacy.

Arginine and lysine modification enhanced DNA binding capacity, complex formation and uptake. Addition of histidine improved endosomal escape. Among all amino acids, the modification with hydrophobic amino acids – tyrosine, tryptophan and phenylalanine - were highlighted as most promising for improved nucleic acid delivery. Hydrophobic, aromatic amino acid modifications performed better as compared to their hydrophobic, aliphatic amino acid counterparts. It could be related to the more hydrophobic structure of aromatic ring, enhancing the interaction between complexes and membrane phospholipids and additionally improving endosomal escape (Creusat et al., 2010; Liu et al., 2010).

Every nanoparticle administrated to the blood is immediately covered by proteins, affecting pharmacokinetics, bio distribution and metabolism (Hidalgo et al., 2017; Klepac et al., 2018). The composition of proteins also affects the fate of polymeric nanoparticles after administration, thus protein-polymer interactions must be well studied. The protein corona on the NP surface dynamically changes over time, although some proteins - human serum albumin (HSA), fibrinogen and immunoglobulins - are ubiquitously found in the hard protein corona (De Paoli Lacerda et al., 2010). The protein corona is mainly composed of two different types, so-called 'hard corona' and 'soft corona'. The hard protein corona exhibits higher affinity to the nanoparticle, determines cellular interaction and is pivotal for drug/nucleic acid release. The soft protein corona contains proteins with lower affinity to the nanoparticle surface. This type of the corona and is characterized by higher exchange ratio (Konduru et al., 2017).

Albumins represents the most abundant group among all serum proteins. Their role is mainly the delivery of different substances including drugs and nanoparticles (Klajnert et al., 2003). Human serum albumin is a single peptide chain, consisting of 585 amino acids with a molecular mass around 66,000 Da. HSA contains three α-helical domains stabilized by 17 disulfide bonds (Yin et al., 2020). HSA is a heart shaped structure with length around 8 nm and 3 nm height (Picó, 1997). The presence of a hydrophobic cavity in the HSA structure is particularly important in drug delivery. Albumin is able to bind a wide range of compounds, including hormones, bilirubin, fatty acids and hemin. Commonly used drugs are able to bind to HSA in subdomains IIA and IIIA (Hegde et al., 2011; Klajnert et al., 2003; Mahesha et al., 2006). Protein adsorption onto the nanoparticle surface may change the conformation and alter protein function (Millan et al., 2018; Saptarshi et al., 2013). The type of interaction

between proteins and nanoparticles strongly depend on the nanoparticle's surface and protein properties (De Paoli Lacerda et al., 2010; Klepac et al., 2018). Loss of activity, denaturation, unfolding, aggregation and presence of intermediate states are the most frequently described conformational changes (Millan et al., 2018; Picó, 1997; Shcharbin et al., 2018; Wright et al., 2017). Some studies revealed that interactions could cause protein fibrillation (Sukhanova et al., 2019).

The aim of the work

The aim of the experiments was to compare the properties of linear and branched, tyrosine-modified and non-modified polyethyleneimines with different molecular mass. The comparison included biophysical and biological properties of PEIs. The ability to interact with human serum albumin was also tested for the most promising polymers, using biophysical methods. siRNA binding ability and biological relevance of siRNA delivery to the cells was assessed *in vitro* and *ex vivo*. The main research hypothesis assumed that tyrosine addition to the PEI structure significantly alters the properties of PEIs and makes them good candidates for small nucleic acid delivery. Figure 1 represents the comparison of polymers based on the proposed methodology.

In order to confirm the research hypothesis, the scientific work was divided into three tasks:

1. Investigation of biophysical properties of all PEIs, in order to identify and select the most promising polymers for further experiments.
2. Investigation of biophysical properties of siRNA/polymer complexes and their effectiveness *in vitro* and *ex vivo*.
3. Evaluation of the polymers' ability to interact with human serum albumin.

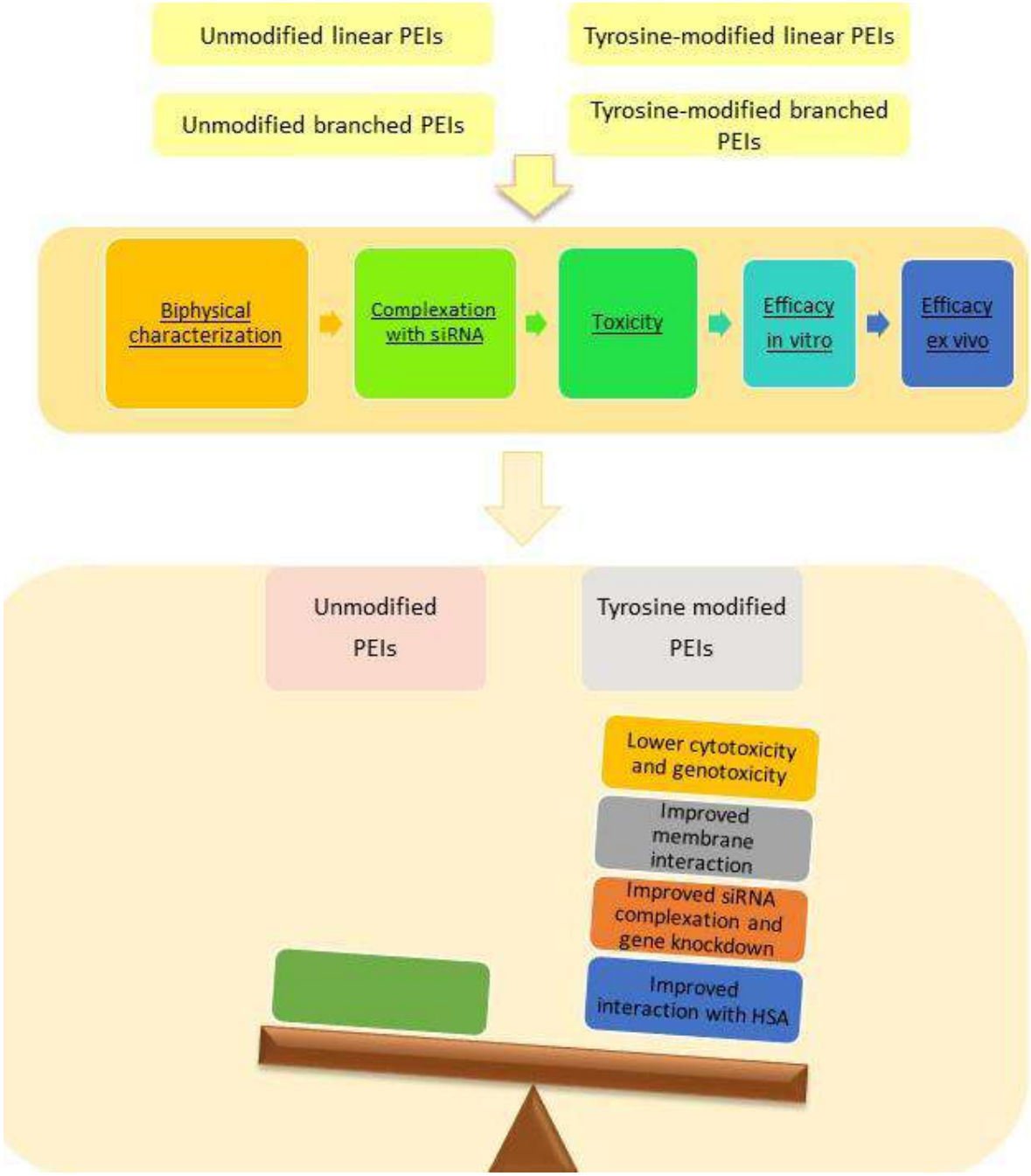

Figure 1 Schematic representation of the methods used in the study and the outcome from the direct comparison between non-modified and tyrosine modified polymers.

Materials

All polyethyleneimines (Tab. 1) were provided by Prof. Dr. Achim Aigner's group in aqueous solutions.

Table 1. Description of polymers used in the study.

Polymer description:	Abbreviation	Polymer description:	Abbreviation
Low molecular weight linear PEI with molecular mass 2.5 kDa	LP 2.5 kDa	Low molecular weight branched PEI with molecular mass 2 kDa	BR 2 kDa
Low molecular weight linear PEI with molecular mass 2.5 kDa, modified with tyrosine	LP 2.5Y	Low molecular weight branched PEI with molecular mass 2 kDa, modified with tyrosine	P 2Y
Low molecular weight linear PEI with molecular mass 5 kDa	LP 5 kDa	Low molecular weight branched PEI with molecular mass 5 kDa	BR 5 kDa
Low molecular weight linear PEI with molecular mass 5 kDa, modified with tyrosine	LP 5Y	Low molecular weight branched PEI with molecular mass 5 kDa, modified with tyrosine	P 5Y
Low molecular weight linear PEI with molecular mass 10 kDa	LP 10 kDa	Low molecular weight branched PEI with molecular mass 10 kDa	BR 10 kDa
Low molecular weight linear PEI with molecular mass 10 kDa, modified with tyrosine	LP 10Y	Low molecular weight branched PEI with molecular mass 10 kDa, modified with tyrosine	P 10Y
Low molecular weight linear PEI with molecular mass 25 kDa,	LP 25 kDa	Low molecular weight branched PEI with molecular mass 25 kDa	BR 25 kDa
Low molecular weight linear PEI with molecular mass 25 kDa, modified with tyrosine	LP 25y	Low molecular weight branched PEI with molecular mass 25 kDa, modified with tyrosine	P 25Y

In task 1 and 3, siRNAs purchased from Dharmacon (Lafayette, CO) were used for complexation (sequence presented in Tab. 2).

Table 2. Sequence of siRNA used in the experiments.

	Sense	**Antisense**
siLuc 2	5’-CGUACGCGGAAUACUUCGAdTdT-3’	5’-UCGAAGUAUUCCGCGUACGdTdT-3’
siLuc 3	5’-CUUACGCUGAGUACUUCGAdTdT-3’	5’-UCGAAGUACUCAGCGUAAGdTdT-3’
siEGFP	5’-GCAGCACGACUUCUUCAAGdTdT-3’	5’-CUUGAAGAAGUCGUGCUGCdTdT-3’
siGAPDH	5’-CCUCAACUACAUGGUUUACdTdT-3’	5’-GUAAACCAUGUAGUUGAGGdTdT-3’

Research models included:

- ✓ Human immortalized cancer cell lines from ATCC (MCF-7).
- ✓ The stably expressing luciferase/GFP cell lines H441/Luc, PC3/Luc/EGFP, HCT 116/Luc/EGFP (obtained from Prof. Dr. Achim Aigner’s group).
- ✓ Erythrocytes from peripheral human and mice blood for hemolysis assay.

- ✓ Liposomes DMPC:DPPG (9:1) were prepared for fluorescence anisotropy measurements.
- ✓ Tumor xenografts from A549 cell line were obtained from Balb/c mice for complex efficiency testing in an *ex vivo* model.
- ✓ Human serum albumin (fatty acid free, >98% purity) from Merck Millipore (Darmstadt, Germany).

Methods

Methods used during the study:

- ✓ Task 1 included: measurements of zeta potential and zeta size of polymers, transmission electron microscopy, hemolysis assay, fluorescence anisotropy, MTT assay, comet assay in alkaline version, agarose gel electrophoresis, LDH assay, JC-1 assay and luminometric measurements.
- ✓ Task 2 included: measurements of zeta potential and zeta size of polymer/siRNA complexes, circular dichroism, agarose gel electrophoresis, LDH assay, caspase-3/7 induction, mitochondrial membrane potential measurements, reactive oxygen species measurements, qPCR analysis of gene expression from in vitro and ex vivo experiments, luciferase activity measurements, confocal microscopy and flow cytometry.
- ✓ Task 3 included: measurements of human serum albumin, tryptophan and 1- nilinonaphthalene-8-sulfonic acid (ANS) fluorescence intensity, circular dichroism, transmission electron microscopy and Z-size measurements.

Results

In the first task, the properties of LP 2.5 kDa, LP 5 kDa, LP 10 kDa, LP 25 kDa, LP 2.5Y, LP 5Y, LP 10Y, LP 25Y, BR 2 kDa, BR 5 kDa, BR 10 kDa, BR 25 kDa, P 2Y, P 5Y, P 10Y and P 25Y were evaluated. Their biophysical features and toxicity were compared. Next, the ability to form complexes with siRNA were tested. Complex toxicity and efficacy were evaluated. Our results revealed that polymers formed small aggregates. Hydrodynamic diameter measurements revealed the linear, unmodified PEIs exhibited lower size values than tyrosine

modified linear PEIs. On the contrary, branched, unmodified polymers, were larger than tyrosine-modified, branched PEIs. All sixteen polymers were positively charged. The highest zeta potential values were observed for branched, tyrosine-modified PEIs.

In order to analyze the polymers' interaction with lipid components, hemolysis assays and anisotropy fluorescence measurements were applied. Linear, unmodified PEIs exhibited some slight interaction with hydrophilic parts of the liposomes and very weakly interacted with the hydrophobic region. In contrast, branched unmodified PEIs showed some hydrophobic interactions, whereas no effect on the hydrophilic region was detected. The introduction of tyrosine modification caused an increase in these interactions. Linear and branched, tyrosine-modified PEIs exhibited the potential to interact with both, the hydrophobic and hydrophilic region. Hemolysis assays confirmed the results from fluorescence anisotropy measurements. Linear and branched, tyrosine-modified PEIs caused stronger hemolysis than unmodified polymers, except for LP 25 kDa, which exhibited stronger hemolytic properties than LP 25Y. Generally, in lower concentrations, hemolysis rates were < 10%, after 3 hours incubation.

Cytotoxicity studies of polymers revealed that linear, tyrosine-modified PEIs were less toxic than linear, unmodified PEIs. Linear, tyrosine-modified PEIs showed significantly lower toxicity than their unmodified counterparts. Branched PEIs (unmodified and tyrosine modified) were generally less toxic than linear PEIs. Similarly, tyrosine modification almost completely abolished cytotoxicity. Comet assays revealed a significant genotoxic effect of linear, unmodified PEIs. DNA damage was reduced when linear, tyrosine modified PEIs were used. Tyrosine modification of branched PEIs did not affect the DNA damage potential, since unmodified, branched PEIs showed very low genotoxicity.

The next part of task 1 included complexation experiments with siRNA. It was confirmed by agarose gel electrophoresis that unmodified polymers did not form complexes with siRNA. In turn, all tyrosine modified polymers bound siRNA at the investigated ratios. The complexation process was confirmed by fluorescence polarization assay. Presented results indicated that tyrosine modified polymers bound siRNA at a polymer/siRNA ratio of 2.5 and increasing concentration of polymers did not alter binding efficacy.

PEI/siRNA complexes of tyrosine modified PEIs caused a low toxicity in H441 cell line where the damage was comparable to untreated cells. On the other hand, the time-

dependent decrease in mitochondrial membrane potential was detected for LP 5Y/siRNA and LP 10Y/siRNA complexes.

A concentration-dependent transfection efficacy of tyrosine-modified PEI/siRNA complexes was evaluated. Complexes of linear, tyrosine modified polymers were more efficient in luciferase gene expression knockdown than complexes with branched, tyrosine modified polymers. LP 5Y/siRNA, LP 10Y/siRNA, P 5Y/siRNA and P 10Y/siRNA complexes exhibited a 50% decrease in luciferase activity even when the lowest concentrations were used. The LP 10 kDa/siRNA complex was tested as a control. Some knockdown effect was observed, but the toxicity was very high.

During the first task we confirmed that tyrosine modified PEIs serve as a better nanocarrier for siRNA delivery than unmodified polymers, thus they were chosen for the experiments in the next tasks.

Task 2 aimed to further investigate the properties, toxicity and efficacy of the complexes. Dynamic light scattering was performed to evaluate the size of formed complexes. It was revealed that tyrosine modified polymers formed stable complexes with siRNA at a ratio 2.5:1, under different buffer conditions. Complexes at ratio 2.5:1 generally exhibited a positive surface charge. At higher ratios, all complexes were positively charged.

Electrophoretic studies reveled that complexes were formed at 2.5:1 ratio. They exhibited strong stability, with heparin only partially being able to release siRNA from the complex. SiRNAs in the complexes were also protected against RNAse digestion.

Circular dichroism was applied for a better understanding of polymer interactions with the siRNA structure. Changes in siRNA secondary structure were seen with increasing polymers concentrations. Again, it was confirmed that a polymer:siRNA ratio 2.5:1 was sufficient for complexation.

As we established the biophysical properties of complexes and the optimal ratio for complexation, toxicity and efficacy studies were performed. The cytotoxic potential of complexes was assessed by measuring the level of membrane damage. All tyrosine modified PEIs/siRNA complexes exhibited low levels of membrane damage. The linear, unmodified 10 kDa PEI/siRNA complex was used as a control and it caused a membrane damage comparable to the positive control (cells treated with Triton X-100). Complexes exhibited a low level of

caspase-3/7 induction, thus their apoptotic effect was considered very low. For comparison, linear, unmodified LP 10 kDa/siRNA complex showed >3-fold increase in caspase-3/7 induction over control cells. The effect of complexes on mitochondrial membranes was generally minimal, except for LP 5Y/siRNA complexes, which caused some mitochondrial membrane disruption. Complexes from LP 10Y and P 10Y caused an increase in the level of oxidative stress, while LP 5Y and P 5Y complexes led to slightly elevated ROS level, but the increase was not significant.

The efficacy of gene expression knockdown was analyzed by RT-qPCR, flow cytometry, confocal microscopy and luminescent measurements. All methods confirmed that tyrosine modified PEIs/siRNA complexes at a ratio 2.5:1 were very efficient *in vitro*. In *ex vivo* experiments, the most effective complexes were the ones containing LP 10Y/siRNA.

Summarizing task 2, we revealed that all tyrosine-modified polymers could complex siRNA, with sizes and positive surface charges allowing them to enter the cells. During the interaction, changes in the secondary structure of siRNA occurred. Complexes were stable under physiological conditions and were resistant to RNAse digestion. Their toxicity and efficacy profile confirmed that they may serve as promising vectors for siRNA. Due to their relatively high activity ex vivo, LP 10Y was chosen as the best candidate for siRNA delivery among the other tyrosine modified polymers.

Our last goal was to provide more information regarding the polymer interaction with human serum albumin. Spectrofluorometric methods were applied to investigate the binding affinity and the nature of interactions between tyrosine modified polymers and albumin. The most promising polymers from previous studies (LP 5Y, LP 10Y, P 5Y and P 10Y) were chosen for this task. It was observed that linear PEIs exhibited generally higher binding affinity towards albumin ($> 10^5\ M^{-1}$) than branched PEIs ($> 10^4\ M^{-1}$). Stern Volmer quenching constants for albumin and tryptophan alone were comparable, suggesting that polymers could bind to the protein region near the tryptophan residue. Bimolecular quenching constant values were higher than the maximum of diffusion-controlled quenching mechanism ($10^{10}\ M^{-1}s^{-1}$), thus a static mechanism of fluorescence quenching occurred. Binding affinities decreased with increasing temperatures, thus we decided to evaluate thermodynamic parameters for polymer interactions with albumin.

The results revealed that enthalpy and entropy values for LP 5Y, P 5Y and P 10Y complexes were negative, suggesting that Van der Waals and hydrogen bonding are a main forces engaged in complex formation. For polymer LP10Y, the entropy value was positive and enthalpy was negative, indicating that hydrophobic interactions and hydrogen bonding are the predominant molecular forces. Gibbs free energy values were negative for all polymers, thus we assumed that complex formation was spontaneous. Additionally, polymers changed the fluorescence intensity of ANS, what suggests the polymer interactions to occur around the hydrophobic pocket of albumin. Circular dichroism measurements of the protein secondary structure revealed that the changes occurred when increasing amounts of polymers were added to the protein. The changes were more profound in the case of branched polymers, with a higher level of α-helix loss and increase in random coil structures, than in the case of linear polymers. Size measurements and transmission electron microscopy analysis confirmed that polymers formed larger structures with human serum albumin. On the contrary, size measurements for unmodified polymers showed that they did not form such structures with albumin.

Conclusions

Summarizing all obtained results, we can conclude that:

1. Tyrosine modification alter the properties of polyethyleneimines by reducing the toxicity, improving the interactions with lipid structures and facilitating siRNA complexation.
2. Tyrosine modified polymers form stable complexes with siRNA, resistant to digestion and with low potential towards cell damage and mitochondrial membrane disruption as well as induction of apoptosis and oxidative stress. Furthermore, complexes show high gene expression knockdown potential *in vitro*. LP 10Y/siRNA complexes were also useful for *ex vivo* gene knockdown.
3. Tyrosine modified polymers show an ability to interact with human serum albumin what may affect their toxicity, bio distribution and biological activity *in vivo*.

Summary

RNA interference (RNAi)-based therapy is a promising treatment option for many diseases, including cancer, neurodegenerative disorders and auto-immune diseases. RNAi

may target a selected gene, allowing to avoid adverse effects related to standard treatment options. Due to its specificity, RNAi technology may be useful for the treatment of so-called 'undruggable' genes. Strategies based on RNAi have been broadly explored. Currently, increasing numbers of studies focus on the improvement of nanomedicines. Among many available nanocarriers for siRNA, cationic polymers gain special attention, due to efficient and non-toxic delivery mechanisms. As positively charged particles, they show improved interaction with negatively charged cell membranes and enhanced cellular uptake.

The aim of the current work was to describe the properties of polyethyleneimines (PEIs) and their tyrosine modified counterparts. The first step focused on the evaluation of the biophysical properties of PEIs, their toxicity and ability to form complexes with siRNA. We found that the tyrosine modification altered PEI properties, facilitated the interaction with membranes, reduced cytotoxicity and genotoxicity. Linear and branched, tyrosine modified polymers complexed siRNA at favorable mass ratios which allowed significant gene expression knockdown with a limited cytotoxicity. In step 1, we confirmed that tyrosine modification is crucial for improving PEI properties and complexation with siRNA. Due to these favorable properties, tyrosine modified PEIs were investigated in the further steps.

The second task included a better understanding of complex formation, toxicity mechanisms and gene knockdown efficacy. We revealed that the siRNA secondary structure underwent conformational changes upon addition of polymers. Complexes exhibited a positive charge, what suggests that they would be able to enter the cells. Stability of complexes and protective effects against nuclease digestion were confirmed. Toxicity studies revealed that complexes did not affect cellular and mitochondrial membranes, and did not induce apoptosis. In knockdown experiments, levels of GAPDH, EGFP and Luc3 genes were significantly decreased in vitro. In ex vivo studies, the level of GAPDH was significantly decreased with LP 10Y/siRNA complexes, making them the most promising nanovectors among tyrosine modified PEI.

The third task aimed to describe biophysical properties of tyrosine modified polymer: human serum albumin interactions. Human serum albumin is known as transporter protein for many chemical compounds that are administered to the blood stream. Our goal was to compare complex formation when using different types of polymers. Tyrosine modified polymers were able to interact with human serum albumin; size measurements and

transmission electron microscopy confirmed this complexation, which was not seen for unmodified polymers. A tryptophan residue was engaged in complex formation. Polymers were also able to interact with the hydrophobic pocket of human serum albumin. Based on thermodynamic studies, we assumed that Van der Waals and hydrogen bonding were a predominant molecular forces. Analysis of Gibbs free energy values revealed that complex formation is a spontaneous process. We believe that for a better understanding of the mechanism of interaction of tyrosine modified polymers with other human plasma components, further studies must be performed.

Summarizing, tyrosine modification significantly affected the properties of polyethyleneimines, changing their toxicity profile, siRNA binding capacity and interaction with human serum albumin. Tyrosine modified polymers performed better as siRNA carriers, exhibiting acceptable toxic effects and a very promising knockdown efficiency. The above-mentioned properties and the ability to interact with human serum albumin indicate that such polymers may be efficient in more advanced experimental models.

Streszczenie w języku polskim

Terapia oparta na interferencji RNA (RNAi) jest obiecującą opcją w leczeniu wielu chorób nowotworowych, zaburzeń neurodegeneracyjnych i chorób autoimmunologicznych. Interferencja RNA może być ukierunkowana na wybrany gen, co pozwala uniknąć działań niepożądanych związanych ze stosowaniem standardowych metod leczenia. Ze względu na mechanizm działania, technologia RNAi może znaleźć zastosowanie w terapii tzw. chorób „nieuleczalnych”. Rozwiązania oparte na wyciszeniu ekspresji genów od wielu lat pozostają przedmiotem szeroko zakrojonych badań. Obecnie coraz więcej badań koncentruje się na udoskonalaniu nanocząstek. Spośród wielu dostępnych nanonośników dla siRNA na szczególną uwagę zasługują polimery kationowe, ze względu na wydajne i nietoksyczne mechanizmy dostarczania siRNA do komórek. Jako dodatnio naładowane cząstki poprawiają interakcję siRNA z ujemnie naładowaną błoną komórkową i zwiększają wychwyt komórkowy.

Celem niniejszej pracy było opisanie właściwości polimerów polietylenoiminowych (PEI) oraz ich odpowiedników modyfikowanych tyrozyną. W pierwszym etapie skupiono się na ocenie właściwości biofizycznych PEI, ich toksyczności oraz zdolności do tworzenia

kompleksów z siRNA. Potwierdzono, że modyfikacja tyrozyną zmieniła właściwości PEI, ułatwiła interakcję z błonami, zmniejszyła cytotoksyczność oraz genotoksyczność polimerów. Liniowe i rozgałęzione, modyfikowane tyrozyną polimery kompleksowały siRNA w korzystnych stosunkach masowych, co umożliwiło obniżenie poziomu mRNA wybranych genów przy ograniczonej cytotoksyczności względem komórek. Podsumowując ten etap, potwierdzono, że modyfikacja tyrozyną ma kluczowe znaczenie dla właściwości PEI i kompleksowania z siRNA. Ze względu na ujawnione właściwości polimery modyfikowane tyrozyną przeznaczono do dalszych analiz.

Drugie zadanie badawcze obejmowało lepsze poznanie mechanizmów kompleksacji, toksyczności i wyciszenia ekspresji genów. Potwierdzono, że struktura drugorzędowa siRNA uległa zmianom konformacyjnym pod wpływem polimerów. Kompleksy polimer/siRNA wykazywały ładunek dodatni, co sugerowało, że będą w stanie wniknąć do komórek. Dodatkowo, potwierdzono stabilność kompleksów oraz działanie protekcyjne przed trawieniem nukleazą. Badania toksyczności wykazały, że kompleksy nie uszkadzają błony komórkowej i błony mitochondrialnej oraz nie indukują apoptozy. Przy zastosowaniu kompleksów LP 5Y/siRNA, LP 10Y/siRNA, P 5Y/siRNA oraz P 10Y/siRNA w warunkach in vitro poziom ekspresji genów GAPDH, EGFP, Luc3 istotnie zmalał. W badaniach ex vivo poziom GAPDH znacząco spadł w przypadku zastosowania kompleksów LP 10Y/siRNA. To właśnie liniowy, modyfikowany tyrozyną polimer 10 kDa wykazał najbardziej obiecujące właściwości w dostarczaniu siRNA do komórek.

Trzecie zadanie miało na celu opisanie interakcji polimerów modyfikowanych tyrozyną i albuminy ludzkiej. Albumina jest znana jako transporter większości związków chemicznych, które są podawane do krwioobiegu. Naszym celem było porównanie kompleksów tworzonych między albuminą a polimerami LP 5Y, LP 10Y, P 5Y oraz P 10Y. Polimery modyfikowane tyrozyną były zdolne do interakcji z ludzką albuminą. Pomiary średnicy hydrodynamicznej i transmisyjna mikroskopia elektronowa potwierdziły kompleksowanie, czego nie zaobserwowano dla niezmodyfikowanych polimerów. Wykazano, że tryptofan w pozycji 214 (Trp 214) jest zaangażowany w tworzenie kompleksu. Polimery były również zdolne do interakcji z kieszenią hydrofobową albuminy. Na podstawie badań termodynamicznych zasugerowaliśmy, że dominującymi siłami molekularnymi są oddziaływania Van der Waalsa i wiązania wodorowe. Analiza wartości energii swobodnej

Gibbsa wykazała, że tworzenie kompleksów jest procesem spontanicznym. Uważamy, że dla lepszego zrozumienia interakcji polimerów modyfikowanych tyrozyną z innymi składnikami osocza ludzkiego należy przeprowadzić bardziej zaawansowane badania.

Podsumowując, modyfikacja tyrozyną znacząco wpłynęła na właściwości polimerów polietylenoiminowych, zmieniając ich profil toksyczności, zdolność wiązania siRNA oraz interakcję z albuminą ludzką. Polimery modyfikowane tyrozyną działały efektywniej jako nośniki siRNA, wykazując niski efekt toksyczny i bardzo obiecującą skuteczność wyciszenia poziomu genu. Powyższe właściwości oraz zdolność do interakcji z albuminą wskazują, że kompleksy polimerów modyfikowanych tyrozyną mogą być skuteczne w bardziej zaawansowanych modelach eksperymentalnych.

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Academic achievements

Research papers included in PhD thesis:

1. **Kubczak, M**, Michlewska, S, Karimov, M, Ewe, A, Noske, S, Aigner, A, Bryszewska, M, Ionov, M, "Unmodified and tyrosine-modified polyethylenimines as potential carriers for siRNA: biophysical characterization and toxicity", *International Journal of Pharmaceutics*, 121468, 0378-5173, **2022**. **IF = 6.51, 100 p. MEiN**

2. **Kubczak, M**, Michlewska, S, Karimov, M, Ewe, A, Aigner, A, Bryszewska, M, Ionov, M, "Comparison of tyrosine-modified low molecular weight branched and linear polyethylenimines for siRNA delivery", *Nanotoxicology*, Nov-Dec;16(9-10): 867-882, **2022**. **IF= 5.881. 140 p. MEiN**

3. **Kubczak M**, Grodzicka M, Michlewska S, Karimov M, Ewe A, Aigner A, Bryszewska M, Ionov M, „The effect of novel tyrosine-modified polyethyleneimines on human albumin structure –thermodynamic and spectroscopic study”, *Colloids and Surfaces B: Biointerfaces*, 227,113359, **2023**. **IF = 5.999, 100 p. MEiN**

4. **Kubczak, M**, Michlewska, S, Bryszewska, M, Aigner, A, Ionov, M, "Nanoparticles for local delivery of siRNA in lung therapy", *Advanced Drug Delivery Reviews*, 114038, 0169-409X, **2021. IF = 16.04, 200 p. MEiN**

Total IF: 34.43

Total MEiN points: 540

Other research papers, not included in PhD thesis:

1. Michlewska S, Garaiova Z, Šubjakova V, Hołota M, **Kubczak M**, Grodzicka M, Okła E, Naziris N, Balcerzak Ł, Ortega P, de la Mata F.J, Hianik T, Waczulikova I, Bryszewska M, Ionov M, "Lipid-coated ruthenium dendrimer conjugated with doxorubicin in anticancer drug delivery: Introducing protocols", *Colloids and Surfaces B: Biointerfaces,* **2023. IF= 5.999, 100 p. MEiN**
2. Michlewska S, Maly M, Wójkowska D, Karolczak K, Skiba E, Hołota M, **Kubczak M**, Ortega P, Watala C, Javier de la Mata F, Bryszewska M, Ionov M, "Carbosilane ruthenium metallodendrimer as alternative anti-cancer drug carrier in triple negative breast cancer mouse model: A preliminary study", *International Journal of Pharmaceutics*, Apr 5;636:122784, **2023. IF= 6.51, 100 p. MEiN**
3. **Kubczak M**, Khassenova AB, Skalski B, Michlewska S, Wielanek M, Skłodowska M, Aralbayeva AN, Nabiyeva ZS, Murzakhmetova MK, Zamaraeva M, Bryszewska M, Ionov M, "Hippophae rhamnoides L. leaf and twig extracts as rich sources of nutrients and bioactive compounds with antioxidant activity" *Scientific Reports*, Jan 20;12(1):1095, **2022. IF= 4.996, 140 p. MEiN**
4. **Kubczak M**, Szustka A, Rogalińska M, "Molecular Targets of Natural Compounds with Anti-Cancer Properties", *International Journal of Molecular Sciences*, Dec 20;22(24):13659, **2021. IF= 6.208, 140 p. MEiN**
5. Karimov M, Schulz M, Kahl T, Noske S, **Kubczak M**, Gockel I, Thieme R, Büch T, Reinert A, Ionov M, Bryszewska M, Franke H, Krügel U, Ewe A, Aigner A, "Tyrosine-modified linear PEIs for highly efficacious and biocompatible siRNA delivery in vitro and in vivo" *Nanomedicine: Nanotechnology, Biology and Medicine*, Aug;36:102403, **2021. IF= 6.14, 140 p. MEiN**
6. Michlewska S, Maroto M, Hołota M, **Kubczak M**, Sanz Del Olmo N, Ortega P, Shcharbin D, de la Mata FJ, Bryszewska M, Ionov M, "Combined therapy of ruthenium dendrimers and anti-cancer drugs against human leukemic cells", *Dalton Transactions*, Jul 13;50(27):9500-9511, **2021. IF= 4.569, 140 p. MEiN**
7. **Kubczak M**, Khassenova A.B, Skalski B, Michlewska S, Wielanek M, Aralbayeva A.N, Murzakhmetova M.K, Zamaraeva M, Skłodowska M, Bryszewska M, Ionov M,

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8. **Kubczak M**, Szustka A, Błoński JZ, Gucký T, Misiewicz M, Krystof V, Robak P, Rogalińska M, "Dose and drug changes in chronic lymphocytic leukemia cell response in vitro: A comparison of standard therapy regimens with two novel cyclin-dependent kinase inhibitors" *Molecular Medicine Reports*, May;19(5):3593-3603, 2019. **IF= 2.100, 70 p. MEiN**
9. Hołota M, Magiera J, Michlewska S, **Kubczak M**, Del Olmo NS, García-Gallego S, Ortega P, de la Mata FJ, Ionov M, Bryszewska M. "In Vitro Anticancer Properties of Copper Metallodendrimers" *Biomolecules*, Apr 18;9(4):155, **2019. IF= 4.694, 100 p. MEiN**
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Total IF (calculated based on IF in the year of publication): 83.757

Total IF of publications included in PhD thesis: 34.43

Total IF of publications not included in PhD thesis: 49.327

Total MEiN points: 1740

Total MEiN points of publications included in PhD thesis: 540

Total MEiN points of publications not included in PhD thesis: 1200

H-index (according to WoS): 6

Citation number: 84

Number of conference abstracts: 18

Active participation on the conferences:

1. **Kubczak M**, Szustka A, Gucký T, Krystof V, Błoński J, Robak T, Rogalińska M, Nowe pochodne roskowityny jako induktory apoptozy w komórkach przewlekłej białaczki limfocytowej, III Ogólnopolska Konferencja Doktornatów Nauk o Życiu, Bioopen, 11-12.05.2017, Lodz, poster
2. Szustka A, **Kubczak M**, Błoński J, Góralski P, Robak T, Rogalińska M. Analiza potencjału antynowotworowego związków pochodzenia naturalnego. III Ogólnopolska Konferencja Doktornatów Nauk o Życiu, Bioopen, 11-12.05.2017, Lodz, poster
3. **Kubczak M**, Szustka A, Gucký T, Krystof V, Błoński J, Robak T, Rogalińska M, Novel roscovitine derivatives as promising drugs for CLL treatment, 9th International Conference of Contemporary Oncology, 22-24.03.2017, Poznan, poster
4. Szustka A, **Kubczak M**, Błoński J, Góralski P, Robak T, Rogalińska M. Natural compounds as a potential anticancer agents for CLL. 9th International Conference of Contemporary Oncology, 22-24.03.2017, Poznan, poster
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10. **Kubczak M,** Aleksandra Szustka A, Czy sygnały wysyłane przez komórki apoptotyczne mogą działać oknogennie? X Interdyscyplinarna Konferencja Naukowa TYGIEL. 17-18.03.2018, Lublin, oral presentation
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15. **Kubczak M**, Grodzicka M, Ionov, M, Polimery modyfikowane tyrozyną jako nośniki siRNA, "XIV Ogólnokrajowa Konferencja Młodzi Naukowcy w Polsce Badania i Rozwój" 26.11.2021 (on-line), poster
16. **Kubczak M**, Karimov M, Ewe A, Miłowska K, Aigner A, Ionov M, Bryszewska M, "Tyrosine-modified polyethyleneimines as promissing sirna carriers in lung cancer terapy", 18th Congress of the Polish Biophysical Society, Warsaw, 06-09.09.2022, poster
17. **Kubczak M**, Michlewska S, Karimov M, Ewe A, Aigner A, Bryszewska M, Ionov M, „The efficacy of vasohibin 2 gene expression knockdown and biological relevance in pancreatic cancer cells and tumor associated macrophages", ESMO Immuno-Oncology Annual Congress, Geneva, 07-09.12.2022, poster
18. **Kubczak M**, Michlewska S, Miłowska K, Karimov M, Ewe A, Aigner A, Bryszweska M, Ionov M. Tyrosine-modified polyethyleneimines serve as efficient and non-toxic sirna

carriers. 25th International Conference on Redox Medicine 2023 June 21-23 – Paris, France, poster

Other scientific activities:

Participation in the research projects:

1. Tyrosine-modified polyethylenimine polymers as a promising vectors for siRNA delivery in synergistic cancer immunotherapy. 01.02.2021 - 31.01.2024 – NCN PRELUDIUM 19 - 2020/37/N/NZ5/02359 – Principal Investigator
2. Novel polymeric nanoparticles for pulmonary nucleic acid therapy – synthesis, toxicological analysis and biological / therapeutic assessment, 01.01.2021 – 30.11.2013, BEETHOVEN Life 1 – Polish-German Funding Initiative – Researcher.
3. New drug delivery nanosystem combining liposomal and dendrimeric technology (liposomal locked-in dendrimers) for development of cytostatic drugs, 01.01.2019 – 31.12.2021, Bilateral exchange of researchers between Poland and Slovakia. NAWA, PPN/BIL/2018/1/00150/U/00001.

Internships:

1. A three-month research internship at the Institute of Pharmacology and Toxicology. Rudolf Boehm, University of Leipzig, Leipzig, Germany.

Workshops and events:

- Research assistance for an intern student in cooperation with the Department of Biotechnology, Faculty of Food Production, Almaty University of Technology, Kazakhstan, October-December 2018
- Workshop on gamification and IT tools, Lodz, 11 - 12.02.2019
- Research assistance for an intern student as part of international cooperation with the Institute of Pharmacology and Toxicology of the University of Leipzig, Germany, February - April 2019

- Training on writing research projects, Lodz, March/April 2019
- Workshop “Measure binding affinities and protein stability with Nanotemper Technologies”, Lodz, 24-25.04.2019
- Workshop ”Innovative solutions for mammalian cell culture”, Lodz, 23.05.2019
- Workshop „Using real time PCR technique in gene expression analysis", Warsaw, 24-26.10.2019
- Workshop on creativity and innovation, Lodz, 29.10.2019
- English workshop with native speaker, Lodz, 22.10 - 20.12.2019
- Workshop on writing scientific papers in English, Lodz, 04 - 05.11.2019
- Workshop on commercialization of research results, Lodz, 13.11 – 18.12.2019
- Workshop on self-presentation and public speaking, Lodz, 02. - 04.07.2020
- Workshop "NGS sequencing and data analysis", (on-line), 21-23.03.2022

Prizes and awards:

- Pro-quality scholarship for the best PhD Students: 2018/2019, 2021/2022, 2022/2023
- Rector’s scholarship for the best PhD Students: 2018/2019, 2019/2020

Advanced Drug Delivery Reviews 179 (2021) 114038

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/adr

Nanoparticles for local delivery of siRNA in lung therapy

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ARTICLE INFO

Article history:
Received 1 September 2021
Revised 26 October 2021
Accepted 1 November 2021
Available online 3 November 2021

Keywords:
Drug delivery
Nanoparticles
Nucleic acids administration
Lung cancer therapy

ABSTRACT

An overview of the application of natural and synthetic, non-viral vectors for oligonucleotide delivery into the lung is presented in this review, with a special focus on lung cancer. Due to the specificity of the respiratory tract, its structure and natural barriers, the administration of drugs (especially those based on nucleic acids) is a particular challenge.

Among widely tested non-viral drug and oligonucleotides carriers, synthetic polymers seem to be most promising. Unique properties of these nanoparticles allow for essentially unlimited possibilities regarding their design and modification. This gives hope that optimal nanoparticles with ideal nucleic acid carrier properties for lung cancer therapy will eventually emanate.

Contents

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https://doi.org/10.1016/j.addr.2021.114038
0169-409X/

1. Introduction

Lung cancer is one of the most common lethal diseases and equally affects men and women [1]. The two main types of lung cancer are non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). According to the literature, around 85% of all lung cancers consist non-small cell lung cancer, with its three subtypes adenocarcinoma, squamous cell carcinoma and large-cell carcinoma. Adenocarcinomas account for about 40% and originate from peripheral lung tissues, while 25% are squamous cell carcinomas originating from proximal airway epithelial cells and 15% are large cell carcinomas from epithelial cells [2]. Surgery, radio- and chemotherapy are the conventional and most widely used methods to treat lung cancer. However, due to limitations such as poor therapeutic efficacy, toxicity and adverse effects towards normal tissues, novel treatment strategies are needed. Beyond targeted therapies based on specific inhibitors, gene or oligonucleotide therapies are very promising [3–5]. In particular, this includes gene knockdown strategies.

Since its discovery in 1998, the therapeutic potential of RNA interference (RNAi) has been extensively studied [6–12]. Small interfering RNA (siRNA) for the induction of RNAi seem to be among the best candidates for nucleic acid-based therapeutics [13]. siRNA therapeutics have some benefits over conventional chemotherapies, including lower toxicity, less side effects and the possibility of targeting any gene of interest, also covering otherwise 'undruggable' genes [6,14,15] and thus making them very powerful therapeutic tools. Currently, various genes involved in cell growth and proliferation, cell survival, cell cycle, tumor vascularization, metastasis, and drug resistance are targets for RNAi approaches in lung cancer. Among others, siRNA-based therapeutics have been explored against PLK-1, Bcl-2, Akt1, BIRC5/CCNB, WT-1 or MDM2. Indeed, the silencing of these genes in lung cancer cells *in vitro* and *in vivo* has been shown to lead to cancer inhibition [16–22].

However, siRNA is negatively charged with a molecular weight around 13 kDa and thus unable to cross the cellular membrane. Additionally, siRNA is very sensitive to digestion by nucleases and to enzymatic/non-enzymatic degradation. Therefore, the therapeutic application of siRNA requires a delivery system which protects from nucleases, facilitates cellular uptake and mediates intracellular siRNA trafficking to its site of action [23–26]. With the exception of certain siRNA conjugates like e.g. GalNAc-siRNA for selective uptake in hepatocytes, most systems rely on nanoparticle systems, which are the topic of this review.

Different types of nanoparticles are intensively studied as potential oligonucleotide carriers. Nanoparticles may be transported into the cells by endocytosis [27,28]. For efficient endocytosis, nanoparticles should be around 150 nm in size; however, larger nanoparticles have been found to be efficiently taken up by cells as well [27]. Larger aggregates entering the respiratory tract must be redispersed into smaller particles prior to endocytotic uptake.

Mechanistically, several types of endocytosis can be distinguished, which may affect the eventual intracellular fate of the nanoparticle. Clathrin-mediated endocytosis is the best-characterized way found in all mammalian cells. Nanoparticles are enclosed in so-called 'clathrin-coated vesicles' and transported to the early endosomes. These early endosomes are then transformed into late endosomes and finally into lysosomes. Endosomal/lysosomal escape of nanoparticles may be limiting for nanoparticle efficacy and is thus a major issue to be solved, and since lysosomes are acidified with the pH value decreasing to pH 5, siRNA must be protected from degradation [27].

Caveolae-mediated endocytosis is another mechanism that is able to mediate nanoparticle uptake. After the internalization process, the nanoparticles are enclosed in caveolin-coated vesicles called caveosomes. The caveosomes are non-acidic and do not possess any hydrolytic enzymes, and nanoparticles may be translocated directly to the Golgi or the endoplasmic reticulum, avoiding lysosomes [28]. Caveolin is expressed in many types of cells, including pulmonary cells. Therefore, caveolae-mediated endocytosis may be an important mode of nanoparticle uptake the in pulmonary system. It may provide an attractive alternative for cell entry, especially when the delivery vectors cannot escape from the acidic environment of clathrin-coated endosomes [29,30].

The last mechanism of cellular uptake that may occur in the lungs is phagocytosis. This process can be carried out only by specialized cells and it is believed that the role of phagocytosis in nanoparticle delivery is limited, with the exception of therapies aiming to target alveolar macrophages. However, according to the literature, nanoparticles taken up by macrophages are eventually digested in phagocytosomes, thus highlighting that intracellular processing rather than uptake efficacy may be the limiting factor [31].

The systemic delivery of naked, unmodified siRNAs is practically impossible, while some efficacy was found upon their direct, local administration into the lungs [32–36]. In these studies, the siRNA was administered intranasally or intratracheally to mice and silencing of endogenous and viral genes was achieved. The fact that lower nuclease activity has been reported for lung tissues may have influenced the efficiency of naked siRNA [34]. Still, the use of naked siRNA directly applied to the lung will be limited to certain cases and for example not applicable for diseases related to deeper parts of the lungs. Aerosol inhalation is an administration route of choice, requiring a delivery agent [37]. Beyond the requirements stated above, nanoparticles should also protect the siRNA from shear force during the nebulizing process. The use of delivery vectors may also enhance the cell specific targeting, they should improve pharmacokinetics and facilitate the uptake [38–40]. Therefore, the development of powerful delivery vectors for pulmonary siRNA application is still a challenge.

2. Histological organization and function of the respiratory tract

The knowledge of the respiratory tract structure provides the basis for designing effective pulmonary delivery systems. The respiratory tract can be divided into two regions, i.e., the conducting airways and the respiratory area (Fig. 1).

The conducting airways are an air transport system and include the mouth/nasal cavity, pharynx, larynx, trachea, bronchi and bronchioles [55]. The respiratory zone is made up of respiratory bronchioles and alveoli [39].

The most prominent feature of the respiratory tract is its high degree of branching. Lungs resemble an inverted tree, where the conducting airways branch systematically over more than 20 generations, before reaching the alveoli. This allows to deliver warmed, humidified and filtered air to the gas exchange area [56]. More than 40 types of cells have been found in the pulmonary structures [57]. The conducting airway epithelium consists of basal, ciliated and secretory cells. Ciliated cells (20–60 μm) average approximately 50% of all cells from the conducting region. Each cell is coated by about 250 cilia [58]. Their function is moving the upper layer of mucus to the proximal airways, providing clearance and metabolism. The basal cells can be found near the basement membrane [58,59]. They were proposed to be progenitors of ciliated cells. The family of secretory cells includes Clara cells, mucous, goblet and serous cells. These cells secrete the molecules found in the mucus layer [59]. The structure of the airway epithelium in distal bronchioles is different. The cells are more cuboidal and non-ciliated [60]. Also, the thickness of the protective mucus

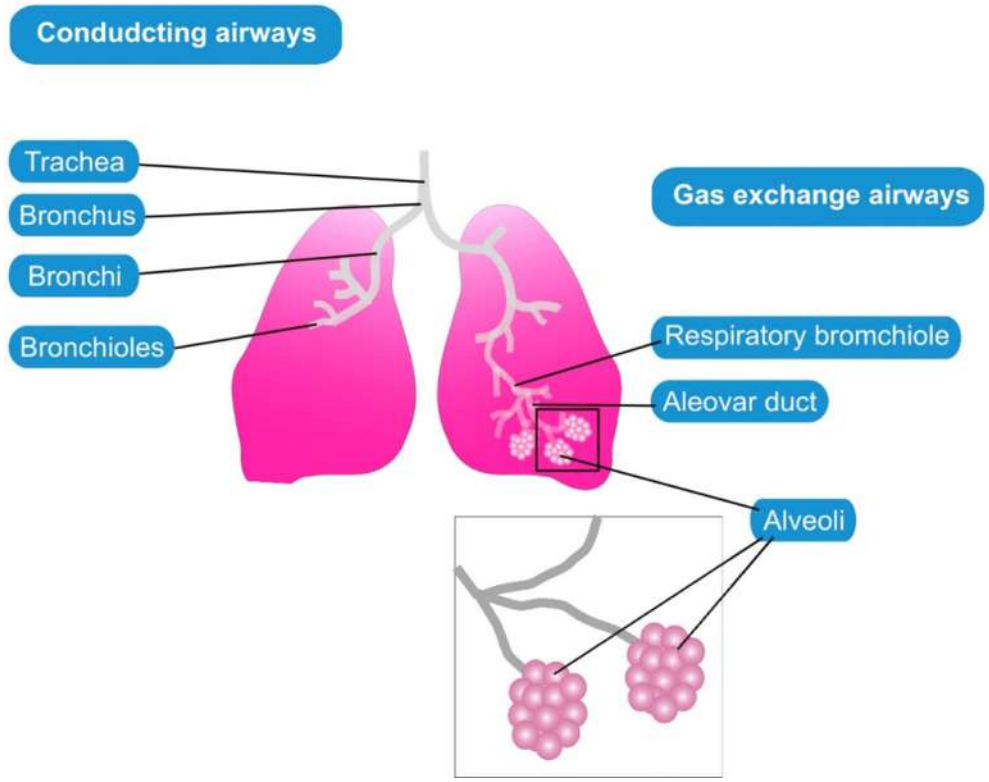

Fig. 1. The organization and structure of the respiratory tract.

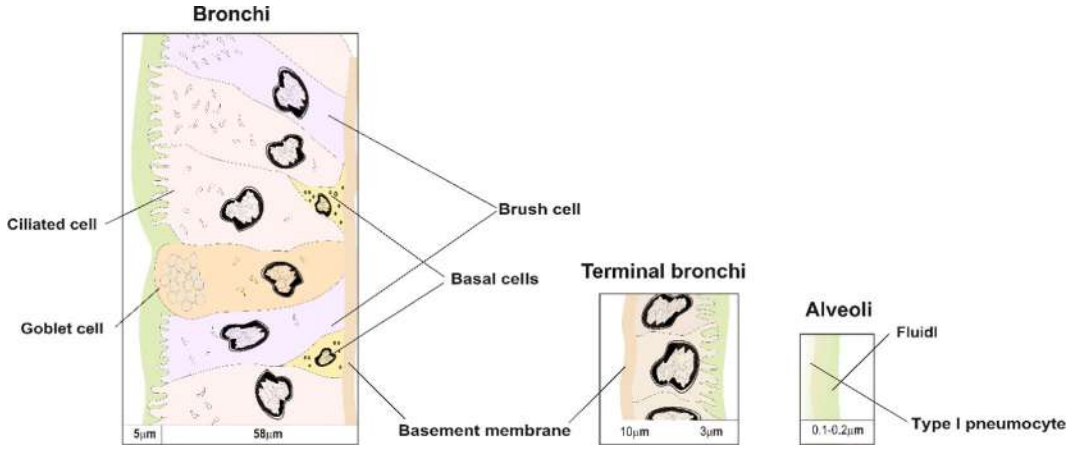

Fig. 2. Scheme of epithelial morphologies within different lung regions. The morphology of exemplary parts of the lung epithelium in bronchi, terminal bronchi and alveolar region is shown.

layer progressively decreases from 10 – 30 μm on the level of the trachea to 2–5 μm in the smaller alveoli (Fig. 2) [61].

Two cell types are mainly found in the alveolar region: type I and type II pneumocytes. Type I pneumocytes show a flattened shape with a thickness of about 2–3 μm around the perinuclear region and only 0.02 μm in the cell periphery [57]. Although type I pneumocytes account for only 10% of all alveolar cells, they cover more than 90% of the alveolar surface due to their structure [62,63]. They provide an excellent gas exchange platform between the alveolar zone and capillary vesicles [57].

Type II pneumocytes play a secretory role in the alveolar region [63–65]. Their shape is more cuboidal and for this reason, although they are more abundant than type I pneumocytes, they cover only 5% of the alveolar surface [63–65].

The histology of lungs in disease conditions is frequently altered, terminally leading to lung obstruction. Airway epithelium is composed of many cell types which exhibit impaired morphology during disease like chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), asthma or lung cancers. In COPD, obstruction and disappearance of bronchioles with a subsequent emphysema occur. Basal cell hyperplasia was the first lesion described for COPD. Recent studies also confirmed impaired stemness of basal cells during COPD. Goblet cell hyperplasia and metaplasia was identified in patients, as well as reduced numbers of ciliated cells. Remaining ciliated cells exhibited dysfunctional cilia, limited beating frequency and shortening. The appearance of squamous metaplasia in epithelium is related with COPD obstruction as well [66,67].

The main histological changes in asthma refer to changes in the cell composition in airway epithelium, where the most prominent lesion is goblet cell hyperplasia and metaplasia. Hypertrophy of submucosal glands and impaired goblet cell morphology result in excessive mucus production. Mucus under asthma conditions is enriched in overexpressed MUC5AC and MUC5B. Similarly to asthma, cystic fibrosis is also related to changes in the mucus content. Due to the stasis of viscous mucus, the mucociliary clearance is decreased, leading to higher risk of infection. The main lesions in the epithelium refer to the hyperplasia of goblet and basal cells [58,68–70].

As mentioned previously, lung cancer is a very heterogenic group, with each type exhibiting different morphological and histological patterns. Most lung cancers originate from airway epithelium. Due to their proliferative capacity, secretory and basal cells were proposed as progenitor cells for bronchogenic cancer types [71]. The main lesions in the cell morphology described for lung cancer pathology are squamous dysplasia, atypical adenomatous hyperplasia and *carcinoma in situ* [72]. Squamous cell carcinoma is typically located in the central zone of the lungs. It appears in the area of bronchus with altered, dysplastic, squamous epithelium. Small cell undifferentiated carcinomas originate from the bronchial epithelium in their first few branchings. Their morphology is very homogenous, they present as comma-shaped cells, small, round cells, fusiform cells and larger intermediate cells. On the other hand, large cell undifferentiated carcinomas are localized in the central part of the lungs. Their major morphological feature is the large size of cells and lack of differentiated histology. Adenocarcinomas are located in the central part of the lungs as well and originate mainly from the bronchial epithelium. They represent a broad range of secretory and glandular patterns [2,5,73].

The localization of cancer lesions in the lungs have been extensively studied. Most cancer lesions appeared in the bronchi (first, second, third-order), the rest was found in the distal parts [74]. Interestingly, cancers located peripherally were largely described as adenocarcinomas [75]. In another study, it was revealed that 50–60% of lung cancers are located in distal bronchi and bronchioles. Another group found that peripherally located tumors consisted only 8% among all lung tumors [73].

3. Pulmonary routes of nucleic acid administration

Three methods of delivering drugs to the lungs can be distinguished: intratracheal, intranasal and inhalation. However, the intratracheal route of administration is an invasive method and thus not practicable in humans. Gene or oligonucleotide delivery via the intranasal route has been tested in rodents, but results are difficult to extrapolate to humans due to their more complex respiratory system. The most promising non-invasive way for delivering drugs to the lung seems to be the inhalation route, also because of an efficient and rather simple setup [40,76–79]. Direct delivery of drugs to the respiratory system is achieved by inhalation of aerosols [80,81].

Several devices for inhalation exist, such as dose inhalers, dry-powder inhalers and nebulizers [67,69,82–85]. Dose inhalers are the most popular devices for inhalation. The therapeutic agents are delivered as a pressurized solution dissolved or suspended in propellants [67,83]. Propellants serve as substances that provide a driving pressure to aerosolize the therapeutic agents for inhalation. However, this method cannot be adapted to siRNA delivery since lung deposition after the usage of dose inhalers is poor [86]. Additionally, the propellants exert negative effects on the environment [67] and their poor compatibility with the therapeutic formulation may be an additional problem.

Thus, dry powder inhalation (DPI) is the more promising way for pulmonary administration of siRNA. In this system, drugs are delivered to the lungs as a cloud of dry particles [67,69,82,85]. Also, based on its successful use for the *in vivo* delivery of substances like insulin, heparin or hormones, this seems to be an appropriate tool for pulmonary siRNA delivery. However, the formulation of biological molecules as a dry powder has some limitations and requires stability of the biomolecules [87,88]. The major problem that arises when the DPI technique is applied relates to differences in drug deposition, which was found to be dependent on the patients' inspiration flow rate. De-aggregation of dry powders is another limitation for DPI formulations [69]. Furthermore, a proper nanocarrier and formulation technique is necessary for siRNA formulation, in order to protect the oligonucleotide from shear force and high temperature during the drying process [82,89]. Despite the challenges in optimizing DPI formulations, their advantages for pulmonary drug delivery are significant. They increase the stability and sterility of the therapeutic agents and do not require the use of propellants [82,89–91].

Finally, another method is based on using nebulizers for producing liquid aerosols, suitable for the inhalative delivery of large volumes of drug solutions. They are used for drugs that are not suited for dose inhalers or dry powder inhalers and can be also adapted to siRNA formulations [67,69,82,84]. However, high shear forces may contribute to oligonucleotide degradation and biomolecules are less stable in liquid environment. Therefore, nanovectors protecting the siRNA from physical and chemical degradation are required, regardless of the method used for administration [92,93].

4. Biological barriers of the alveolar region

Presently, nanoparticles are considered as proper tools for crossing biological barriers in the respiratory system. Despite promising studies using different types of nanoparticles as carrier systems, research still aims at the design of new and more efficient vehicles.

In the last years, the knowledge regarding the design of nanoparticles suitable as optimal delivery systems has significantly increased. Researchers found that manipulating the particle diameter may help to control the kinetic profile of polymeric vectors. Three mechanisms are responsible for nanoparticle sedimentation in the lungs: Brownian diffusion, gravitational sedimentation and inertial impaction [55,94,95]. Particles with a diameter > 1 μm are deposited mainly by gravitational sedimentation and inertial impaction. In turn, for nanoparticles < 0.2 μm the Brownian diffusion is the main deposition mechanism. Particles with a diameter of about 0.1 μm and between 1 and 5 μm are mainly localized in the alveolar region, whereas larger particles (>5 μm) are destined to the upper lung region. Nanoparticles with a diameter between 0.1 and 1 μm are mostly exhaled during breathing and their deposition in the alveolar region is very poor [94,95]. Likewise, nanoparticles accumulate in different parts of the lung in a size-dependent manner. For instance, almost 90% of nanoparticles with a diameter around 1 nm were found to be localized in the nasopharyngeal area, 10% in the tracheobronchial area and almost none reached the alveolar system. In turn, 50% of nanoparticles 20 nm in diameter were accumulated in the alveolar system, while 15% were localized in nasopharyngeal and tracheobronchial areas [94].

Biological barriers in the respiratory system, such as mucus, pulmonary surfactant or alveolar macrophages, are main obstacles for transport to the lung cells (Fig. 3). These factors severely impair an accumulation of nano-therapeutics at their designated target sites and, as a result, response towards treatment may differ from what is expected.

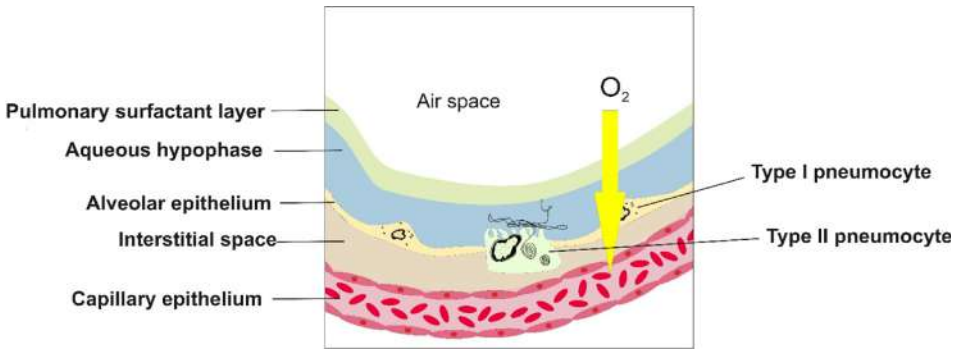

Fig. 3. The organization of the blood gas exchange barrier in the alveolar region.

4.1. Mucus

The presence of mucus in the respiratory system is a factor that severely affects the transport of nanoparticles. Mucus is composed of water (95%), mucins (2–5%), non-mucin proteins, DNA, enzymes, salts, bacteria, and others [64,80,96] (Fig. 4).

Mucins are a class of glycoproteins containing large amounts of O-glycosylated repeats in the polypeptide backbones [97]. The presence of 40–80% glycans make mucins negatively charged under physiologic conditions [97,98]. Among the five mucins identified in respiratory mucus, MUC5A and MUC5B are responsible for maintaining the viscosity in the airways [99,100]. Mucins are able to form a complex mesh, due to their affinity to interact with other mucin molecules via disulfide bonds [101]. However, the evaluation of pore size is a controversial issue, because of some difficulties in the sample preparation. Pore sizes can differ significantly, from 100 nm to several micrometers [96,102,103].

Moreover, due to its negative charge provided by glycans, the respiratory mucus is prone to electrostatic interactions with positively charged (nano-)particles. Additionally, some non-glycosylated polypeptides are able to interact with nanoparticles via hydrophobic interactions. The mucus pore size as well as electrostatic and hydrophobic interactions are thus the main mechanisms of particle entrapment in the mucus layer. These properties of mucus efficiently protect the respiratory system against any unfavorable particles. Therefore, the detailed knowledge about the mucus structure and composition is very important for developing new delivery systems [98,104,105].

Particles useful for pulmonary disease treatment should overcome the mucus barrier prior to releasing their payload from the nano-vector. Nanotherapeutics should penetrate at least the upper layer of mucus towards epithelial cells in order to avoid the mucociliary clearance mechanism [80]. One strategy for passing the mucus barrier is the use of mucolytic agents as adjuvant therapy [106]. Mucolytic agents are able to disrupt the mucus structure. The addition of N-acetylcysteine may effectively reduce the viscosity, due to its high affinity to disulfide bonds [107]. Similarly, the degradation of DNA that accumulates in the mucus layer under some physiological conditions may lead to decreased viscosity of the respiratory mucus [100,106]. On the other hand, increased contact with mucus results in prolonged residence times of therapeutic agents in the lung tissue [98]. It is known that mucoadhesive agents alter the mucus rheology by increasing the viscosity. This may lead to decreased mucociliary clearance rates and prolonged residence in the respiratory tract [100,104]. However, using mucoadhesive or mucolytic substances always affects the morphology and protective function of respiratory mucus. The use of such substances may thus result in undesired effects, like the penetration of unwanted factors into the mucus layer in parallel with the therapeutic nanoparticles [100]. Currently, one of the most innovative approaches in overcoming the mucus layer relies on mucus-penetrating nanoparticles [108,109]. These particles should cross the mucus gel at rates similar to their diffusion in water. If the nanoparticles are smaller than the pores in the mucus mesh and the fluid in the pores has a viscosity similar to water, it is possible for nanoparticles to penetrate the mucus layer. However, this also requires that they do not interact with mucins [102]. Uncoated nanoparticles with hydrophobic domains may interact with non-glycosylated parts of mucin fibers, which exhibit a high potency for hydrophobic interaction. In turn, cationic residues are able to interact with mucus based on electrostatic forces, due to their high affinity to glycosylated, highly anionic regions of mucins [98]. Thus, for efficient penetration particles should be hydrophilic and the surface charge should be negative or neutral [110].

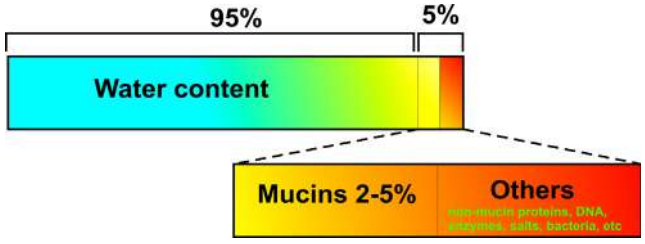

Fig. 4. The biochemical composition of mucus.

4.2. The pulmonary surfactant

The pulmonary surfactant (PS) is located at the air-aqueous interface of the aqueous hypophase lining in the alveoli [111]. Nanoparticles have to overcome PS prior to reaching the pneumocytes (Fig. 5). PS consists of lipids (90%) and proteins (10%) [112,113]. An essential part of PS are saturated phosphatidylcholines, including dipalmitoylphosphatidylcholine (DPPC) – the most important lipid for maintaining the interfacial surface tension at levels down to nearly zero. Other lipid components are unsaturated phosphatidylcholines (PC), anionic phosphatidylglycerol (PG) and phosphatidylinositol (PI) as well as neutral lipids, such as cholesterol (Chol) [39]. The 10% protein content of pulmonary surfactant comprises two families of proteins, hydrophilic SP-A and SP-D and hydrophobic SP-B and SP-C [114].

The large, hydrophilic proteins SP-A and SP-D were first described in the 1980s. Both proteins contain collagen-like regions at the N-terminus and ligand-binding C-type lectin domains at the C-terminus. Based on their structure, they were assigned to the collagen-lectin (collectin) family [115]. They are weakly attached to the surfactant surface and play a primary role in host defense by binding to particles and pathogens, enhancing phagocytic clearance. In turn, hydrophobic proteins are smaller and tightly bound

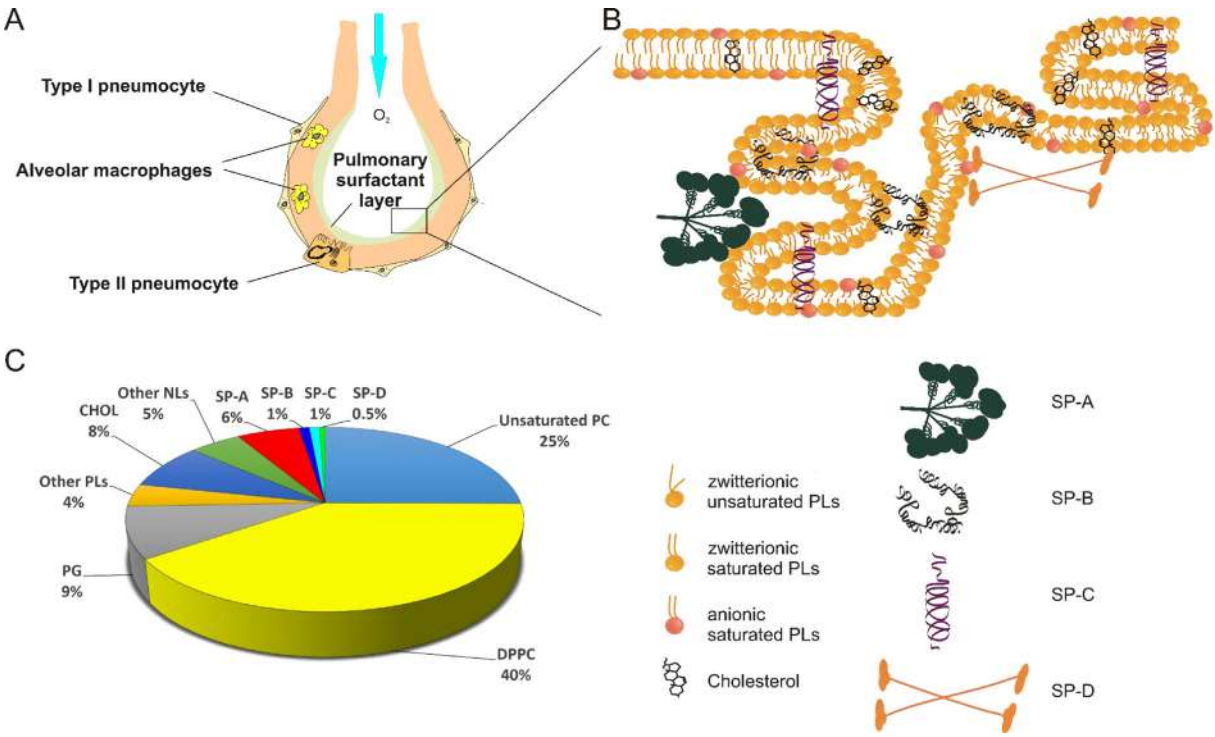

Fig. 5. The biochemical composition and the functional organization of the pulmonary surfactant. (A) The surfactant layer is localized in the alveolar region. (B) Schematic depiction showing the complexity of the surfactant layer including the main components that can be found there. (C) The main biochemical components are lipids (PL, phospholipids; PC, phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; PG, phosphatidylglycerol; Chol, cholesterol; NL, neutral lipids) and surfactant proteins (SP-A, SP-B, SP-C, SP-D).

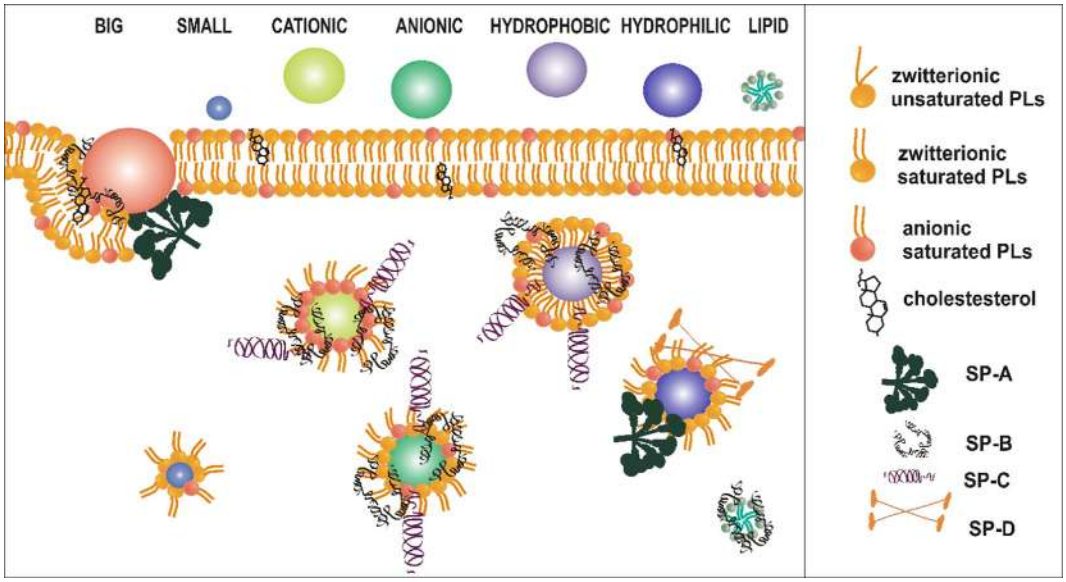

Fig. 6. The interactions of different types of nanoparticles with pulmonary surfactant.

to the surfactant film. Their crucial role is regulating surface tension in the lungs to very low, near to zero levels [114].

While the PS is a crucial barrier nanoparticles have to overcome in order to reach the alveoli region, it may enhance the bioavailability of inhaled therapeutics (Fig. 6). Pulmonary surfactant may facilitate nanoparticle displacement to the alveolar lining fluid by increase of nanoparticle solubility. If the nanoparticles display a size lower than the surfactant thickness, their interaction with the PS phospholipids may cause a rapid displacement to the alveolar region. The transport of larger molecules is determined by the cross-talk of SP-A and SP-D proteins, alveolar macrophages and epithelial cells [116]. Previous studies have shown that SP proteins were able to bind magnetic nanoparticles. Moreover, the activity of proteins promoted the clearance of magnetic nanoparticles by alveolar macrophages [116,117]. Hydrophobic nanoparticles may rapidly penetrate the surfactant layer, whereas hydrophilic nanoparticles are trapped by the surfactant layer [112,114,118,119]. In turn, hydrophobic nanoparticles were found

to inhibit the properties of pulmonary surfactant more efficiently than the hydrophilic ones [118].

The charge of nanoparticles is an important factor that also determines their interaction with protein components of the pulmonary surfactant. It is known that anionic particles can interact with surfactant protein B, but not with surfactant protein C. Thus, anionic, hydrophilic particles are easily entrapped in the surfactant monolayer by SP-B. Hydrophobic, anionic nanoparticles, although trapped in the PS monolayer, interact with SP-B in the surfactant film as well. Additionally, negatively charged nanoparticles were shown to affect the activity of pulmonary surfactant, while similar effects were not observed in the case of positively charged nanoparticles [119].

All features characteristic for nanoparticles will also determine the formation of the so-called 'protein corona'. Upon contact with biological materials and media, nanoparticles will be rapidly covered by biomolecules [120]. This corona on the surface of nanoparticles further determines their interaction with other molecules and tissue structures in the body [121].

The protein corona is usually composed of two distinct types, the so-called 'hard corona' and the 'soft corona'. The hard corona shows higher affinity to the nanoparticle surface, determines the interaction with cells and is crucial for drug/nucleic acid release. The soft corona consists of proteins with lower affinity to the nanoparticle surface and is characterized by higher exchange ratios [122]. The pulmonary surfactant is a first endogenous barrier which nanoparticles must overcome. It has been demonstrated that a surfactant corona is formed within a few minutes, regardless of nanoparticle composition, size and charge [117–119]. The corona was shown to be formed in accordance with the Vroman theory (high abundance proteins with lower affinity were bound first, then being displaced by less abundant proteins with higher affinity) [123]. Protein composition and lipid amounts were found to differ between different types of nanoparticles. In the case of hydrophilic nanoparticles, proteins were bound first and then attracted lipids from the surfactant layer. In turn, hydrophobic particles preferentially bound lipids and lipid-associated proteins from the pulmonary surfactant layer, with subsequent impairment of the surfactant function [124,125]. This is of relevance since, for safety reasons, the design of therapeutic nanoparticles which would have minimal effects on the pulmonary surfactant function is crucial. Additionally, they should elicit very little, if at all, inflammation response, in order to avoid pulmonary surfactant activation by the inflammatory cascade. These effects on PS function should be assessed *in vitro* prior to *in vivo* use [113,122,123].

4.3. Alveolar macrophages

Alveolar macrophages, which are localized in the deeper areas of the lung, are part of the clearance system [126]. They are an important component of the biological barrier in the respiratory system because they influence the number of particles entering the alveolar space. Additionally, they were found to degrade particles in the 1–5 μm size range. Simultaneously, it is known that the optimal size of therapeutic particles able to reach the alveolar region after aerosol inhalation is in the same range. Thus, there is a risk that therapeutics tailored for the alveolar space may to a large extent be engulfed and digested by macrophages prior to being able to exert their therapeutic potential. In turn, particles > 5 μm or with a diameter < 240 nm are very poorly taken up by macrophages [127]. Therefore, the optimal particle size is very difficult to define. The chemical properties and shape of a given particle can have a significant impact on its uptake as well. Notably, alveolar macrophages modulate the immune response after particle administration, by releasing pro-inflammatory cytokines TNFα, IL-1α, IL-β and the anti-inflammatory IL-10 [39,76].

5. Nanoparticles for oligonucleotide delivery to the respiratory tract

Exploring ligand-decorated nanoparticles for targeted delivery may provide an avenue towards highly specific and more efficient uptake of nanoparticles by cancer cells with reduced side effects in non-target cells. Some of surface receptors are exclusively or predominantly expressed on (lung) tumor cells or at least overexpressed in comparison to normal cells. By conjugation, nanoparticles can be decorated with specific ligands, mediating nanoparticle retention at the tumor site and, at least in some cases, enhanced ligand-mediated cell uptake. The success of targeted delivery depends on several conditions, e.g., ligand receptor binding affinity, ligand concentration and presentation on the nanoparticle surface as well as stability and tissue penetration of the nanoparticles. In recent years, several receptors have been described as overexpressed in lung cancer. Different types of small molecules, peptides and antibodies have been explored for optimal targeted delivery [41,42].

Epidermal growth factor receptor (EGFR) was found to be overexpressed in lung cancer. This overexpression of EGFR was related with poor prognosis and resistance to chemotherapy in patients with lung cancer [43,44]. Chitosan nanoparticles conjugated with an EGFR antibody were used to deliver siRNA (siMad 2) to A549 cells. The targeted nanoparticles efficiently silenced gene expression, whereas non-targeted complexes showed lesser silencing potential [45].

Another receptor which may be targeted in lung cancer is CD44. This integral glycoprotein is involved in cell growth, adhesion, migration, metastasis and drug resistance. It is overexpressed in lung cancer and is related with poor outcome [46]. Polymeric nanoparticles based on PEGylated polyethylenimine (PEI-PEG) were decorated with hyaluronic acid in order to deliver siRNA to lung cancer cells. The study demonstrated higher cellular uptake of targeted nanoparticles by cancer cells. Gene knockdown efficacy was also demonstrated. Biological activity was confirmed *in vitro* in sensitive and resistant A549 cells as well as *in vivo*, in solid tumors and in metastatic sites [47].

Many types of lung cancer cells exhibit high CD71 (transferrin receptor) expression, whereas in normal lung cells levels of CD71 are low [48]. Thus, ligands for the CD71 receptor can be used for targeted nanoparticles. Lipid-based nanostructured material was conjugated with transferrin in order to deliver a pDNA/paclitaxel payload to lung cancer cells. The results showed successful DNA transfection with simultaneous delivery of paclitaxel to the cancer cells [49].

The folate receptor was found to be overexpressed in many types of tumors as well, including lung cancer [50,51]. Nanoparticles conjugated with folate were used to deliver shRNA to the cancer cells. PEI-FA nanoparticles showed improved condensation of the nucleic acid and its protection from DNAses. Also, the uptake by lung cancer cells was found to be improved *in vitro* and *in vivo* in comparison to the non-targeted nanoparticles [52].

Another class of receptors which can be used in targeted lung cancer nanotherapy are integrins, This group represents transmembrane receptors containing α and β subunits. The composition of the subunits is cell-specific and may vary between different types of cancers. Integrins were found to be overexpressed in lung cancer cell lines and in patient samples [53]. For targeted lung cancer therapy, αvβ3 receptor was chosen. This receptor can be targeted by a peptide containing arginine (R), glycin (G) and aspartic acid (D). RGD-decorated chitosan-PEI nanoparticles were

used to deliver siRNA (siGFP) to the cancer cells. EGFP silencing reached 90% when RGD-decorated nanoparticles were used instead of the non-modified polyplex [54].

Various types of nanoparticles such as lipid-based nanoparticles, inorganic nanoparticles, peptide-based delivery vectors, exosome-based delivery vectors, dendrimers and polymeric nano-vectors have been explored for gene, oligonucleotide or drug delivery to the lung. Major groups of nanocarriers that have been used for nucleic acid delivery to the lung are shown in Fig. 7. A detailed comparison of properties, gene knockdown efficacies, toxicity and proinflammatory potential of selected nanoparticles for pulmonary delivery of siRNA is presented in Table 1.

Synthetic linear or branched polymers are non-viral nanovectors of particular interest. Due to their versatility, they can be easily modified to obtain delivery vectors with well-defined properties [22,92,175,176]. Advantages of some of these systems over other nanocarriers are low cytotoxicity, high biocompatibility and biodegradability. Additionally, they tend to cause weak immune responses [177,178].

Cationic polymers seem to be the most promising nanovectors. Due to their positive charge, polycations and polymeric nanoparticles have been used for nucleic acid delivery for a long time [38,179–181]. Cationic polymers may cause higher toxicity than anionic polymers, which are considered as relatively safe [182,183]. Similarly, other features responsible for efficient nucleic acid delivery such as their size, charge density, and chemical composition are frequently related to their cytotoxicity [112,114,118,119].

Degradability, size, hydrophobicity and concentration in the targeted tissues play a role in pulmonary toxicity [112,114,118,119]. Smaller particles are considered as more toxic than larger particles, due to their large surface area [112,119]. The shape and structure of nanoparticles are important factors determining toxic effects as well. For example, the response towards carbon nanotubes, carbon black and graphite has been found to be different, despite them sharing the same chemical composition [120]. Also, biodegradable polymers are less toxic than non-degradable ones. On the other hand, PLGA, which is perhaps among the best nano-vectors for pulmonary drug delivery, showed slow degradation rates. This increased toxic effects, especially when repeated doses of treatment were administered [121,184,185].

The hydrophobicity of nanoparticles is another factor contributing to toxicity. It has been demonstrated that hydrophobic nanoparticles may be trapped in the lining layer of the pulmonary surfactant, thus generating increased levels of reactive oxygen species (ROS) and causing an inflammatory response [119,186]. The prolonged exposure to the nanoparticles may trigger immune response (irritation, cellular injury, edema, suppression of phagocytosis, and eventually, the breakdown of immune defense). When using hydrophobic nanoparticles, oxidative stress and inflammatory response were observed in most cases [57,119,121,182,183]. Enhanced cytokine production, LDH release and increased numbers of inflammatory immune cells in different parts of the lungs were detected after nanoparticle administration [57,121,182,183].

The availability of nanoparticles in the systemic circulation and their penetration into other organs is another significant issue regarding toxicity. Particles absorbed by lung epithelium may be distributed via the blood and lymph to all organs, including bone marrow, heart, liver, kidneys and the central nervous system [187–189].

A controversial issue is the carcinogenicity caused by nanoparticles in the lung [190]. Tumorigenic effects related to primary genotoxicity or secondary genotoxic reactions (inflammation and oxidative stress) after nanoparticle administration have been discussed [191].

5.1. Lipid-based nanoparticles in pulmonary delivery

Lipid-based delivery systems are commonly used *in vitro* and *in vivo*. In the case of cationic lipids, siRNA/lipid particles are generated based on electrostatic interactions of the negatively charged nucleic acid with the cationic lipid [128–131]. To avoid premature siRNA release and subsequent degradation, the stability of the formed lipoplexes in the pulmonary route of administration is of major importance [129]. In order to obtain efficient lipid vectors for pulmonary delivery, the lipid composition, lipid to siRNA ratio and method of nanoparticle generation must be well defined [132].

The main limitations of lipid-based nano-vectors are their toxicity and their potential to activate the immune system, including cytokine and interferon activation [133]. Cationic liposomes and lipoplexes are generally more toxic than neutral lipids [131,134]. The structure of cationic head groups and the length of hydrophobic tails determine the transfection efficacy and the toxicity of

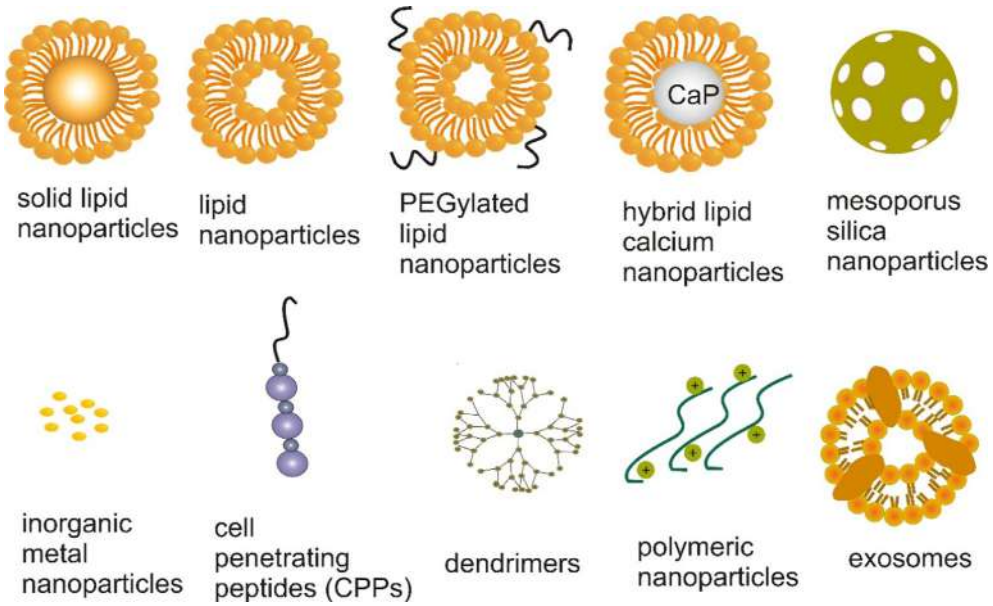

Fig. 7. Nanoparticles used for nucleic acid delivery to the lung.

Table 1
Properties of selected nanocarriers for pulmonary siRNA delivery.

Nanocarrier	Gene silencing efficacy	Toxicity	Inflammatory properties	Properties	Ref.
Cationic lipid	Up to 30% gene knockdown	n.d	n.d	Rapid translocation to the intended cells, siRNA-lipid complexes were mainly found in macrophages	[134]
Cationic lipid	85% in A549 cells 82% in primary human bronchial epithelium	n.d	OAS1, IL-8 low gene expression level	Formulation facilitated mucus and PS penetration and crossing the cellular membrane Protection from degradation by pulmonary enzymes	[136]
PEGylated DOTAP:CHOL liposomes	Up to 50%	n.d	IL-6 and IL-12 levels similar to non-treatment	PEG modification prolonged retention in alveolar mucous, protecting from alveolar macrophages and absorption into the blood stream. Targeting ligand facilitated sustained distribution	[139]
Lipid coated dextran nanogel	95% eGFP expression for DPPC:DOPC:eggPG; 30% eGFP expression for Curosurf and non-coated nanogel	>90% for all formulations	Increased levels of proinflammatory cytokines and chemokines in BAL (IL-1α, IL-1β, Il-6, TNF α, MIP-1 α)	SP-B facilitates intracellular delivery of siRNA *in vitro* and *in vivo* Most of siRNA traffics to the alveolar macrophages	[144]
Mesoporus silica nanoparticles decorated with LHRH ligand	40 – 60% knockdown of gene expression for Bcl-2 and MDR1	>80%	n.d	Size of pores and nanoparticles can be adjusted to effective cellular uptake and accumulation in the lungs	[146]
Calcium phosphate-PLGA-PEI nanoparticles	40–55% knockdown of gene expression	Not shown	Not shown	Most of siRNA was found in alveolar macrophages; PEI modification improved cellular uptake and endosomal escape	[148]
Gold nanoparticles with PEG and RGD targeting peptide	c-myc expression level decreased up to 20% in an adenocarcinoma cell line	Not shown	Lymphocyte and neutrophil infiltration was neglible	AuNPs were rapidly taken up by the cells. RGD targeting peptide facilitated trafficking to tumor sites	[149]
CPP	Knockdown of gene expression was significant (approximately 90% after 48 h)	Not shown	Not shown	Size of the complexes facilitates prolonged deposition in the lung, HA2 conjugate facilitates endosomal escape, optimal N/P ratio enhances complex stability and protection from degradation	[154]
CPP	Approximately 50% knockdown of gene expression in the whole lung	Not shown	Not shown	The average size of complexes less than 100 nm facilitate deposition in the deeper parts of lungs	[155]
HMG/oligoarginine micells	Approximately 25% knockdown of gene expression when siRNA/OR was used, 75% when siRNA/OR/HMG was used	Low toxicity towards THP1 cells	Not shown	Complexes were taken up mainly by macrophages	[156]
Exosomes	Knockdown of gene expression up to 50% in macrophages	>90% cell viability	Decreased levels of proinflammatory cytokines TNF-α, IL-2, IL-3, IL-6, and chemokines CCL-2, MCP-5, and CCL-5	The size of complexes facilitated uptake by alveolar macrophages	[158]
Chitosan	10–35% gene knockdown efficacy	>80% cell viability	Not shown	Chitoans exhibit mucoadhesive and mucosa penetration properties. The size of complexes < 100 nm facilitated deposition in the lungs.	[172]
Chitosan	Approximately 70% gene knockdown efficacy	>90% cell viability when siCRTL was used in the complex	Not shown	Chitoans exhibit mucoadhesive and mucosa penetration properties.	[173,221]
PAMAM	Up to 70% eGFP knockdown efficacy	>80% cell viability	Not shown	The size of microparticles was suitable for lung deposition and the uptake by alveolar macrophages was minimal.	[201]
PEI	60–70% knockdown on the protein level	Toxicity was not statistically significant	TNF-α level was 2 times higher than in control but not statistically significant	DPPC-PLGA core facilitated mucus penetration, the size around 150 nm led to the easier diffusion through gel-like mucus, PEI enhanced encapsulation efficacy and endosomal escape, nanoparticles were able to reach alveolar epithelium	[219]
PEI	Approximately 42% gene knockdown efficacy in mouse lungs	Approximately 20% cell toxicity for PEI, 5–25% cell toxicity for PEI-PEG complex	Elevated level of proinflammatory cytokines CCL2, CXCL1, CXCL10, CCL5, IL-6, TNF-α	PEI enhanced encapsulation efficacy and endosomal escape, nanoparticles were able to reach alveolar epithelium	[222–224]

cationic liposomes [135]. Additional features that can lead to higher liposome toxicity are their charge density and their potential for lipid interaction.

Cationic lipid nanocarriers were shown to facilitate cellular siRNA uptake via endocytosis and siRNA release at the intended site. siRNA against the subunit α of the epithelial sodium channel was formulated in a liposome and delivered in BALB/c mouse model via the intranasal route of administration. This resulted in the inhibition of the expression and function of ENaCα *in vivo*. This delivery system was proposed as a promising therapeutic tool in cystic fibrosis treatment [136].

In order to avoid the toxicity and immune response of cationic lipid vectors, PEG chains have been added to the nanoparticles. PEGylation was used to prolong the liposome circulation in the bloodstream and to prevent them from opsonization by the mononuclear phagocyte system [82,137]. The addition of neutral components such as cholesterol to the lipid reduces toxicity and immune response of lipid nanoparticles as well [138,139]. However, this delivery system has some limitations, including the weak interaction between siRNA and the lipid component as well as between the lipid complex and the cell membrane. To overcome these problems, a chemical conjugation of siRNA and a lipid instead of electrostatic interactions has been introduced. A cholesterol-conjugated siRNA showed higher gene knockdown efficacy compared to naked siRNA. Moreover, reduced inflammation was observed *in vivo* [36]. An alternative approach towards reducing the toxic effects of lipid-based nanoparticles is the encapsulation of siRNA inside neutral lipid particles. An important advantage, responsible for the unique properties of such lipid systems, is their behavior at different pH [140]. For example, they are positively charged at acidic pH, while being uncharged at neutral pH [140].

So-called stable nucleic acid lipid particles (SNALP) with well-defined size, high siRNA encapsulation efficacy and efficient delivery ratio have been developed as well [141]. The evaluation of pKa values of cationic lipids is a crucial step for designing the SNALP delivery platform. This parameter determines the charge of the lipid particles under different pH conditions and the ability of protonated particles to induce a non-bilayer phase structure, after being mixed with anionic lipids [142,143]. These characteristics in turn determine the membrane destabilizing capacity and *endo*-osmolytic potential necessary for nucleic acid delivery.

Recently, siRNA against oncoprotein Eph2 encapsulated in 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine neutral liposome entered Phase I of clinical trials in lung cancer. Additionally, another SNALP-mediated siRNA delivery platform entered Phase I as a carrier for siRNA targeting PLK1 [8].

The addition of pulmonary surfactant to the siRNA-containing nanoparticles may allow to control toxicity, efficacy and distribution of particles as well. Merckx et al. studied an siRNA-loaded nanogel based on dextran and coated with the artificial pulmonary surfactant (PS) Curosurf® [144]. Their results showed that the PS-coated hydrogel was more stable and exhibited improved siRNA delivery *in vitro* and *in vivo*. In this study, the SP-B protein was identified as a crucial component for improving siRNA delivery, and the SP-C protein as an additional enhancer of siRNA delivery. The impact of the lipid composition on the siRNA nanogels was studied as well and revealed preferential binding of SP-B when the level of cholesterol did not exceed the physiological level. Furthermore, the function of this protein was retained in the presence of different classes of anionic lipids. In contrast, the addition of SP-B did not enhance siRNA delivery when cationic DOTAP:DOPE liposomes were used [145].

5.2. Inorganic nanoparticles in pulmonary delivery

Inorganic materials have been developed for therapeutic and diagnostic purposes. They are rather simple to prepare and have also been explored as siRNA delivery platforms. For the pulmonary route of administration, hybrid lipid-calcium phosphate nanoparticles, gold nanoparticles and silica mesoporus nanoparticles are best characterized [81].

Mesoporus silica nanoparticles exhibited a high efficiency in delivering siRNA against MRP-1 in a lung cancer model that was resistant to conventional treatment. The efficacy of administration via the pulmonary route was 73.6%, in comparison to 5% when administered intravenously. Also, the accumulation in liver and kidneys was significantly lower when the pulmonary rote of administration was chosen [146].

Hybrid lipid-calcium nanoparticles effectively delivered siRNA against Bcl-*2 in vitro* and in a lung cancer xenograft mouse model *in vivo*. They exhibited a higher accumulation of anticancer siRNA in tumor tissue and protected it from degradation [147]. In another study, calcium phosphate/siRNA nanoparticles were used for decreasing an inflammatory response. For this, calcium phosphate solution was mixed with siRNA against pro-inflammatory mediators, then it was encapsulated in PLGA and finally in a polyethylenimine (PEI) layer. In a mouse model, the levels of IP-10, CCL-2 and INF-γ were found reduced after intranasal administration of the nanoparticles [148].

Gold nanoparticles showed promising efficacy in the delivery of siRNA against the c-myc oncogene in mice. Thiolated siRNA was covalently bound to the gold nanoparticles modified with PEG and with arginine-glycine-aspartate peptides for targeting (RGD). The proliferation rate and the size of lung tumors in mice was successfully reduced upon administration of the nanoparticles via intratracheal administration [149].

5.3. Peptide-based delivery vectors

Cell penetrating peptides (CPPs) are of special interest for siRNA delivery due to their low cytotoxicity, versatility and favorable transfection properties [150,151]. CPPs consist of a short amino acid chain and can interact with membranes. The sequence of less than 30 amino acid residues determines the features of CPPs, such as structure, charge, polarity and solubility [151]. All these characteristics are pivotal for the efficacy of peptide/siRNA complex formation, their toxicity and cellular uptake [152]. Peptides can bind to the siRNA via disulfide bond formation and electrostatic interaction. The biggest disadvantage of using CPPs are the lack of cell selectivity and the degradation of L-amino acids from the CPPs chains. Due to their variety, they exhibit different mechanisms of action. Some of them improve cellular delivery by efficient transport through the cell membrane, whereas others facilitate the endosomal escape and protect from lysosomal degradation [153].

Some CPPs have been identified as attractive nano-vectors for siRNA delivery via the pulmonary route [36,154–157]. Moschos et al. investigated the efficiency of p38 MAP kinase knockdown using naked siRNA, TAT-conjugated siRNA and penetrin-conjugated siRNA *in vitro* and *in vivo* [36]. siRNA-conjugates reduced p38 MAP kinase mRNA level up to 30–45% in the L929 mouse fibroblast cell line. In the same study, the efficiency of naked siRNA was evaluated *in vitro* and *in vivo*. Interestingly, naked siRNA caused gene knockdown *in vivo* after intra-tracheal administration, whereas it had shown low efficiency *in vitro*. [36].

A silencing of chitinase-3-like-1 protein in the lung using CPP-siRNA reduced lung metastasis and induced cytotoxic T-

lymphocyte response [154]. Welch et al. developed cyclic, amphipathic peptides that formed complexes with siRNA. The cyclic peptides with disulfide constraints were reduced by intracellular glutathione which enhanced the presentation of siRNA to the RNAi system. Gene knockdown was also observed in lung cells when the pulmonary route of administration was chosen [155]. Choi et al. described a poly-arginine peptide with siRNA and an HMG peptide ligand that targets activated alveolar macrophages, and found promising biological effects in an asthma model [156]. Qui et al. synthesized a peptide (KL-4) which mimics the surfactant protein SP-B. The PEGylated KL-4 peptide was used as inhalable dry powder formulation for mRNA delivery [157].

5.4. Exosome-based delivery vectors

Exosomes are extracellular vesicles secreted by many cell types. They are engaged in cell–cell communication through the transfer of lipids, proteins, nucleic acids or others [158]. Due to their high biocompatibility, low toxicity and low immunogenicity, they are considered as promising siRNA nano-vectors [158–161]. Zhang et al. used siRNA loaded into serum-derived exosomes via calcium-mediated transfection. The complexes were successfully administered via the intratracheal route. They were mainly deposited in the alveolar region and macrophages were the most affected cells. Exosomes from human pluripotent stem cells were used to knock down ICAM-1 levels for acute lung injury (ALI) treatment [162]. Although the use of exosomes in oligonucleotide delivery offers many benefits, there are still some drawbacks, mainly associated with large scale production. Additionally, storage may influence their stability and biological function [163,164]. Therefore, long-term safety and efficacy of the application of exosomes still requires exploration [165].

5.5. Chitosan

Chitosan is a polysaccharide-based polymer derived from chitin [166]. As a naturally occurring polymer, it exhibits good biocompatibility and biodegradability. Chitosan may be an attractive vector for nucleic acid delivery, due to the positive charge density of amino groups [167–169]. It is able to form complexes with DNA or siRNA via electrostatic interactions [170]. A sufficient nitrogen/phosphate (N/P) ratio in the complexes supports complex stability and mediates protection from nucleases [170,171]. The use of chitosan nanoparticles for the pulmonary delivery of therapeutic agents is of special interest. Sharma et al. investigated cross-linked chitosan/siRNA nanoparticles, formed at different N/P ratios, after nebulization. They found that complexes were spherical (all ~150 nm), positively charged and non-toxic for lung-mucoepithelial cells. The complexes did not show a tendency to aggregate at physiological pH and remained stable after exposure to shear forces. The results indicate that chitosans may serve as safe and efficient nanocarriers [93]. In another study, Ni et al. studied the use of chitosan derivatives, i.e. guanidinylated O-carboxymethyl chitosan and N2-hydroxypropyltimethyl ammonium chloride chitosan, for efficient siRNA delivery to the lungs using nebulizers. They found that complexes comprising guanidinylated O-carboxymethyl chitosan, N2-hydroxypropyltimethyl ammonium chloride chitosan and survivin siRNA significantly decreased the level of survivin in A549 cells, caused the inhibition of cell proliferation and led to apoptosis. The nebulization process did not influence the properties of the complexes and ~60% were detected as fine particle fractions (around 130 nm for unlabeled nanoparticles) [92]. Guanidinylated chitosan was used by Luo et al. [172]. They proved that guanidinylated chitosan at a ratio of 40:1 was able to condense siRNA and to form positively charged (15 mV), spherical particles of ~100 nm. The addition of guanidine reduced the toxicity of complexes and significantly improved siRNA uptake. These results confirmed the experiments performed by Ni et al. [92]. Due to their positive charge, guanidine groups facilitate hydrogen bond formation with phospholipids on the cell surface and improve cellular uptake [92]. In addition, nanoparticles were modified with the β2 adrenoceptor agonist salbutamol that increased the targeting specificity *in vitro* and *in vivo*. It was also found that the modified chitosan particles were able to protect the siRNA from high shear forces generated by a mesh-based nebulizer. Modified chitosan was also used in a study by Capel and coworkers. They synthetized piperazine-substituted chitosan for intratracheal siRNA delivery. The complexes were water-soluble at physiologic pH and were found non-toxic for the cells. They also exhibited a high siRNA encapsulation efficacy into 300 nm spherical nanocapsules, at a low chitosan/siRNA ratio of 5:1. The level of gene knockdown reached even 80%. No changes in the stability and integrity were found after their conversion into aerosols. *In vivo* studies revealed that complexes did not cause adverse effects after repeated administration, while tumors were found significantly reduced in an atopical lung cancer model in mice [173]. Carriers based on chitosan and its derivatives seem to be very promising due to their favorable properties. However, these compounds also have limitations, such as low solubility and low stability of complexes inside cells, possibly leading to a premature release of the payload after cellular uptake [174].

5.6. Synthetic polymeric nano-vectors in pulmonary nucleic acid delivery

5.6.1. Dendrimers

Dendrimers are synthetic, poly-branched, monodisperse, low-immunogenic spherical nanoparticles [192]. Nucleic acids may be placed inside the cavities of dendrimers or attached to the chemical groups on the surface of nanoparticles [193–196]. Due to their good biocompatibility, the most widely used dendrimers in nucleic acid delivery are polyamido amine (PAMAM) and poly(propylene imine) (PPI) dendrimers [197–200]. Since PAMAM and PPI possess amine groups, they are able to form complexes with negatively charged nucleic acids via electrostatic interactions [199]. Dendrimers facilitate cellular uptake and protect nucleic acids from digestion by nucleases. Due to the physicochemical properties of dendrimers, they have been extensively studied as potent oligonucleotide carriers in lung cancer disease [22,79,89,201–203].

Conti at al. suggested PAMAM dendrimers (G4NH2) as potential siRNA carriers which would be administered as an aerosol [201]. The dendriplexes were encapsulated in mannitol, and hydroxyfluoroalkane (HFA) was used as a propellant in a pressurized metered-dose inhaler. 40% knockdown of gene expression was determined as a consequence of complex activity *in vitro*. The preparation of the inhaled formulation did not change the properties of the complexes. Moreover, the complexes reached the deeper region of the lungs to 77% of the respirable fraction. What is important, the complexes maintained their integrity and stability towards the hydrofluoroalkane, even after longer exposure [201]. Similarly, PAMAM G4 with a triphenylphosphonium (TPP) modification was investigated as siRNA vector [89]. The authors showed that modified PAMAM exhibited better transfection properties and was suitable for metered-dose inhaler and dry-powder inhaler formulations. The gene knockdown efficacy increased with TPP density and with N/P ratio. Most effective were dendrimers modified with 12 TPP molecules on the surface, complexed with siRNA at ratio 30. For inhalation, the aerosols were prepared using mannitol microparticles for dendriplex encapsulation. The complexes were efficient in both, in aerosols from metered-dose inhalers as well as in dry powder inhalers (fine particle fractions were 50–53% and 39%, respectively). The preparation of inhalation formulas

did not affect gene knockdown efficacy [89]. In another study, G4 PAMAM dendrimers were complexed with siRNA and then embedded in microparticles built from trehalose, mannitol and inulin. The dry powder-based microparticles were produced for inhalation using the spray drying technique. The obtained results indicated that complexes maintained their integrity and bioactivity. Additionally, the complexes could be reconstituted from the dry powders. Trehalose and inulin provided better stability of nanocomplexes than mannitol. The best properties were found for the mixed formula of trehalose and inulin, where high gene knockdown efficacy, stabilization of the complexes and enhanced cellular uptake were observed [79].

5.6.2. PLGA xxxx

PLGA (polylactic-co-glycolic acid) is a co-polymer of lactic acid and glycolic acid, coupled through ester linkages [204]. Due to its good biocompatibility and biodegradability, PLGA has been approved by the FDA and the European Union as a drug delivery system [205]. Several methods for obtaining PLGA exist, the most popular being emulsion-solvent evaporation, where sphere-shaped nanoparticles are formed [206,207]. The drugs or nucleic acids can be encapsulated inside the nanoparticle or adsorbed onto the surface [205,207,208]. PLGA is able to deliver siRNA, facilitate its cellular uptake and protect from degradation [24,25,204]. PLGA nanoparticles can enter the cell through pinocytosis or clathrin-dependent endocytosis [204,209]. As PLGA is negatively charged, cationic or hydrophilic modification is required for efficient siRNA complexation [176,210]. Several studies describing the use of PLGA for pulmonary delivery have been conducted. Jensen et al. generated a dry powder formulation based on lipid-modified PLGA and siRNA. The gene knockdown efficacy was correlated with the ratio of DOTAP in the formulation. The mixture containing 15% DOTAP was then spray dried with mannitol into a particles with a diameter suitable for lung deposition. It was demonstrated that spray drying neither affected the physicochemical properties of the complexes nor their *in vitro* activity. The content of siRNA was similar to the theoretical values and the siRNA remained intact, indicating that the nucleic acid was protected during the spray drying process. The X-ray diffraction studies confirmed that the use of mannitol as excipient supported the stability of the complexes in the dry powder formulation by inhibiting interactions between neighboring nanoparticles [90]. The same group suggested that PLGA-based nanoparticles with siRNA and with mannitol as an excipient may serve as a promising tool in pulmonary nucleic acid delivery [211].

Wu et al. investigated how the route of administration affects the deposition of nanoparticles in the lung lobes. The siRNA/PLGA nanoparticles were administered by intratracheal instillation, intratracheal spraying or intranasal instillation. Over 95% or 85% of the therapeutic siRNA, respectively, reached the lungs when intratracheal instillation or intratracheal spraying were performed. However, the distribution of the nanoparticles was inhomogeneous in the lung lobes. In turn, the intranasal instillation deposited only 28% of nanoparticles in the lung. These results thus indicated that intratracheal instillation and spraying are promising administration routes for pulmonary delivery in pre-clinical animal studies, while the intranasal route should not be considered [212].

5.6.3. PEI xxxx

PEIs (polyethylene imines) are linear or branched cationic polymers with characteristic water-soluble and protonatable amine groups [213–215]. Due to their positive charge, PEIs are able to form complexes with DNA or siRNA via electrostatic interactions [216]. The amine groups present in the PEI structure also facilitate the interaction with the negatively charged cell membrane, thus enhancing cellular uptake of nanoparticles via endocytosis [175,213,215,217]. Due to the 'proton sponge effect', PEIs exhibit protective functions in the lyso-endosomes [218]. More specifically, nitrogens act as proton acceptors, thus buffering the otherwise acidic pH of lyso-endosomes. In consequence, proton pumping into the endosomes occurs, leading to the passive entry of chloride ions and an excess influx of water. Eventually, this endosome swelling can lead to their disruption and the release of their content into the cytoplasm [214,215,218].

While PEIs are widely used especially in DNA delivery, their potential to deliver siRNA has been studied as well. Some research groups found PEIs to be also applicable as nano-vectors in pulmonary oligonucleotide delivery. However, the optimization of parameters that are crucial for inhaled formulas is a challenge. Keil et al. tested the excipient matrix for PEI/DNA complexes as pulmonary delivery platform prepared via spray drying [91]. The excipient impact on size, bioactivity and stability was assessed after generating nano-embedded microparticle powders. The results showed that integrity, bioactivity and reconstitution from the dry powder were maintained when trehalose was used as stabilizer. Moreover, the particles reached a diameter of 1 to 5 μm, which allows them to be deposited in the deeper parts of the lungs. Studies aiming at the evaluation of cellular uptake revealed that redispersed macroparticles containing trehalose were more effective than non-spray-dried polyplexes [91].

The use of polymer/siRNA complexes containing PEI was assessed by D'Angelo et al. [219]. They created hybrid lipid-polymer nanoparticles conjugated with siRNA against the sodium transepithelial channel. Additionally, one fraction of the nanoparticles was modified with PEI. The obtained nanoparticles displayed a diameter of ~150 nm, with a zeta potential of around −25 mV. The siRNA release lasted for up to 6 days, with the presence of PEI in the complex additionally prolonging this time period. Aerosols were generated by a vibrating mesh nebulizer and the complexes exhibited promising activity after nebulization. Moreover, they showed high stability upon incubation with artificial mucus, were internalized into lung epithelial cells and caused neither harmful toxic effects nor pro-inflammatory reactions. The levels of the sodium transepithelial channel, especially the β form, were successfully decreased when DPPC/PLGA/siRNA or DPPC/PLGA/PEI/siRNA complexes were used [219].

A biokinetic study aimed to investigate and compare the properties of PEI/siRNA complexes with naked siRNA upon intratracheal administration into mice. Complexes based on the low molecular weight branched PEI F25 LMW provided the protection of siRNA from degradation by nucleases. It also prolonged its retention time. Moreover, PEI complexes did not cause high toxicity or acute immune response [220].

Spray-dried PEI polyplexes were also produced for DNA delivery using a nanoparticle-in-microparticle delivery system. Polyethyleneimine-based polyplexes or lipoplexes were encapsulated into polyvinyl alcohol (PVA) microparticles. This maintained complex stability for a prolonged time due to the PVA corona on the complex surface which inhibited nanoparticle aggregation. The spray-dried, (lipo-)polyplex-containing PVA microparticles exhibited reduced toxicity and improved transfection efficacy compared to the parent (lipo-) polyplexes. *In vivo* studies revealed a significant level of EGFP-expressing cells after one administration of spray-dried polyplexes encapsulated in PVA microparticles [78].

6. Conclusion and future prospects

This review paper has covered various aspects related to nanoparticle delivery to the lung, from the histological organization and functions of the respiratory tract, the presence of biolog-

ical barriers in the alveolar region and their relevance in (nanoparticle) drug application towards the discussion of the broad spectrum of natural or synthetic, non-viral vectors for oligonucleotide formulation. Oligonucleotide delivery using the pulmonary route of administration has turned out to be very promising for lung diseases, including cancers. It possesses advantages over conventional treatment, by combining the possibility to target any gene of interest (siRNA) with the approach of direct delivery into the lung (nanoparticle). While this strategy avoids systemic side effects by minimizing the drug action on off-target organs, the nucleic acid formulation must overcome several very specific barriers prior to reaching its site of action. Obviously, this requires the development of proper nanocarriers which may well differ from their counterparts used for example as siRNA delivery reagents to the liver. Beyond general properties like efficient encapsulation of the siRNA and its protection from nucleases, cellular internalization and proper intracellular processing, the nanoparticles must also penetrate and survive the mucus layer and pulmonary surfactant, avoid alveolar clearance and remain non-toxic even upon local treatment by inhalation, in a very sensitive organ system. While many natural and synthetic vectors have been proposed for inhaled drug/gene delivery, these systems come - not surprisingly - each with a range of advantages and disadvantages in pulmonary delivery.

Liposomal nanoparticles have probably been most widely used and described in the literature. Main advantages of lipid-based delivery vectors are the enhancement of their internalization into pulmonary cells through their interaction with phospholipids, and the availability of many well-established systems, in part developed over decades. On the other hand, efficacy and stability issues as well as comparatively high production costs are still among the obstacles in their translation into therapy. Previous work for lipid-mediated siRNA delivery into organs other than lung has also shown that the identification of optimal liposomal systems may in fact still rely on very defined yet minor modifications of their lipid structures and lipid composition, leading to somewhat complicated multi-component assemblies with rather delicate properties. Not surprisingly, this also applies nanoparticles for delivery to the lung. Solid-lipid nanoparticles (SLNPs) are considered as easier in scaling up and as more stable when compared to liposomes. One problem with SLNPs, however, relates to low drug/gene loading. Exosomes have been investigated as 'natural' nanocarriers due to their unique properties which provide the basis for their physiological and pathophysiological roles, thus rendering them attractive systems for siRNA delivery. Among others, they seem to exhibit some target cell specificity which could be well explored with regard to targeted delivery into a given of interest. One main limitation for their use as inhaled formulation, however, may lie in their rather complicated large-scale production, especially when considering the need of batch-to-batch stability.

Polymeric nanovectors cover a very wide range of various types and compositions, with several being of interest due to their high encapsulation efficacy, siRNA protection, cell uptake, sustained intracellular payload release profile and ability to avoid alveolar macrophage clearance. In several cases, efficacy vs. toxicity remains a challenge that is closely related to nanoparticle properties like surface charge. This also applies, for example, to PEI-based carriers where the gene transfection efficacy increases with molecular mass, which is paralleled, however, by increased toxicity and pro-inflammatory potential. On the other hand, polymers allow for particular straightforward and diverse chemical modifications, which can be beneficial for creating novel nanoparticles with markedly improved properties. This also allows for setting up structure–function relationships for somehow predicting the direction towards optimized systems. For example, PEGylated nanoparticles based on PEI show decreased toxicity/improved biocompatibility and the avoidance of mucus clearance, but also less complexation efficacy, poorer stability and impaired transfection properties. Many of these alterations, however, have been found to be dependent on the precise pattern of PEGylation and again highlight the necessity for the precise definition of optimal nanocarrier systems. Also, apparent disadvantages of a given nanoparticle in 2D cell culture *in vitro* with regard to low efficacies may not necessarily indicate its inferiority for *in vivo* applications, thus emphasizing the importance of relevant *in vitro*, *in vivo* and ex vivo systems for nanoparticle testing. This is particularly so in the case of lung application and has been addressed for example by sophisticated air–liquid interface culture systems.

In contrast to PEG-PEIs, studies introducing tyrosine modification to linear and branched PEIs successfully improved their efficiency while in parallel reducing toxicity and improving other nanocomplex properties. Again, however, this was found to rely on the definition of optimal parameters, like here the degree of tyrosine-grafting. Still, these systems are comparably simple and straightforward which may offer advantages. On the other hand, hybrid delivery systems containing more complex compositions of polymers, lipids, peptides, exosomes and/or other components are extensively investigated as well, aiming at the defined improvement of nanoparticle properties for siRNA formulation and delivery. While they may offer solutions for overcoming certain limitations characteristic for single or less complex nanocarriers, their clinical translation may be hampered by their inherent complexity as multi-component systems, with difficulties in production, standardization and upscaling.

Beyond the development of ideal nanocarrier systems for (lung) delivery, the definition of 'ideal' drugs remains an issue as well. This is true e.g. for tumor therapy where the identification of optimal target genes for siRNA-mediated knockdown often turns out to be remarkably difficult. Also, considering many pathologies including cancer as non-monogenetic disease, the delivery of a single siRNA may not be sufficient. Consequently, combinational therapies are more and more considered as a promising strategy in the context of cancer, for hitting cancer cells more efficiently. This may also include the combination of drugs with nanoparticle therapies, and can thus lead to additional requirements in nanoparticle development with regard to making them capable of simultaneously co-delivering compounds as different as a low molecular weight drug and an siRNA. On the other hand, this emerges as particularly promising since the siRNA-mediated knockdown may sensitize the cells to a cytostatic drug, e.g. by inhibiting a given oncogene or active efflux mechanism related to multidrug resistance. Again, polymeric nanoparticles may be particularly suited for chemical modification and adaptation, aiming at the implementation of defined nanoparticle properties required for these strategies. Inhaled targeted therapies designed for treating lung cancer or lung (micro-) metastases will be promising in this regard, especially when considering that small lung metastases may be poorly vascularized and may thus not be easy to reach from the systemic circulation. Obviously, it will be of special interest then to create nanoparticles which can reach cancer cells without unwanted or toxic effects on non-target cells.

Once developed, however, nanoparticle systems for local siRNA delivery to the lung may be well useful in different pathologies. Perhaps more than ever, this has become clear since the onset of the COVID-19 pandemic, where many efforts for developing nanoparticles for various (lung) pathologies have been shifted towards their exploration in the context of treatment or prevention of coronavirus infection by local drug administration. Thus, beyond the development of (m)RNA drugs, the pandemic situation may also help booster the research towards identifying optimal nanoparticle formulations for inhalative siRNA application.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors' own work related to the topic of this review article is supported by grants from the "Beethoven Life 1" program of National Science Centre of Poland, No: 2018/31/F/NZ5/03454 (M. K., M.I., M.B., A.A.), partly supported by NAWA IAP Program "EURO-PARTNER" (M.I., M.B., A.A.) and the Deutsche Forschungsgemeinschaft (DFG; AI 24/24-1 to A.A.).

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International Journal of Pharmaceutics 614 (2022) 121468

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Unmodified and tyrosine-modified polyethylenimines as potential carriers for siRNA: Biophysical characterization and toxicity

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ARTICLE INFO

Keywords:
Polyethyleneimines
Tyrosine modification
siRNA delivery
Electrokinetic potential
Membrane fluidity
Cytotoxicity
Genotoxicity

ABSTRACT

Polyethylenimines (PEIs) are being explored as efficient non-viral nanocarriers for nucleic acid delivery *in vitro* and *in vivo*. To address limitations regarding PEI efficacy and biocompatibility, modifications of the chemical structure of linear and branched PEIs have been introduced, including grafting with tyrosine. The aim has been to compare linear and branched polyethylenimines of a wider range of different molecular mass with their tyrosine-modified derivatives. To do so, physico-chemical and biological properties of the polymers were investigated.

Even in the absence of a negatively charged nucleic acid counterpart, PEIs form particle structures with defined size and surface potential. Tyrosine modification of PEI led to significantly reduced toxicity, while simultaneously increasing interaction with cellular membranes. All the effects were also dependent on the PEI molecular weight and structure (i.e., linear vs. branched). Especially in the case of linear PEIs, the improved membrane interaction also translated into slightly enhanced hemolysis, whereas their genotoxic potential was essentially abolished. Due to the improvement of properties critical for nano-vector efficacy and biocompatibility, our data demonstrate that tyrosine-modified PEIs are very promising and safe nanocarriers for the delivery of small RNAs, like siRNAs and miRNAs.

1. Introduction

Gene and oligonucleotide therapies are among the most promising new strategies for the treatment of numerous diseases, including as cancer, neurodegenerative disorders, genetic diseases and viral infections (Falkenhagen and Joshi, 2018; Fleifel et al., 2018).

They are based on the delivery of nucleic acids to target cells, which, however, is still a challenge due to the size and negative charge of nucleic acids. Modern strategies focus on improving the delivery of different types of nucleic acids, such as DNA, mRNA, siRNA and miRNA into cells (Höbel and Aigner, 2013; Ibraheem et al., 2014; Scherman et al., 2017). Currently, viruses seem to be the most efficient gene carriers due to their unique properties. However, there are safety concerns and other major limitations regarding viral vectors. Therefore, non-viral gene delivery systems based on lipids, amino acids or polymers are considered as alternatives for nucleic acid delivery. Ideal gene carriers should mimic the viral infection process, condense genetic material and protect it from degradation. They should internalize into cells, while being non-toxic and non-immunogenic (Pandey and Sawant, 2016). Cationic polymers seem to be among the most promising synthetic gene carriers. The positive charge of polymers not only facilitates their interaction with nucleic acids, but also allows for efficient uptake of the respective polymeric nanoparticles by cells (Wong et al., 2007).

Polyethylenimines (PEI) are synthetic branched or linear polymers widely investigated as vehicles for non-viral gene delivery (Lungu et al., 2016; Pandey and Sawant, 2016). The synthesis of branched PEIs relies on acid catalyzed ring-opening polymerization of aziridine monomers, whereas linear PEIs are synthesized by cationic ring-opening polymerization of 2-oxazolines (Ogris and Wagner, 2012; Wiseman et al., 2003). There is a wide range of PEIs applications in science and medicine; they are used as a drug, as an anti-coagulant agent which prevents fibrin formation and in photodynamic therapy for treating localized infections and cancers. Interestingly, polyethylenimines are also employed as non-invasive optical imaging devices (Vicennati et al., 2008). They are being extensively studied as non-viral vectors for genetic material and drug delivery (Kircheis et al., 2001; Kunath et al., 2003).

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https://doi.org/10.1016/j.ijpharm.2022.121468
Received 23 September 2021; Received in revised form 20 December 2021; Accepted 9 January 2022
Available online 11 January 2022

The structure and molecular weight of PEIs determine their biological activity for nucleic acid delivery. Their buffering capacity depends on the amount and type of amino groups. Linear PEIs have secondary as well as primary amino groups at the end of the chain, whereas branched PEIs have primary, secondary and tertiary amino groups in a 1:2:1 proportion, which enhances their complexation capacity. Several PEIs with a wide range of different molecular weights have been investigated. The findings suggest that PEIs between 600 and 1800 Da do not have significant efficiency in gene delivery. In contrast, PEIs with molecular weights of 25 kDa or higher make good carrier candidates for gene delivery, with 25 kDa PEI having better transfection activity than 50 kDa PEI (Godbey et al., 1998; Kircheis et al., 2001). The mechanism of PEI/nucleic acid transfection has been intensively studied. PEI-based complexes enter cells through syndecan receptors. Complexes bind to heparan sulfate proteoglycans (HSPGs) on syndecan, prior to syndecan clustering into rafts and mediating PKC-dependent phosphorylation and actin binding through mediator proteins. As a result, actin-mediated phagocytosis occurs. Since PEI is only partially protonated under physiological conditions, it can act as a proton acceptor in the acidic environment of lysosomes, thus buffering the low pH and protecting the genetic material from degradation. The concomitant influx of chloride ions and water may eventually lead to endosome/lysosome disruption, with subsequent release of the complexes into the cytoplasm (the proton sponge effect) (Kopatz et al., 2004).

Despite being very promising as non-viral vectors, the use of PEIs is associated with several issues, including cytotoxicity, which increases with higher molecular weight. However, certain modifications can enhance their biocompatibility and can additionally improve transfection efficacy. PEIs grafted with carbohydrates (chitosan, dextran, pullulan), amino acids (leucine, histidine, arginine, tyrosine) or fatty acids have been reported as more efficient and safer than their unmodified counterparts (see e.g. (Aldawsari et al., 2011; Ewe et al., 2016)). Likewise, biodegradable PEIs have a lower cytotoxicity and a higher transfection efficiency (Jere et al., 2009) Commercially available polymers can be modified or grafted with cross linking agents like 1,3-butanediol (Forrest et al., 2003), disuccinidyl suberate, ethylene glycol bis[succinidyl succinate] (Thomas et al., 2005) or oligo (L-lactic-co-succinic acid) (Petersen et al., 2002).

We recently described tyrosine-modified linear or branched PEIs as particular efficient for small RNA transfection *in vitro* and therapeutic delivery *in vivo* (Ewe et al., 2016, 2019; Karimov et al., 2021; Noske et al., 2020). Initial data also indicated favorable biocompatibility of the complexes comprising these tyrosine-grafted PEI derivatives and small RNAs. Despite the high stability of these complexes, their decomposition, with subsequent release of the free polymer, must be taken into consideration, thus requiring independent evaluation of the free modified or unmodified PEIs. We have compared important physicochemical/biophysical and biological properties of linear and branched polyethylenimines with their tyrosine-modified counterparts.

2. Materials and methods

2.1. Polyethylenimines (PEIs) and tyrosine-modified derivatives

Linear polyethylenimines 2.5 and 25 kDa (Polysciences, Eppelheim, Germany) purchased as hydrochloride salts were neutralized using a 1 M sodium hydroxide solution and lyophilized to obtain the non-ionic form. 5 kDa and 10 kDa linear PEIs, as also 2 kDa and 25 kDa branched PEI, were from Sigma-Aldrich (Taufkirchen, Germany). 10 kDa branched PEI was from Polysciences (Eppelheim, Germany) and 5 kDa branched PEI was a kind gift from BASF (Ludwigshafen, Germany). Tyrosine modified derivatives were synthetized as before (Ewe et al., 2016, 2019; Karimov et al., 2021). All polymers we tested are listed in Suppl. Table 1.

2.2. Measurement of zeta potentials and hydrodynamic diameters

For measuring the hydrodynamic diameter and the zeta potential of particles from unmodified and tyrosine-modified polyethylenimines, a Photon Correlation spectrometer Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) was used. Zeta potential measurements were made in 0.9% NaCl solution at 25 °C and the values were calculated from the Helmholtz-Smoluchowski equation. Data from 18 to 25 measurements were collected and averaged for each sample. For the determination of hydrodynamic diameters, the wavelength was set at 633 nm, the detection angle 90° and the refraction factor 1.54. Measurements were made in 75 mmol/L NaCl solution at 25 °C, with 7 measurements in 5 cycles for each sample. The data were analyzed using Malvern software.

2.3. Transmission electron microscopy (TEM)

The morphology of the polymers was analyzed by TEM. 20 µl of a 5 mg/ml PEI solution were placed on a 200-mesh carbon-coated copper grids, prior to drying and staining with saturated uranyl acetate for 20 min. The samples were washed in distilled water and dried at room temperature. A JEM 1010 transmission electron microscope (JEOL, Japan) was used to take the images.

2.4. Hemolysis assay

Erythrocytes from healthy mice were isolated by several washings (0.5% sodium citrate in Ringeŕs solution; m/V)) and centrifugation (5 min, 5000 rpm) steps. The erythrocytes were used immediately after isolation. PEIs and their tyrosine-modified derivatives were added to erythrocyte samples at 5–25 µg/ml and incubated for 3 h at 37 °C. After incubation, samples were centrifuged (5 min, 1000 rpm) and the supernatant transferred into a 96-well plate (100 µl/well, in triplicates). Absorbance measurements were performed in an ELISA plate-reader at 550 nm (reference $\lambda = 620$ nm), with pure PBS as the negative control, while a lysate prepared by adding 2% Triton X-100 was used for the determination of maximum hemoglobin release (positive control = 100% value).

The hemolysis percentage was calculated by the formula:

$$H_{(\%)} = \left(A_{pb}540_{nm}/A_{Pos.CTRL}540_{nm}\right) \times 100\%$$

where $H_{(\%)}$ is the percentage of hemolysis, A_{pb} 540 $_{nm}$ is the absorbance of samples and $A_{Pos.CTRL}$ 540 nm is the absorbance of erythrocytes incubated with 2% Triton X-100 (100% hemolysis).

2.5. Evaluation of changes in membrane fluidity

The interaction of modified and unmodified PEIs with membranes were determined by measuring alterations in the membrane fluidity of artificial lipid membranes. For this, DMPC:DPPG (9:1) liposomes were prepared by an extrusion technique using an Avanti Polar Lipids mini-extruder. Lipid mixtures dissolved in chloroform were evaporated under vacuum, and the thin lipid film was watered with PBS (pH 7.4, 10 mmol/L), prior to heating the lipid suspension to 45 °C and passing it > 21 times through a polycarbonate membrane (pore diameter 100 nm) installed in the extruder. After extrusion, the liposome suspension (5 mg/ml lipids) was stored at 4 °C and used for experiments within 1 week after preparation at a final lipid concentration of 25 µg/ml.

The fluorescence anisotropy technique was applied using 2 fluorescent probes, DPH (1,6-diphenyl-1,3,5-hexatriene and TMA-DPH (1-(4-(trimethyloamino)phenyl)-6-phenylhexatriene), at 1 µmol/L. Fluorescence anisotropy intensities were measured in the presence of increasing polymer concentrations using LS-50B (Perkin Elmer, UK) spectrofluorimeter. Excitation and emission wavelengths were 348 nm and 426 nm for DPH and 358 nm and 428 nm for TMA-DPH, respectively. Slit widths

were 2.5 nm for the excitation monochromator and 4 nm for the emission monochromator. The fluorescence anisotropy was measured at 37 °C and calculated using the Perkin Elmer software from Jablonski's equation:

$$A = (I_{VV} - GI_{VH})/(I_{VV} + GI_{VH})$$

where A is fluorescence anisotropy, I_{VV} and I_{VH} are vertical and horizontal fluorescence intensities, respectively, to its vertical polarization of the excitation light beam, and G is the grating correction factor I_{VH}/I_{VV}, correcting the polarization effects of the monochromators.

2.6. MTT assay

To evaluate polymer cytotoxicity MCF7 and H441 cell lines were used. MCF 7cells were maintained in DMEM medium, H441 were kept in RPMI 1640 (Gibco, Dublin, Ireland) supplemented with heat-inactivated 10% FBS (Sigma Aldrich) and 1% antibiotics (penicillin/streptomycin). Cells were kept at 37 °C in air plus 5% CO_2.

MCF 7 cells were seeded at 1×10^4 cells per well in 96-well plates and cultivated as described above. The next day, PEIs or their tyrosine-modified derivatives were added to the medium at concentrations ranging from 1 to 25 μg/ml. H441 cells were prepared as described in Section 2. After 24 h incubation, the medium was aspirated and the cells washed with 100 μl PBS, prior to adding 100 μl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thermo Fisher Scientific, UK), dissolved in PBS at 0.5 mg/ml to each well and incubating for 4 h. Then, MTT was removed and 100 μl DMSO was added to dissolve the insoluble formazan crystals. The absorbance was measured in a BioTek plate reader, with the wave-length set at 580 nm and the reference wave-length at 720 nm. Cell viabilities were calculated from the formula:

$$\text{Viability}(\%) = A/A_s \times 100\%$$

with A = absorbance of control and A_s = absorbance of the sample. Results are given as mean +/− standard deviation (SD) from at least 3 independent experiments.

2.7. Comet assay

The alkaline version of the comet assay was used to estimate the DNA damage in cells treated with modified or unmodified PEIs. The cells were seeded on 24-well plate, at 50,000 cells/well. After overnight incubation the cells were treated with polymers (polymers concentration were equal IC20 values, calculated from GraphPad Prism Software). DNA damage was checked after 24 h incubation. Subsequently, the positive control was prepared (MCF 7 cells were treated with 80 μM H_2O_2, for 30 min at 37 °C). Cells were resuspended in 0.75% low melting-point agarose in PBS, pH 7.4, and spread at 5×10^4 cells per dish on microscope slides pre-coated with a layer of 1% normal melting-point agarose. The slides were treated with lysis buffer (2.5 mmol/L NaCl, 100 mmol/L EDTA, 1% Triton X-100 and 10 mmol/L Tris, pH 10) at 4 °C for 1 h, prior to placing them in the electrophoresis solution (300 mmol/L NaOH, 1 mmol/L EDTA) at 4 °C for 20 min. Electrophoresis was carried out at 0.73 V/cm, 300 mA for 30 min in electrophoresis solution. After electrophoresis, DNA was stained with DAPI (2 μg/ml) in the dark. 100 randomly selected cells were image-analyzed by microscopy (Nikon E200, Tokyo, Japan), attached to a COHU 4910 video camera (Cohu, San Diego, CA) and equipped with UV filter, and by using the image analysis system Lucia-Comet v. 7.3. The percentage of DNA in comet tails was measured.

2.8. Fluorescence polarization

Fluorescence polarization of FITC-labelled siRNA was measured with a PerkinElmer LS-50B spectrofluorometer (Perkin-Elmer, Waltham, MA, USA). 30 pmol of siRNA were complexed with PEI polymers in different mass ratios (form 1.25 to 5.0), in HN buffer, in room temperature. Excitation wavelength of 488 nm was with an excitation-slit width set at 2.5 nm, and emission wavelength 518 nm with an emission-slit set of 3.5 nm. Data were presented as mean ± standard deviation (SD) of a minimum of 3 independent experiments.

2.9. Gel electrophoresis

0.2 μg siRNA were complexed with PEI polymers in different mass ratios (from 1.25 to 5.0) in HN buffer for 40 min in room temperature. Complex formation was monitored by gel electrophoresis carried out on 3% agarose with GelRed dye in TAE buffer (Tris:acetate:EDTA) for 45 min in 90 V, 35 mA. Gels were visualized using ChemiDoc-it2 camera.

2.10. Preparation of the complexes and cell culture

PEI/siRNA complexes were prepared as previously described (Karimov et al., 2021). Briefly, 0.4 μg (30 pmol) of siRNA were complexed with 1 μg of non-modified or tyrosine-modified PEI in HN buffer prior to incubation for 40 min. For luciferase activity complexes were prepared in mass ratio 2.5 and four different concentrations of siRNA (siLuc3) were tested (7.5 pmol, 15 pmol, 22.5 pmol and 30 pmol). siLuc2 was used as a negative control.

H441 cell line were maintained in RPMI 1640 cell culture medium supplemented with 10% FBS and 1% antibiotics, in 37 °C, 5% CO_2.

2.11. LDH and JC-1 assays

LDH assay was performed using post-treatment cell medium. Cytotoxicity Detection Kit (Roche, Mannheim, Germany) was applied in this study according to the manufacturer's manual. Cells lysed with Triton X-100 were served as positive control. Post-treatment medium from untreated cells served as negative control. 50 μl sample medium was mixed with 50 μl reagent and incubated for 30 min in the dark. The readout was performed on 96-well plate at wavelengths 490/620 nm. Fresh medium was mixed with reagent and subtracted from all values for blank values correction. For mitochondrial membrane disruption JC-1 assay was performed. Cells were seeded at a density 7 000/well and left for an overnight incubation. At the next day cells were treated with PEI/siRNA complexes (siRNA concentration was 6 pmols on 96 well plate what corresponds to the treatment at 30 pmols on 24-well plate). The changes in mitochondrial membrane potential were monitored after 24 and 40 h. 5,5 ,6,6 -tetrachloro-1,1 ,3,3 - tetraethylbenz-imidazolcarbocyanine iodide (JC-1) was added at the final concentration 5 μM and the cells were incubated for 20 min in the dark. Fluorescence values were collected for JC-1 monomers (ex. 485/em. 538 nm) and for JC-1 dimers (ex. 530/em. 590 nm). The fluorescence factor for mitochondrial membrane potential was calculated as follows:

$$\Delta\Psi m = F_D/F_M$$

where Ψm is a fluorescence factor proportional to the changes in mitochondrial membrane potential, F_D is a fluorescence values for JC-1 dimers and F_M is a fluorescence values for JC-1 monomers.

2.12. Measurement of luciferase activity

Luciferase activitiy were measured after 72 h incubation using the Beetle-Juice Kit (PJK, Kleinblittersdorf, Germany). The medium was removed and lysis buffer (Promega, Mannheim, Germany) was added, for 30 min at room temperature. In a test tube cell lysate was mixed with luciferin substrate and luminescence was immediately measured in a luminometer (Berthold, Bad Wildbad, Germany).

2.13. Statistical analysis

Results were obtained from a minimum of 3 independent experiments and are presented as mean ± SD. Statistical analyses were performed using non-parametric ANOVA and the Kruskal-Wallis test. Significance levels were defined as * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

3. Results

3.1. Zeta potential and hydrodynamic diameter of particles

Micro-images obtained by TEM revealed that all analyzed polymers formed small aggregates in 10 mmol/L Na-phosphate buffer, pH 7.4 (Suppl. Fig. 1). PEIs were visible both as single nanoparticle-like small, clumped structures and as larger aggregated formulations with different sizes. Since the drying process for TEM preparation may affect the morphology of the polymers, we then took zetasizer measurements in aqueous solution.

Hydrodynamic diameter measurements of polymers revealed indeed measurable particle sizes (Table 1). When analyzing the linear polymers, these were lower in the case of the unmodified PEIs (from 31.1 ± 11.8 to 73.2 ± 25.9 nm) compared to their tyrosine-modified counterparts (from 68.8 ± 16.4 to 112.6 ± 34.2 nm). The opposite results were found for the branched polymers, where tyrosine-modified, branched PEIs exhibited lower diameter values than unmodified PEIs (from 96.5 ± 6.9 to 157.3 ± 78.6 nm vs. 51.6 ± 14.6 to 129.1 ± 12.3 nm, respectively). The existence of particle structures also allowed determination of zeta potentials. The results show that all our polymeric structures were positively charged. Highest values were detected for branched PEIs modified with tyrosine (Table 1). The zeta potential of the unmodified PEIs ranged from 17.6 ± 2.9 to 28.3 ± 5.0 mV, and 6.5 ± 2.39 mV to 27.1 ± 6.05 mV for linear and branched polymers, respectively. The values of tyrosine modified PEIs ranged from 14.7 ± 2.5 to 34.5 ± 6.5 mV for linear, and 26.4 ± 2.7 mV to 28.8 ± 4.6 mV for branched polymers.

3.2. Polymer interaction with lipid membranes

3.2.1. Polymer effects on membrane fluidity

One critical parameter for biological activity/cellular uptake, and for possible cytotoxicity, is the interaction of polymeric materials with cell membranes. In the case of PEIs, this may well depend on their chemical structure and modification with a partially hydrophobic substituent, i.e., tyrosine. Thus, fluorescence anisotropy was measured to compare the interaction potential between the various PEI-based polymers and lipid membranes. Two fluorescent probes, TMA DPH and DPH, were used to assess alterations in membrane fluidity based on hydrophilic or hydrophobic interactions, respectively. In the case of linear PEIs, relatively weak and largely concentration-independent effects on lipid membranes were observed. Direct comparison between the different probes showed that these membrane effects were essentially dependent on hydrophilic interactions, as seen from a slight increase in fluorescence anisotropy with the TMA DPH probe (Fig. 1A). In contrast, branched unmodified PEIs had some hydrophobic interactions (DPH probe), which were concentration-dependent and slightly more profound with decreasing molecular weight of the PEI (Fig. 1B). Notably, the introduction of tyrosine modifications led to a substantial increase in membrane interactions. This was particularly true for the linear tyrosine-modified PEIs and its hydrophilic interactions with the lipid membranes, which were profoundly increased even at the lowest polymer concentrations. Hydrophobic interactions were also enhanced, but to a lower extent and dependent on the polymer concentration (Fig. 1C). In the case of branched PEIs, tyrosine modification did not lead to major differences in hydrophobic interactions compared to their unmodified counterparts, and a sharp increase in hydrophilic interactions was only seen with the 10 kDa polymer (Fig. 1D).

3.2.2. Hemolysis

In principle, the observed increase in membrane interaction with tyrosine-modification of several PEIs may also translate into adverse effects on erythrocytes or other cells, potentially limiting their use. Hemolysis is an easy and simple method to assess cell membrane damage after incubation with test compounds. In the case of tyrosine-modified very small linear PEIs, a moderate increase in hemolysis over their unmodified counterparts occurred. This effect, however, was not seen for 25 kDa PEI, where tyrosine-modification led to reduced hemolysis (Fig. 2A). Linear PEIs and their tyrosine-modified derivatives generally caused < 10% hemolysis, with the exception of LP5Y (Fig. 2B).

3.3. Polymer cytotoxicity

MCF7 cells were used to evaluate the toxicity of the whole set of unmodified and tyrosine-modified PEIs. Any decrease in viability was concentration-dependent. The tyrosine-modified PEIs were less cytotoxic than their unmodified counterparts. This was particularly true for the higher molecular weight PEIs (Fig. 3A, B). At 5 μg/mL, the linear PEIs 5 kDa, 10 kDa and 25 kDa already showed a marked (40–70%) reduction of viability, which was not seen in the tyrosine-modified polymers. Major differences were also seen at higher concentrations. Only the very low molecular weight 2.5 kDa PEI had some cytotoxicity, which again, however, could be further reduced by its tyrosine modification, as seen at very high (25 μg/mL) concentration (Fig. 3A). Branched PEIs were overall slightly less toxic; however, their tyrosine modification once again markedly reduced or almost completely abolished cytotoxicity (Fig. 3B). This beneficial effect of tyrosine grafting was independent of the molecular weight of the PEI and seen at different concentrations; it was also found for the 2 kDa branched PEI, which showed the least cytotoxicity of the unmodified PEIs.

3.4. Polymer genotoxicity

Genotoxicity due to PEIs were analyzed in Comet assays for DNA fragmentation. Results obtained after 24 h incubation of MCF7 cells indicate some genotoxicity in the case of unmodified linear PEIs (Fig. 4A). This DNA damaging action was largely abolished with tyrosine

Table 1
Size (nm) and zeta potential [mV] of particle structures formed by the tyrosine modified or unmodified PEIs

	Linear polymers	Hydrodynamic diameter [nm]	Zeta potential [mV]	Branched polymers	Hydrodynamic diameter [nm]	Zeta potential [mV]
Unmodified	*LP 2.5 kDa*	48.9 ± 14.0	17.6 ± 2.9	*BR2 kDa*	96.5 ± 6.9	6.5 ± 2.39
	LP 5 kDa	31.1 ± 11.8	20.1 ± 3.3	*BR 5 kDa*	141.7 ± 41.1	15.01 ± 3.39
	LP 10 kDa	73.2 ± 25.9	25.7 ± 2.8	*BR 10 kDa*	152.6 ± 35.9	27.1 ± 6.05
	LP 25 kDa	52.1 ± 19.8	28.3 ± 5.0	*BR 25 kDa*	157.3 ± 78.6	24.1 ± 5.84
Modified with tyrosine	*LP 2.5 Y*	46.5 ± 4.4	17.7 ± 2.5	*P 2Y*	51.6 ± 14.6	28.8 ± 4.6
	LP 5 Y	80.8 ± 24.7	14.7 ± 2.5	*P 5 Y*	65.7 ± 26.2	27.4 ± 11.4
	LP 10 Y	68.8 ± 16.4	34.5 ± 6.5	*P 10 Y*	55.4 ± 24.3	26.4 ± 2.7
	LP 25 Y	112.6 ± 34.2	29.9 ± 6.8	*P 25 Y*	129.1 ± 12.3	26.4 ± 6.6

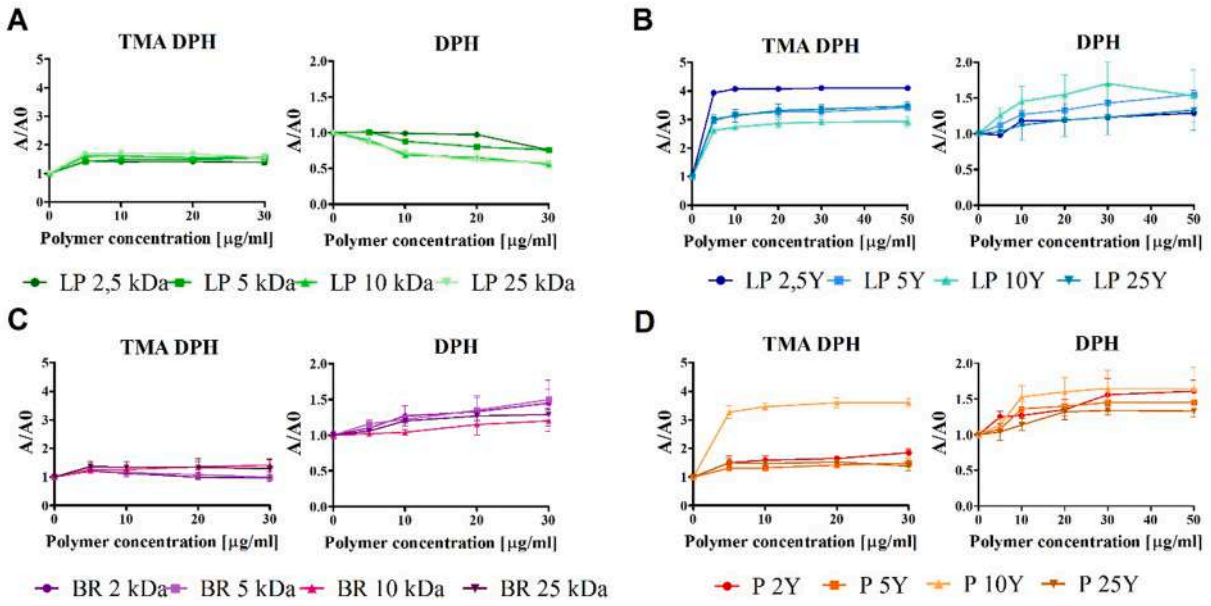

Fig. 1. Determination of the changes in fluorescence anisotropy of TMA DPH and DPH fluorescent probes incorporated into a liposomal DMPC:DPPG (9:1 w/w) lipid matrix in the presence of unmodified linear (A) or branched PEIs (B), or their tyrosine-modified linear (C) or branched counterparts (D).

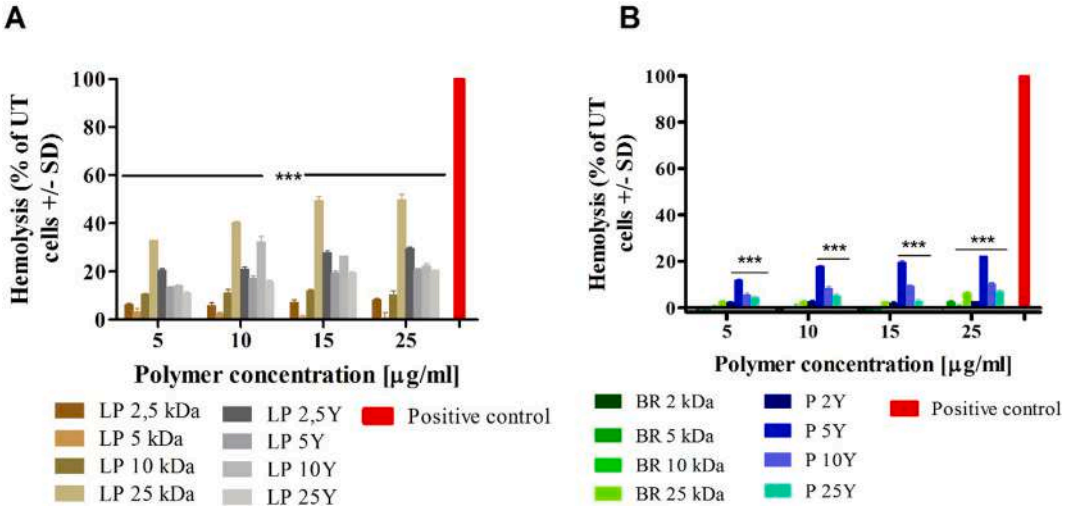

Fig. 2. Percentage of hemolysis of mice erythrocytes treated with linear (A) and branched polymers (B), normalized to UT cells (negative control).

modification of linear PEIs, and branched PEIs showed essentially no genotoxicity, whether or not tyrosine-grafted (Fig. 4B).

3.5. PEI/siRNA complex formation

Tyrosine modified and non-modified polymers were complexed with siRNA in different mass ratios. Results presented on Fig. 5A-B show that non-modified polymers did not create complexes with siRNA in a range of mass ratios from 1.25 to 5.0. In turn all tyrosine-modified polymers completely saturated siRNA in mass ratio 2.5. Complex formation was confirmed also by fluorescence polarization assay (Fig. 5C). Obtained results indicate that tyrosine-modified polymers could bind siRNA at the mass ratio 2.5. Increasing amount of polymers was not altered complexes formation.

3.6. Cytotoxicity and efficacy of tyrosine-modified PEI:siRNA complexes

The toxicity of complexes was evaluated after 24-hour incubation using LDH assay (Fig. 6). Basing on results of previous experiments, complexes were prepared in PEI/siRNA mass ratio 2.5:1, at the siRNA concentration 30 pmol. Formed complexes caused a low toxicity towards H441 cell line. The level of released LDH was comparable to untreated cells. In turn the decrease in mitochondrial membrane potential was observed when LP 5Y and LP 10Y were used after 24 and 48 h of incubation. The decrease in membrane potential was more profound after 48 h for all PEI complexes

Complexes activity and gene knockdown efficiency was evaluated after 72-hour incubation (Fig. 7). In order to observe dose-dependency the complexes were prepared with a different siRNA concentration (from 7.5 to 30 pmol). The ratio between polymers and siRNA remained the same (ratio 2.5). Complexes containing linear polymers more efficiently decreased luciferase activity than complexes with branched polymers. The most efficient in gene knockdown were complexes contained LP 5Y, LP 10Y, P 5Y and P 10Y polymers. They caused a 50 % and over decrease in luciferase activity even in the lowest concentration. LP 10 kDa polymer was tested as well. Some gene knockdown was observed

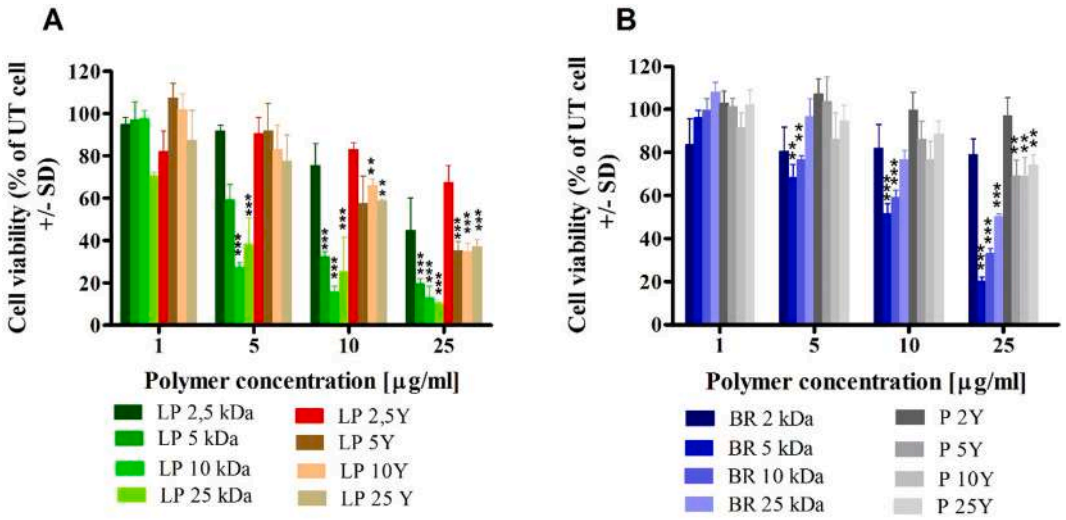

Fig. 3. Cytotoxicity profiles of linear (A) and branched (B) PEIs in MCF7 cells after 24 h incubation. Values are the mean ± standard deviation of 3 independent experiments, with * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ indicating statistically significant differences in comparison to control values.

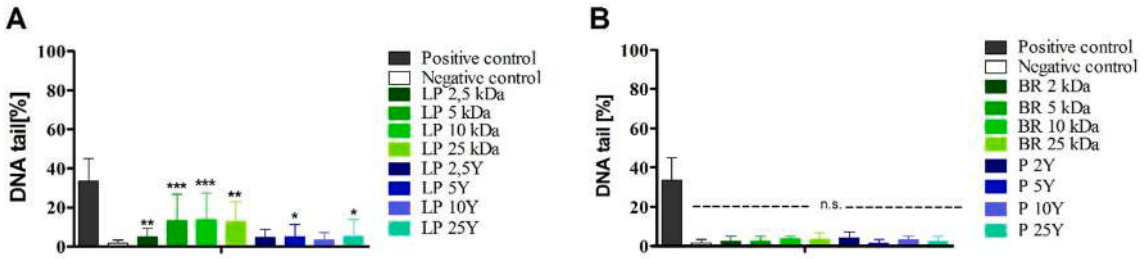

Fig. 4. Genotoxicity profiles of linear (A) and branched PEIs (B) in MCF7 cells. Values are the mean ± standard deviation of three independent experiments, with * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ indicating statistically significant differences in comparison to control values. n.s – not statistically significant.

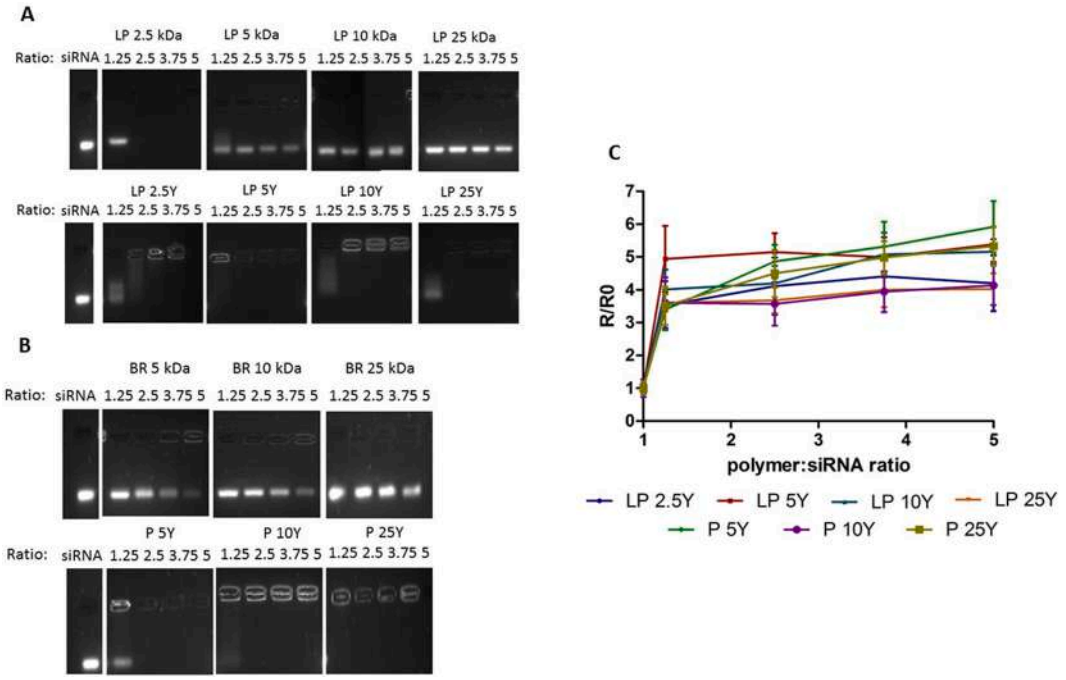

Fig. 5. Biophysical characterization of complex formation by tyrosine modified and non-modified PEI polymers and siRNA. (A) – Gel electrophoresis images representing complex formation between linear PEIs and siRNA in a range of mass ratios from 1.25 to 5.0. (B) – Gel electrophoresis images representing complex formation between branched PEIs and siRNA in a range of mass ratios from 1.25 to 5.0. (C) – fluorescence polarization of FITC-labelled siRNA complexed with tyrosine modified PEIs, R/R_0 – fluorescence polarization ratio of non-complexed siRNA to complexed siRNA.

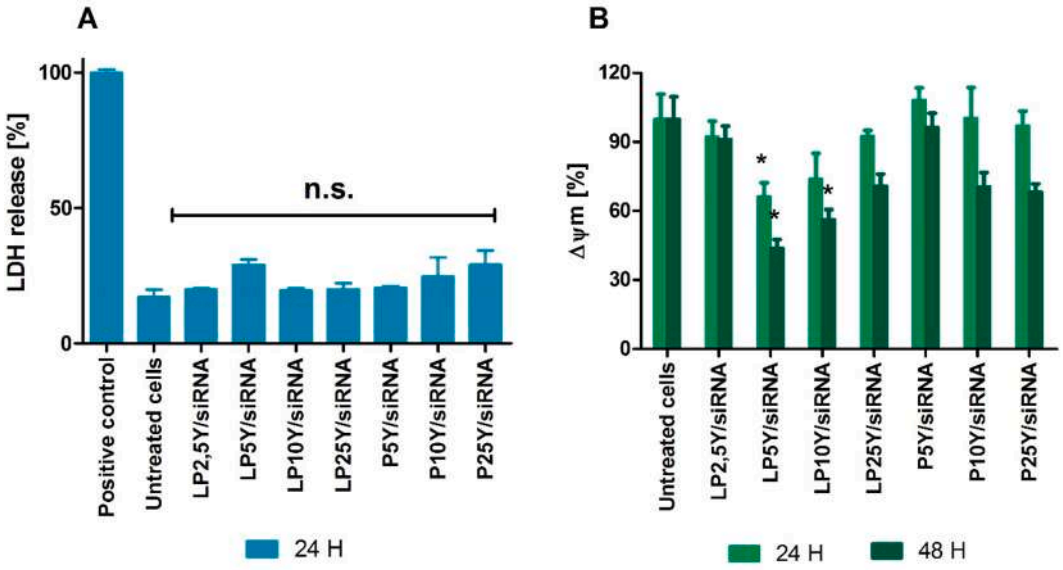

Fig. 6. (A) - H441 cell damage after 24-hour treatment with PEI/siRNA complexes. siRNA concentration 30 pmols, mass ratio 2.5. Positive control was consisted the cells treated with 10% Triton X 100. (B) – changes in mitochondrial membrane potential after 24/48 h treatment with PEI/siRNA complexes. Results presented as mean +/− SD, n = 3, * - p < 0,005. N.s. – non-significant.

but low level of unspecific siRNA indicates that the complex toxicity was high.

4. Discussion

Cationic polymers as non-viral nanocarrier systems are being intensely researched (Liu et al., 2010; Wang et al., 2016). Chemical modifications of polymers can improve their properties by enhancing biological efficacy and biocompatibility (Ewe et al., 2016; Hashemi et al., 2016). We have compared 16 linear or branched PEIs over a wider y molecular weight range and their corresponding tyrosine-modified derivatives. Tyrosine is crucial for maintaining protein structures and constituting the cores of globular proteins (Creusat and Zuber, 2008). It is considered as one of the best amino acids for coupling to nanovectors and improving their activity (Mehta et al., 2014; Ewe et al., 2016; Wang et al., 2016). Tyrosine coupling to the polymers did not impair their potent nucleic acid binding affinity based on electrostatic interactions, since amino groups in the polymer structure essential for complexation are replaced by amino groups from the tyrosine (Creusat and Zuber, 2008; Creusat et al., 2010). Indeed, tyrosine modification of PEIs can further enhance complex stabilities, putatively due to the occurrence of hydrophobic interactions. In line with this notion, we found that tyrosine-modified PEIs interacted with lipid membranes more strongly than their unmodified counterparts (see below). Thus, the combination of (i) introducing additional hydrophobic components, which are often associated with increased potential to interact with and cross cellular membranes, while (ii) retaining a positive charge for the interaction with negatively charged cellular membrane components (Pozzi et al., 2014; Schöttler et al., 2016; Francia et al., 2019) may contribute to the overall high efficacy of tyrosine-modified PEIs as seen previously (Ewe et al., 2019, 2016; Noske et al., 2020; Karimov et al., 2021). In line with this, we found that some tyrosine-modified linear PEIs had a stronger hemolytic potential than unmodified PEIs. Interestingly, however, this was dependent on the molecular weight of the linear PEI and, with the exception of the 5 kDa polymer, was not seen in the case of branched PEIs. Overall, our present and previous data indicate that nanoparticles modified with hydrophobic amino acids, especially when containing an aromatic ring, had increased interactions with phospholipids, thus facilitating cellular uptake (Creusat and Zuber, 2008; Liu et al., 2010; Wang et al., 2016, 2014). The polymer structure also seems to be relevant in this context.

Our fluorescence anisotropy results confirmed that the polymers, in particular the tyrosine-modified PEIs, interact with the lipid bilayer. We used 2 fluorescent probes to monitor changes in membrane fluidity. DPH is a non-polar molecule that may be localized in different parts of the hydrophobic tail region of the lipid bilayer. DPH anisotropy values correlate well with rotational diffusion movement and are sensitive to changes in packing order in lipid chains and membrane fluidity. TMA DPH is a cationic derivative of DPH that binds to hydrophilic regions of the membrane. It is useful to monitor the membrane properties at the fourth carbon atom in the transient part between hydrophobic and hydrophilic regions of the lipid bilayer (Pottel et al., 1983; Kwok and Hart, 2011; Ulrih et al., 2015). Our tyrosine-modified PEIs penetrate better into the membranes, as indicated by higher anisotropy values, while the unmodified polymers only slightly interacted with the lipid bilayer. Tyrosine-modified linear PEIs interacted very well with hydrophilic region of phospholipids, while some hydrophobic interactions also occurred, with especially LP 10Y also reaching the lipid chains in the hydrophobic part. Tyrosine-modified branched PEIs generally interacted less efficiently with the membranes than their linear counterparts, with the exception of P10Y, which strongly interacted with hydrophilic parts of the membrane. Regarding the comparison between tyrosine-modified linear and branched PEIs, and the overall effect of tyrosine modification, these results mostly correlate well with our hemolysis data.

DMPC:DPPG liposomes served as membrane model in this study. Addition of DPPG gave a negative charge to the membranes, to mimic the physiological conditions. However, it is well known that not only phospholipids, but many components from cellular membranes may also interact with nanoparticles. One example of proteins with anionic charge density due to sulfate modifications are glycosaminoglycans (GAGs), which are present on most cells and might compete with nucleic acids for binding to cationic nanocarriers, thus potentially disrupting cellular uptake of complexes (Kwok and Hart, 2011; Miller et al., 2014; Nademi et al., 2020).

Cationic polymers are considered to be toxic for cells, thus limiting their use in medicine (Fischer et al., 2003). As described above, however, tyrosine modification seems to offer an optimal hydrophobic/hydrophilic balance, translating into beneficial properties for gene delivery combined with reduced toxicity (Creusat et al., 2010). Indeed, we found

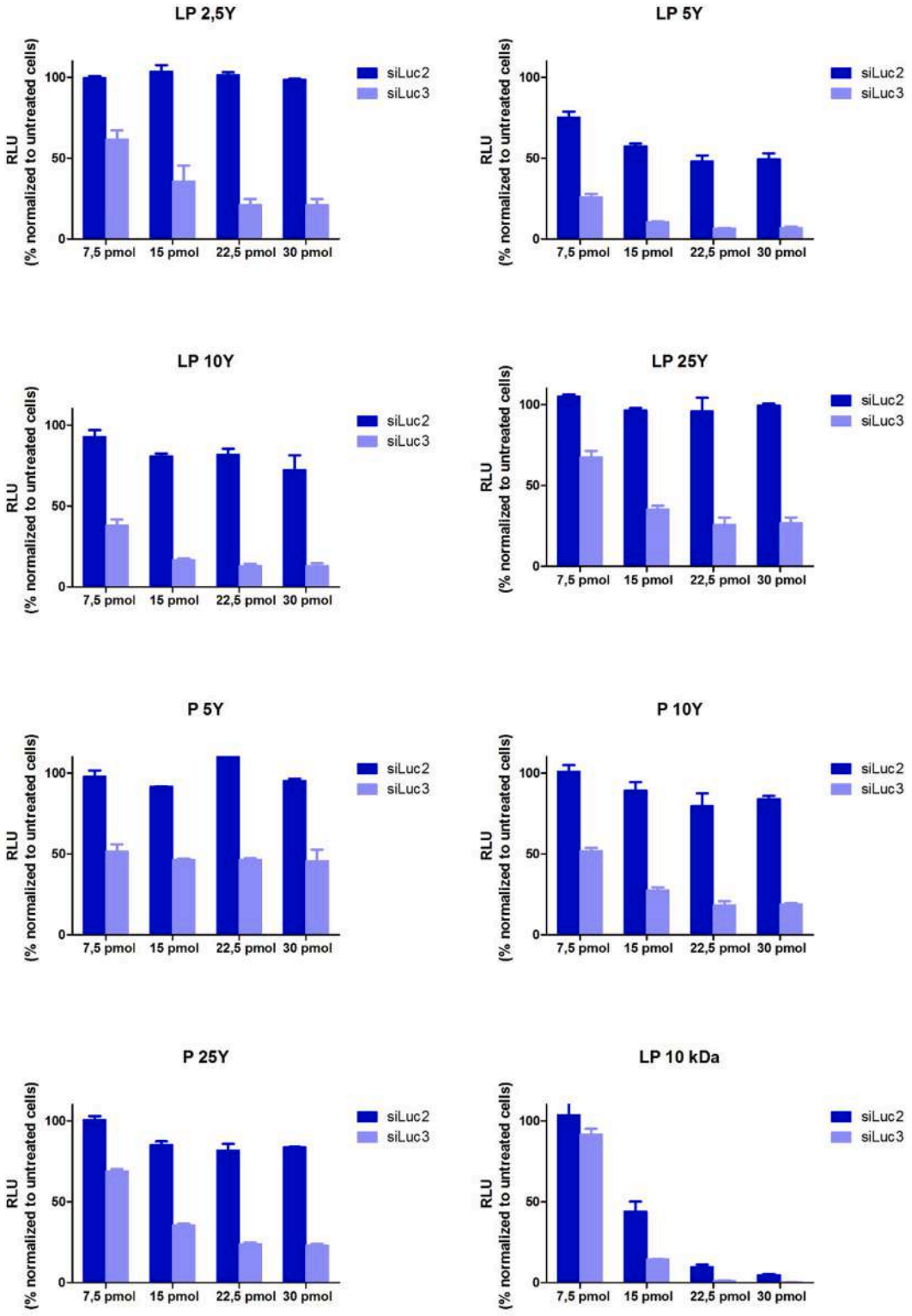

Fig. 7. Knockdown efficacy of luciferase reporter gene in stably Luc-expressing H441 cells after 72 h incubation with complexes containing siLuc 3 (specific siRNA) and siLuc 2 (unspecific siRNA).

that PEIs modified with tyrosine were considerably less toxic than unmodified PEIs. The underlying mechanisms of PEI cytotoxicity are not fully understood. Polycations do not cause apoptotic events, but are more disruptive to cell membranes (Monnery et al., 2017). These authors investigated poly-L-lysine polymers (PLL), and branched and linear PEI, and concluded that these compounds damaged membrane damages and released LDH. In the same study, mitochondrial activity was measured, the results suggesting that polycations may affect cellular metabolism in a time- and dose-dependent manner. Likewise, toxic effects of PEIs reported by Hall et al. included a decrease the mitochondrial potential and cytochrome *c* release (Hall et al., 2013).

Beyond cytotoxic effects of polyethylenimines, genotoxicity of PEI is still a controversial issue. While some reports describe genotoxic effects of PEIs (Choi et al., 2010; Calarco et al., 2013; Gholami et al., 2014),

another group suggested that PEIs do not induce DNA damage (Beyerle et al., 2011). We found some DNA damaging potential in the case of linear unmodified PEIs, which, despite enhanced membrane interaction and uptake, was largely abolished by tyrosine modification. Branched PEIs were essentially non-genotoxic, independent of any tyrosine modification. Again, this indicates the dependence of desired or unwanted cellular effects on the PEI structure. The alkaline version of the comet assay helps detect double-strand DNA breaks. Calarco et al. (2013) improved the method with enzymes that recognize DNA damage caused by oxidative stress. They found genotoxic effects of PEIs were based on oxidative damage of purines and pyrimidines. In line with our results, they also showed that PEI modifications, in this case with acetic groups, reduced genotoxicity. However, it must always be kept in mind that PEI cyto- and geno-toxicity increase with molecular mass (Liu et al., 2010; Gholami et al., 2014; Monnery et al., 2017). For this reason, it is particularly advantageous that the tyrosine modification permits the use of very low molecular weight PEIs, even for delivering small RNA molecules (Ewe et al., 2019, 2016; Noske et al., 2020; Karimov et al., 2021).

The next step of our work was to evaluate the siRNA:polymer interaction, cytotoxicity of formed complexes and activity *in vitro*. Fluorescence polarization and gel electrophoresis studies were performed to choose the optimal ratio, when all siRNA is complexed by polymers. Results of gel electrophoresis have shown that non-modified polymers did not complex siRNA in a range of mass ratios form 1.25 to 5.0. Tyrosine-modified polymers effectively complexed siRNA in siRNA:polymer mass ratio 1:1.25. In comparison, PEI modified with poly-L-lysine complexed siRNA in mass ratio 5:1 (Chen et al., 2015). In turn, optimal ratio for arginine-modified PEI:siRNA was 4:1 (Lu et al., 2019). Tyrosine modification enhance siRNA complexation in lower mass ratio what allows to use smaller amounts of polymers and reduce toxic effect of nano vectors. Fluorescence polarization studies confirmed the results from gel electrophoresis. Increased polarization values are reflected as interaction between nanocarriers and nucleic acids (Sanz del Olmo et al., 2020). Complexation of siRNA with polymers ≠or dendrimers results in decreased movement of siRNA in the suspension and to the increase of fluorescence polarization of FITC-labelled siRNA (Ferenc et al., 2013; Gorzkiewicz et al., 2020).

It is known that PEIs may induce two-stage toxicity. The early stage affects cellular membrane, second stage relies on mitochondria disruption (Hall et al., 2013; Moghimi et al., 2005; Monnery et al., 2017). LDH measurements allow to evaluate acute cell damage on early stage of treatment. Disrupted mitochondria can be analyzed using JC-1 assay. This cationic fluorescent dye can be found in living cells, exclusively in mitochondria. Non-affected cells convert JC-1 into J-aggregates with a fluorescent signal near 590 nm. In disrupted organelles the fluorescence of JC-1 monomers can be observed near 529 nm (Elefantova et al., 2018). The balance between dimers and monomers allows to estimate the changes in mitochondria membrane potential (Michlewska et al., 2019). Tyrosine-modified polymers were generally less toxic than non-modified polymers. Complexes of tyrosine-modified PEIs and siRNA did not caused any significant damage of the cell membrane what was confirmed by LDH assay. In turn, mitochondrial membrane potential was affected by LP 5Y/siRNA after 24 and 48 h and LP 10Y/siRNA complexes after 48 h. Some toxic effect can be also seen when analyzing luciferase knockdown efficacy. LP5Y/siRNA complexes caused the decrease in cell viability even when non-specific siLuc2 was added to the cells. In this case decreased values can be explained by cell death (Karimov et al., 2021). Tyrosine-modified polymers efficiently transported siRNA to the cells even in the low concentrations. Tyrosine enhance polymers interaction with nucleic acid (Dougherty, 1996; Huang et al., 2015; Plyte and Kneale, 1991). Non-modified low molecular PEIs are inactive for siRNA delivery, but tyrosine modification allows to use them as siRNA nanocarriers. Tyrosine-modified PEIs are highly efficient siRNA nano vectors even in low concentrations what is beneficial when considering cytotoxicity. Linear PEIs are considered as a better alternative for nucleic acid delivery due to higher biocompatibility and appropriate chemical composition giving promising biological effects. The availability of linear, non-modified PEIs to interact with siRNA was limited. Tyrosine modification changes their properties and makes them an attractive nano system for siRNA delivery (Ewe et al., 2016; Karimov et al., 2021).

5. Conclusion

The biophysical and biological properties of linear and branched PEIs of different molecular masses in direct comparison with their tyrosine-modified derivatives alone and in the complexes with siRNA have been evaluated in this study. Our data on the free polymers help to explain previous findings on nanoparticles comprising tyrosine-modified PEIs and small RNAs, in regard to their enhanced efficacy of RNA delivery and improved biocompatibility. This relates to the markedly improved membrane interaction of the tyrosine-modified polymers, as well as to the largely reduction or abolition of cytotoxic and genotoxic effects after tyrosine grafting. While in a potential therapeutic setting, the polymers would better be used as nanoscale complexes, i.e., in a combined formulation with nucleic acids, it must be kept in mind that extra- or intra-cellular nanoparticle decomposition may lead to the release of the free polymer. Thus, our study on the biophysical and biological properties of the free polymers provides a contribution to the notion that tyrosine-modified PEIs are particularly promising delivery platforms especially for small nucleic acids like siRNA. The results confirmed that tyrosine modification significantly improves siRNA complexation, with a high efficiency and reduced cytotoxicity.

Funding sources

This work was supported by the grant from the Deutsche Forschungsgemeinschaft (DFG; AI 24/21-1; AI 24/24-1) and Nacional Science Centre of Poland "BEETHOVEN LIFE 1" program, project no: 2018/31/F/NZ5/03454. The authors are also grateful for funding by grants from the project "EUROPARTNER" of the Polish National Agency for Academic Exchange (NAWA).

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. Ethics Committee of the University of Lodz, Poland (NR19/KBBN-UŁ/III/2019).

CRediT authorship contribution statement

Małgorzata Kubczak: Methodology, Writing – original draft, Visualization, Writing – review & editing. **Sylwia Michlewska:** Methodology, Writing – original draft, Visualization, Writing – review & editing. **Michael Karimov:** Methodology, Writing – review & editing. **Alexander Ewe:** Conceptualization, Methodology, Writing – review & editing. **Sandra Noske:** Investigation. **Achim Aigner:** Conceptualization, Methodology, Writing – review & editing. **Maria Bryszewska:** Writing – review & editing. **Maksim Ionov:** Conceptualization, Validation, Visualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2022.121468.

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Comparative study of unmodified and tyrosine-modified low molecular weight polyethylenimines: biophysical characterization and toxicity

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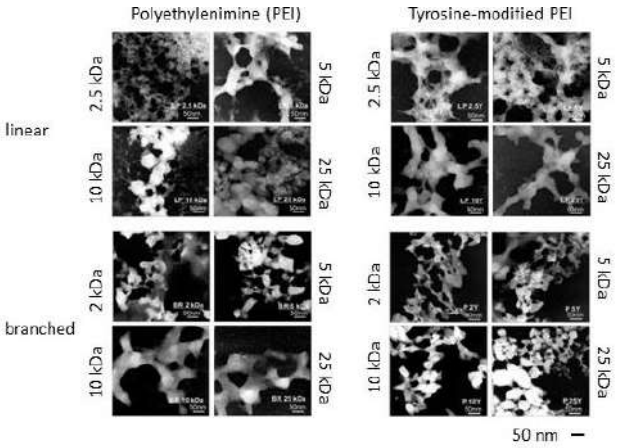
Suppl. Table 1. Linear and branched PEIs, unmodified and modified with tyrosine

	Linear polymers	Branched polymers
Unmodified	LP 2.5 kDa	br 2 kDa
	LP 5 kDa	br 5 kDa
	LP 10 kDa	br 10 kDa
	LP 25 kDa	br 25 kDa
Modified with tyrosine	LP 2.5Y	P 2Y
	LP 5 Y	P 5 Y
	LP 10 Y	P 10 Y
	LP 25 Y	P 25 Y



Suppl. Fig. 1. Ultrastructures of linear (upper panel) and branched PEIs (lower panel), unmodified (left) or with tyrosine-modification (right), visualized by transmission electron microscopy (TEM). Compounds were dissolved in 10 mM Na-phosphate buffer, pH 7.4. Bar = 50 nm.

NANOTOXICOLOGY
https://doi.org/10.1080/17435390.2022.2159891

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ARTICLE

Comparison of tyrosine-modified low molecular weight branched and linear polyethylenimines for siRNA delivery

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ABSTRACT

Polyethylenimines (PEIs) have been previously introduced for siRNA delivery. In particular, in the case of higher molecular weight PEIs, this is associated with toxicity, while low molecular weight PEIs are often insufficient for siRNA complexation. The tyrosine-modification of PEIs has been shown to enhance PEI efficacy and biocompatibility. This paper evaluates a set of tyrosine-modified low molecular weight linear or branched polyethylenimines as efficient carriers of siRNA. Complexation efficacies and biophysical complex properties were analyzed by zeta potential, dynamic light scattering and circular dichroism measurements as well as gel electrophoresis. Biological knockdown was studied in 2 D cell culture and 3 D *ex vivo* tissue slice air-liquid interface culture. The results demonstrate that siRNAs were able to form stable complexes with all tested polymers. Complexation was able to protect siRNA from degradation by RNase and to mediate target gene knockdown, as determined on the mRNA level and in PC3-Luc3/EGFP and HCT116-Luc3/EGFP expressing reporter cells on the protein level, using flow cytometry and confocal microscopy. The direct comparison of the studied polymers revealed differences in biological efficacies. Moreover, the tyrosine-modified PEIs showed high biocompatibility, as determined by LDH release and mitochondria integrity (J-aggregate assay) as well as caspase 3/7 (apoptosis) and H_2O_2 levels (ROS). In 3 D tissue slices, complexes based on LP10Y proved to be most efficient, by combining tissue penetration with efficient gene expression knockdown.

ARTICLE HISTORY
Received 12 September 2022
Revised 13 December 2022
Accepted 14 December 2022

KEYWORDS
Nanomaterials; polyethylenimines; siRNA delivery; gene knockdown cytotoxicity; polymeric nanoparticles

Introduction

Therapies based on RNA interference (RNAi) are currently under special interest (Kim et al. 2015; Song et al. 2018). Due to its mechanism of action, RNAi interference may be directed against any target gene of interest, thus offering certain advantages over traditional drug-based treatment strategies and also allowing for treating otherwise 'undruggable' diseases. RNAi relies on the action of small interfering RNAs (siRNAs) which are incorporated into the RISC complex and mediate RISC binding to the sequence-complementary target mRNA with its subsequent cleavage and degradation. The successful implementation of RNA-based therapies, however, requires nucleic acid delivery vehicles with specific properties (Aigner 2019; Ionov et al. 2015). Major requirements of gene carriers are their ability to protect the cargo from degradation, penetrate the cell membrane and release the nucleic acid 'payload' intracellularly at its site of action (Raval et al. 2019). Viral vectors were proposed as the most suitable delivery vehicles due to their natural feasibility of carrying nucleic acid into the cells. However, their usage is limited to certain nucleic acids, excluding siRNAs, and they are associated with safety concerns and risk of immunogenicity (Kotterman et al. 2015). This highlights the relevance of non-viral carrier systems, including liposomal and polymeric nanoparticles.

Cationic polymers like polyethylenimines (PEIs) have been broadly tested as plasmid DNA carriers (Gosselin et al. 2001; Kichler et al. 2001; Sig et al. 2004). The structure of PEIs is based on nitrogen atoms at every third position, with a certain

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percentage being positively charged even at physiological pH, thus allowing for the complexation of negatively charged nucleic acids. This complexation leads to nanoparticle formation and protection of the nucleic acid from degradation (Rodier et al. 2019). The ability of nucleic acid complexation and delivery as well as the toxicity depend on the molecular mass of the polymers (Godbey et al. 1999; Pandey and Sawant 2016). Higher molecular weight PEIs are efficient, but associated with higher cytotoxicity, while smaller PEIs are essentially nontoxic, but show only poor transfection efficacy (Creusat et al. 2010; Günther et al. 2011; Kichler et al. 2001). The structure of the polymers plays a role in complexation as well. Linear PEIs are able to interact with plasmid DNA whereas the complexation of siRNA is poor. In contrast, branched PEIs are able to bind plasmid DNA as well as siRNA (Kwok and Hart 2011; Sadeghpour et al. 2018).

Polymers like PEI allow the comparatively facile introduction of chemical modifications, and some of those have been proposed for increasing cellular uptake and reducing toxic effects (Abid et al. 2017; Beyerle et al. 2010; Lu et al. 2019; Yu et al. 2017). For example, hydrophobic modifications may improve the interaction the polymers with nucleic acids and enhance the cellular uptake of the resulting nanoparticles. PEGylated polymers would exhibit lower toxicity, prolonged circulation half-lives in the blood stream and increased serum tolerance. On the other hand, the addition of PEG or negatively charged residues may disrupt DNA/RNA condensation and inhibit cellular uptake (Beyerle et al. 2010; Merkel et al. 2009; Saqafi and Rahbarizadeh 2019).

Amino acid residues have been successfully implemented for PEI modification (Chen et al. 2015; Ewe et al. 2016; Karimov, Schulz, et al. 2021; Yu et al. 2017). This approach offers advantages over other modifications. In the case of cationic dendrimers, Wang et al. explored their modification with twenty amino acids (Wang et al. 2016). While anionic or hydrophilic modifications were non-efficient for transfer efficacy, hydrophobic or cationic amino acids increased the complexation efficiency. It was demonstrated that Tyr, Phe, Trp, Arg, Lys or His efficiently increased the transfection efficacy. Arginine and lysine modification improved DNA binding, complex formation and cellular uptake. The addition of histidine promoted endosomal escape. Among all amino acids, hydrophobic, aromatic amino acids were introduced as the most promising modification for nucleic acid delivery (Tyr, Trp, Phe), better acting than hydrophobic, aliphatic amino acids (Ile, Val, Ala). This is related to the more hydrophobic structure of aromatic ring, perhaps facilitating complex interaction with membrane phospholipids and improving endosomal escape (Creusat et al. 2010; Liu et al. 2010).

Tyrosine-grafting has been found to increase efficacy and biocompatibility of linear and branched PEIs (Chen et al. 2015; Ewe et al. 2016; Karimov, Schulz, et al. 2021; Yu et al. 2017). In this study, we aimed to investigate and compare a set of tyrosine-modified linear and branched PEIs with different molecular weights, for siRNA delivery *in vitro* and in a tissue slice model *ex vivo*. Complexation of siRNA, biophysical complex properties, toxicity and gene knockdown efficacy were studied.

Materials and methods

Polymers and siRNA

Tyrosine modified polymers (Table 1) were synthetized as described previously (Ewe et al. 2016; Karimov, Schulz, et al. 2021). Non-modified siRNAs were purchased from Dharmacon (Lafayette, CO) with sequences as follows: siLuc2: sense, 5′-CGU ACG CGG AAU ACU UCG AdTdT-3′; antisense, 5′-UCG AAG UAU UCC GCG UAC GdTdT-3′; siLuc3: sense, 5′-CUU ACG CUG AGU ACU UCG AdTdT-3′; antisense, 5′-UCG AAG UAC UCA GCG UAA GdTdT-3′; siGAPDH: sense, 5′-CCU CAA CUA CAU GGU UUA CdTdT-3′; antisense, 5′-GUA AAC CAU GUA GUU GAG GdTdT-3′.

Table 1. Types of polymers used for complexation (with abbreviations).

Type of polymer used for siRNA complexation	Abbreviation
Linear polymer, 2.5 kDa, tyrosine modified	LP 2.5Y
Linear polymer, 5 kDa, tyrosine modified	LP 5Y
Linear polymer, 10 kDa, tyrosine modified	LP 10Y
Linear polymer, 25 kDa, tyrosine modified	LP 25Y
Branched polymer, 2 kDa, tyrosine modified	P 2Y
Branched polymer, 5 kDa, tyrosine modified	P 5Y
Branched polymer, 10 kDa, tyrosine modified	P 10Y
Branched polymer, 25 kDa, tyrosine modified	P 25Y

Complex preparation

Complexes were prepared as described previously (Noske et al. 2020) at polymer/siRNA mass ratios of 2.5:1 or 5:1, unless indicated otherwise. Briefly, for experiments in a 24 well plate, 30 pmol (0.4 μg) siRNA per well in 12.5 μL HN (150 mM NaCl, 10 mM HEPES, pH 7.4) In a second tube, a calculated amount of polymer was diluted in 12.5 μL HN buffer. siRNA solution was added to the polymer, mixed gently by pipetting and incubated for 30 min at room temperature. For *ex vivo* experiments preparation method was similar with a slight modification. Complexes were prepared in HT/HN buffer (a 1:1 mixture of 20% trehalose, 20 mM HEPES and 150 mM NaCl, 10 mM HEPES, pH 7.4). siRNA was dissolved in HN buffer, whereas polymer was diluted in HT buffer (20% trehalose, 20 mM HEPES).

Biophysical characterization of PEI/siRNA complexes

Dynamic light scattering (DLS) and zeta potential

A Zetasizer Nano ZS-90 (Malvern Instruments, Malvern, UK) was used to determine the hydrodynamic diameter of the nanoparticles by dynamic light scattering. Wavelength was set at 633 nm, at detection angle of 90°. Complexes were prepared as described above, containing 5 μg (375 pmol) siRNA. For each sample seven measurements in five cycles at room temperature were made. Zeta potential values were measured in the same instrument and data obtained from 12 measurements in six cycles at room temperature for each sample were calculated using the Helmholtz-Smoluchowski equation. The data were analyzed using Malvern software and given as mean ± standard deviation (SD).

Gel electrophoresis

Gel electrophoresis was conducted for testing the ability of the PEIs to form complexes with siRNA. 0.5 μg (37.5 pmol) siRNA was complexed in HN buffer as above with polymer/siRNA mass ratios ranging from 1.25 to 5, prior to separation by 3% agarose gel electrophoresis. Electrophoresis was carried out with GelRed stain in Tris-acetate-EDTA (TAE) buffer for 45 min at 90 V/35 mA.

To evaluate the polymers' protective effects from siRNA degradation, the complexes were incubated with 2.5 μg/mL RNase at 37 °C for 30 min, prior to incubation of the samples on ice for 10 min in the presence of 0.082 mg/mL heparin and subsequent gel electrophoresis as above.

Circular dichroism

Circular dichroism (CD) measurements involved a Hellma quartz cells with a thickness of 0.5 cm in a J − 815 CD spectrometer (Jasco, Tokyo, Japan), with software provided by Jasco used for calculating the mean ellipticity values. Complex formation was as above, using 1 μg siRNA (75 pmols) and polymers at mass ratios from 1.25 to 5.0. The wavelength was set from 200 to 300 nm, and the assay parameters were as follows: 0.5 nm step resolution, 1.0 nm bandwidth, 4 s response time, and 50 nm/min scan speed. The slit was set on auto.

Toxicological studies of PEI/siRNA complexes

Cytotoxicity (LDH)

Acute cell damage upon transfection was determined by measuring lactate dehydrogenase (LDH) release, using the Cytotoxicity Detection Kit from Roche (Mannheim, Germany) according to the manufacturer's protocol. Briefly, 35,000 PC3-Luc3/EGFP or HCT-116-Luc3/EGFP cells (Ewe et al. 2019) were seeded in a 24-well plate and the polymer/siRNA complexes were prepared at mass ratio 2.5. Prior to transfection, the medium was replaced with fresh medium in order to remove all dead cells. At 24 h after transfection, the medium was collected and centrifuged for 5 min at 13,000 rpm. Medium from untreated cells served as negative control. For the determination of maximum LDH release (100% value), cells were lysed by adding Triton X-100 to a final concentration of 2% to the medium. From each medium, 50 μL sample was mixed with 50 μL substrate mix and incubated in a 96-well plate for 30 min at RT in the dark. The absorption at 490 nm was determined in a plate reader with 620 nm as reference filter. The background was measured in parallel using fresh medium and subtracted from all values. All samples were run in triplicates.

Induction of caspase 3/7

HCT 116 cells were seeded at a density 7000 cells/well in a 96-well plate. The next day, polymer/siRNA complexes (mass ratio 2.5; siRNA amounts in the complex 3 or 6 pmol) were added to the cells. After 24 or 72 h incubation, 100 µl of Caspase-Glo® 3/7 Reagent (Promega) was added to the cells on the plate. Luminescence intensity was measured after 1 h incubation at room temperature using a plate reading luminometer. Subsequently, the post-treatment number of viable cells was determined by WST-8 assay. 5 µl WST-8 reagent in 50 µl medium was added to each well. After 30 minutes incubation, the absorbance was read using a plate reader ($\lambda = 450$ nm). Caspase 3/7 induction is given relative to WST-8 readings.

Mitochondrial membrane potential measurement ($\Delta\psi m$)

To measure mitochondrial transmembrane potential, PC3-Luc3/EGFP or HCT-116-Luc3/EGFP cells were incubated for 24 or 48 h in the presence polymer/siRNA complexes (mass ratio 2.5), prior to adding 5 µmol/l of fluorescent dye JC-1 (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenz-imidazolcarbocyanine iodide). After 30 min incubation in the dark, fluorescence was measured using a BioTek HTX plate reader, with filters suitable for J-aggregates (l_{ex} = 530 nm, l_{em} = 590 nm). Results were expressed as percentage of the control (100%). Experiments were repeated three times and the results averaged.

Reactive oxygen species measurements

PC3-Luc3/EGFP and HCT116-Luc3/EGFP cells were seeded in a 96 well plate, at a density of 10 000 cells/well and incubated for 24 h. The cells were then treated with polymer:siRNA complexes (mass ratio 2.5, 6 pmol siRNA/well). After 24 and 48 h treatment, the medium was replaced by 50 mmol/L sodium phosphate, 10 mmol/L Amplex Red reagent 50 and horseradish peroxidase (10 U/ml). The cells were incubated for 30 minutes at room temperature in the dark, prior to fluorescence determination (excitation 550 nm and emission 590 nm, respectively). The samples were analyzed in triplicates.

Quantitative and qualitative knockdown efficacy of polymer/siRNA complexes in vitro and ex vivo

RT-qPCR

For the determination of mRNA levels after GAPDH knockdown, cells were seeded and transfected in 24 wells polymer/siRNA complexes at mass ratio 2.5. The total RNA was isolated at 72 h post transfection, using a combined TRI reagent and silica column protocol (Ewe et al. 2019). The RNA concentration and purity were measured in a NanoDrop 2000c (Thermo Fisher, Schwerte, Germany). Total RNA was reverse transcribed with the RevertAid™ H Minus First strand cDNA synthesis Kit (Fermentas Thermo Fisher Scientific). Briefly, 1 µL random hexamer primer was added to 1 µg RNA diluted in DEPC-treated water, prior to incubating for 5 min at 65 °C and cooling to 4 °C. Then, 4 µL 5× reaction buffer, 2 µL 10 mM dNTP Mix, 2.5 µL DEPC-treated ddH2O and 0.5 µL RevertAid™ H Minus M-MuLV Reverse Transcriptase (200 U/µL) were added. The cDNA synthesis mixture was incubated at 25 °C for 10 min and at 42 °C for 60 min, prior to heat denaturing at 70 °C for 10 min. Quantitative PCR was performed using the StepOnePlus PCR system (Thermo) and the ΔΔCt method, with RPLP0 serving as housekeeping gene. The cDNA was 1:10 diluted with DEPC-water, and 4 µL were mixed with 5 µL 2 × PerfeCTa SYBR Green FastMix (Quantabio, Beverly, MA) and 1 µL 5 µM primer mix. Primer sequences were as follows: human GAPDH, for: 5′-GGT GTG AAC CAT GAG AAG TAT GA-3′, rev: 5′-GAG TCC TTC CAC GAT ACC AAA G-3′; human RPLP0, for: 5′-TCT ACA ACC CTG AAG TGC TTG AT-3′, rev: 5′-CAA TCT GCA GAC AGA CAC TGG-3′. The qPCR were run with a pre-incubation at 95 °C for 2 min, followed by 45 amplification cycles (95 °C for 15 s, 55 °C for 15 s, 72 °C 15 s).

Gene expression knockdown in ex vivo model

Tumor tissue slices were generated from A549 tumor xenografts. Briefly, 350-µm slices were obtained using Leica VT 1000 vibratome and with a disposable biopsy punch, pieces of 3 mm in diameter were prepared. These punched tissue slice pieces were then placed on membrane culture inserts with 0.4 µm pore size, located in 6-well plates containing 1 ml of RPMI-1640 medium

supplemented with 10% FCS and 1% antibiotics, for ail-liquid interface culture. After overnight cultivation under standard cell culture conditions, the slices were transferred into a 96-well plate containing 100 µl pre-warmed medium. Polymer/siRNA complexes were added to the slices for 4 h incubation, then the slices were returned onto the membrane inserts and further cultivated, with the medium being replaced every day. After 72 h, slices were harvested for RT-qPCR analysis.

Flow cytometry and confocal microscopy

The efficacy of complexes was studied in PC3-Luc3/EGFP and HCT 116-Luc3/EGFP cells lines. For flow cytometry analysis, cells were seeded in 24-well plates at the density 35,000 cells/well. After an overnight incubation, the cells were treated with polymer/siRNA complexes (mass ratio 2.5; 1 µg siRNA). After 72 h incubation, the cells were trypsinized, centrifuged, resuspended in PBS and transferred to flow cytometry collection tubes. For analyzing changes in EGFP expression, a BD FACS Aria II flow cytometer (ex. 488 nm/em. 530 ± 30 nm) equipped with Flowing Software were used and at least 7000 events were collected per sample.

For confocal microscopy, the cells were seeded in 6-well plates with glass bottom, at a density of 200,000 cells/well. The next day, cells were treated with polymer/siRNA complexes (mass ratio 2.5; 3 µg siRNA). After 72 h, the cells were fixed with 4% formaldehyde at room temperature for 20 min, prior to washing and adding fresh PBS. The samples were analyzed with a Leica TCS SP8 microscope (Wetzlar, Germany), at an excitation wavelength of 495 nm. The Leica software was applied to analyze the data.

Luciferase measurements

Luciferase activity was determined 72 h post transfection using the Beetle-Juice Kit (PJK, Kleinblittersdorf, Germany). The medium was aspirated and 100 µL lysis buffer (Promega, Mannheim, Germany) was added, prior to incubation for 30 min at RT. In a test tube, 25 µL luciferin substrate was mixed with 10 µL cell lysate and luminescence was immediately measured in a luminometer (Berthold, Bad Wildbad, Germany).

Results

Biophysical characterization of PEI/siRNA complexes

Complex sizes

Dynamic light scattering was performed to evaluate the size of formed complexes (Figure 1). Polymer/siRNA complexes were made in two different mass ratios (MR). As long as complexes for *in vitro* studies were made in HN buffer and for *ex vivo* studies in HT/HN buffer, size and zeta potential were checked in two different buffer conditions. The results showed that size of complexes depended on the buffer used for complexation, the polymer and the

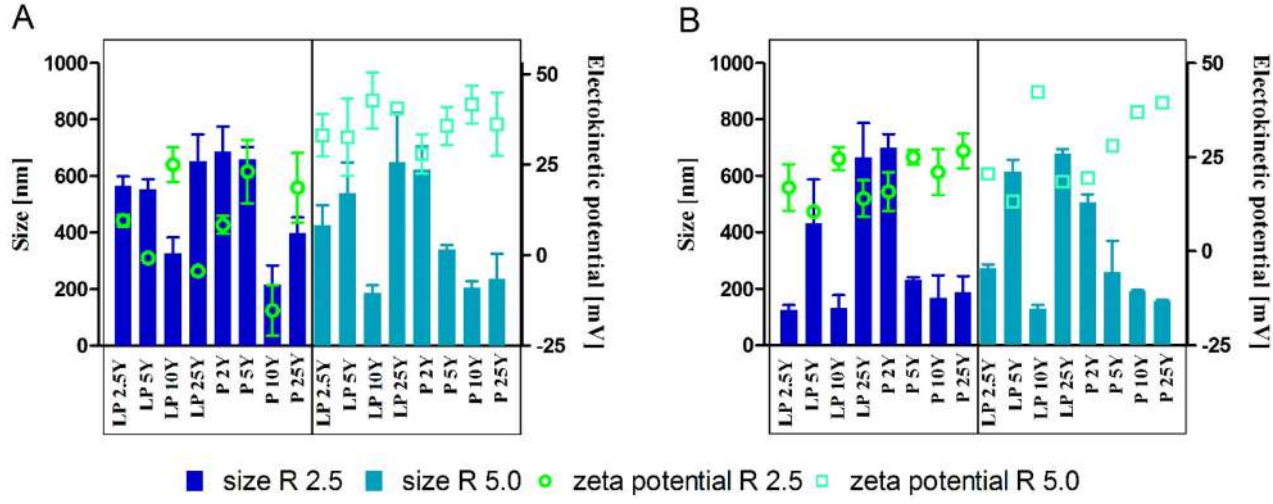

Figure 1. DLS and zeta potential measurements of complexes prepared in (A) HN buffer or (B) HT/HN buffer. Polymer/siRNA mass ratios 2.5 (dark bars) and 5.0 (light bars) are shown. Green circles correspond to zeta potential values at ratio 2.5, whereas pale blue squares correspond to zeta potential values at ratio 5.0.

MR. Sizes were generally lower for the complexes prepared in HT/HN buffer as compared to HN buffer. For example, LP2.5Y-based polyplexes in HN buffer exhibited size values from 600 nm at MR 2.5 to ~400 nm at MR 5, while they were substantially smaller when prepared under HT/HN conditions (150 and 250 nm, respectively). The comparison also revealed that sizes were sometimes dependent on the polymer/siRNA mass ratio, which, however, varied between the different polymers and buffer conditions. For example, the sizes of LP5Y/siRNA complexes in HN buffer were rather comparable at both mass ratios (around 600 nm), while the size of complexes generated with LP10Y decreased from around 300 nm at MR 2.5 to around 200 nm at MR 5. For LP25Y/siRNA complexes, the size values were similar at both mass ratios, similarly to P2Y/siRNA (around 600–700 nm). In the case of P5Y, the size of polyplexes again decreased from 700 nm at MR 2.5 to less than 400 nm at MR 5. Sizes of P10Y/siRNA complexes were around 200 nm for both ratios. In case of P25Y, the complex sizes decreased from about 400 nm to around 250 nm.

When prepared in HT/HN buffer, sizes of LP2.5Y/siRNA complexes were lower at ratio 2.5 (< 200 nm), whereas ratio 5 led to larger complexes around 300 nm. Complexes with LP 10Y exhibited similar sizes of < 200 nm at both studied mass ratios, while the size of LP5Y/siRNA complexes increased with mass ratio from around 400 nm to around 600 nm. Rather large complexes were formed in the case of LP25Y, independent of the MR (600–700 nm). The size of P2Y-based polyplexes decreased from around 700 nm at MR 2.5 to less than 600 nm at MR 5. Complexes generated from P5Y, P10Y and P25Y showed all similar sizes, with values in the 200–300 nm range (Figure 1).

Zeta potential

Regarding zeta potential measurement (Figure 1), all studied formulations in HN buffer exhibited positive charge, except for complexes based on LP5Y, LP25Y and P10Y at the lower mass ratio 2.5. Under the same buffer conditions, however, all complexes were positively charged at mass ratio 5.

In the case of HT/HN buffer and at MR 2.5, zeta potential values were generally higher as compared to their counterparts prepared in HN buffer for all complexes. All complexes were positively charged at both ratios. At MR 5, particularly high zeta potentials above 35 mV were observed in case of complexes based on LP10Y, P10Y and P25Y.

Gel electrophoresis

Electrophoretic studies (Figure 2(A,B)) reveled that siRNA was fully complexed at mass ratio 2.5 by all studied polymers. The addition of heparin led to an at least partial release of the siRNA. While complexes with LP5Y, LP25Y or P10Y were quite stable and the addition of heparin caused a relatively weak siRNA release, this was more profound in the case of the other polymers. Moreover, while free siRNA was completely degraded in the presence of RNase A1, the pre-incubation of the complexes with RNase A1 prior to heparin treatment led to the release of still intact siRNA, indicating the protective effect of siRNA complexation. This protection was seen for all complexes, albeit the direct comparison between the corresponding bands ± RNase A1 revealed lower siRNA band intensities upon RNase A1 treatment. However, this may not necessarily indicate an only partial siRNA protection in the complex, since siRNA may also be degraded by RNase A1 only during the heparin displacement step prior to gel loading.

Circular dichroism

In order to evaluate the interaction between siRNA (siLuc3) and polymers, circular dichroism was performed (Figure 3). The results obtained from CD showed that changes in siRNA secondary structure could be seen upon addition of polymers. While little effects were observed at very low polymer/siRNA mass ratio 0.6, some effects (dependent on the polymer used for complexation) were seen at MR 1.25 and major changes emerged at MR 2.5 for all polymers. Further increase of mass ratios (3.75 and 5.0) did not lead to substantial additional polymer effects on siRNA structure.

Toxicological studies of PEI/siRNA complexes

The cytotoxic potential of complexes was evaluated in HCT116 cells. The level of membrane damage was assessed by LDH assay (Figure 4(A)). Complexes were prepared at mass ratio 2.5 and different complex amounts, corresponding to 7.5–30 pmol siRNA, were applied onto the cells. The results

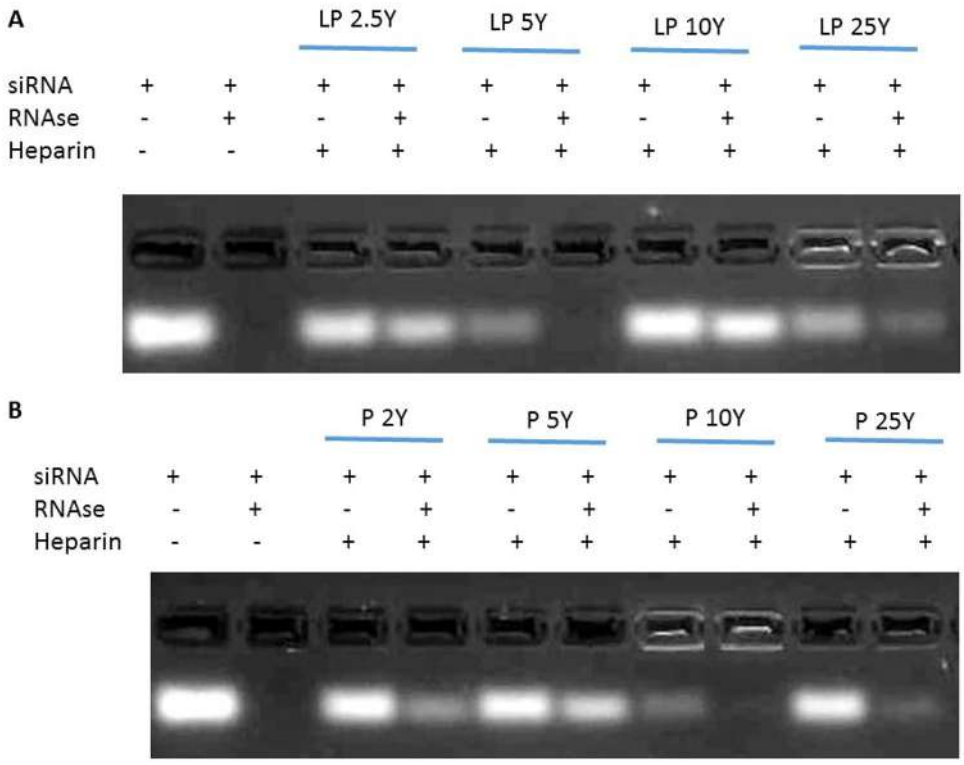

Figure 2. Agarose gel electrophoresis. Complexation of free siRNA (leftmost lane) by (A) LPxY or (B) PxY PEIs. Complexes of polymers and siRNA (siLuc3) were prepared at mass ratio 2.5:1 and then incubated in the presence or absence of RNAse A1. RNAse concentration used in the experiment was 5 U/ml.

demonstrate that in the case of all tyrosine-modified PEIs even the highest complex amounts did not cause membrane damage (Figure 4(A)). In stark contrast, the non-modified 10 kDa linear PEI led to profound, concentration-dependent cytotoxicity. More specifically, LP10 complexes at amounts of 30 pmol siRNA caused cell damage comparable to the positive control (10% Triton X-100).

Caspase 3/7 activation was measured in order to monitor induction of apoptosis. HCT 116 cells were treated with complexes containing 3 pmol siRNA. After 24 h, a marked >3-fold increase over untreated was seen in the case of the unmodified linear 10 kDa PEI, while no apoptosis induction was observed in the case of any of the tyrosine-modified polymers (Figure 4(B)).

The production of J-aggregates was measured in order to evaluate alterations in the mitochondrial activity after treatment with complexes. After 24 h incubation, only LP5Y/siRNA complex caused a significant decrease in J-aggregate production, while little alterations were seen in the case of the other polymers (Figure 4(C)). This was also true at 48 h and 72 h incubation, except for some decrease in the level of J-aggregates in the case of LP10Y/siRNA complex.

For these polymers, the level of oxidative stress was evaluated by measuring H_2O_2 level in cells incubated with complexes containing 30 pmol siRNA (mass ratio 2.5). While after 24 h no major changes in hydrogen peroxide levels were detected (data not shown), values after 48 h showed a significant $\sim 1.5-1.8$ increase in the case of complexes based on LP10Y or P10Y (Figure 4(D)).

Quantitative and qualitative knockdown efficacy of polymer/siRNA complexes in vitro *and* ex vivo

RT-qPCR analyses were performed in order to determine the level of GAPDH expression in cells treated with the complexes containing tyrosine modified polymer and siGAPDH. All complexes based on tyrosine-modified PEIs caused a significant ~70% decrease in GAPDH expression levels after 72 h incubation *in vitro* (Figure 5(A)). Tissue slice ALI cultures preserve the architecture of intact tumor tissue and were thus analyzed next. Since tissue penetration is a major bottleneck in gene

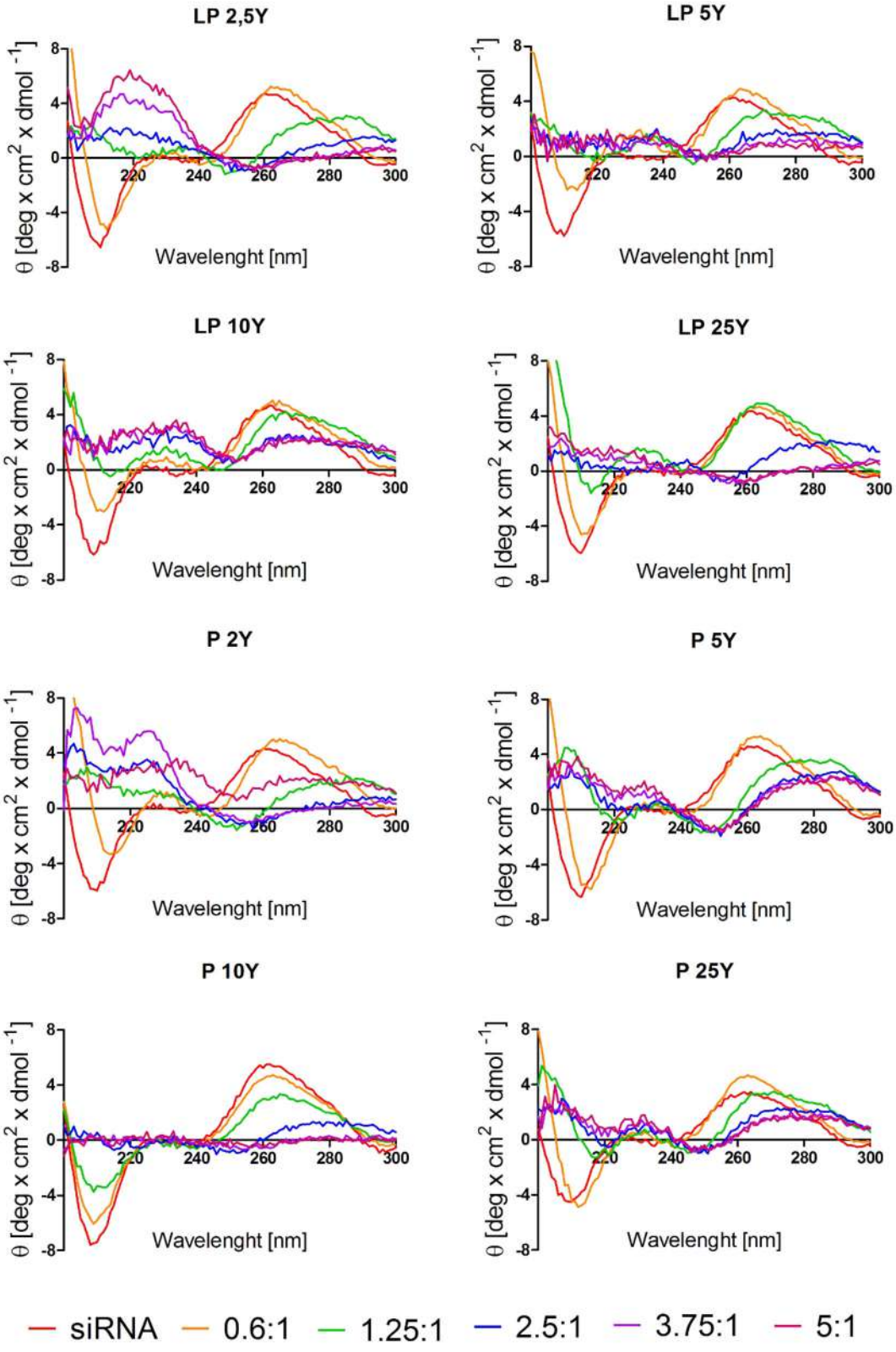

Figure 3. CD spectra of the siRNA alone (black line) as compared to addition of increasing concentrations of tyrosine-modified polymers, in a range of polymer/siRNA mass ratios (MR) 0.6 - 5.0.

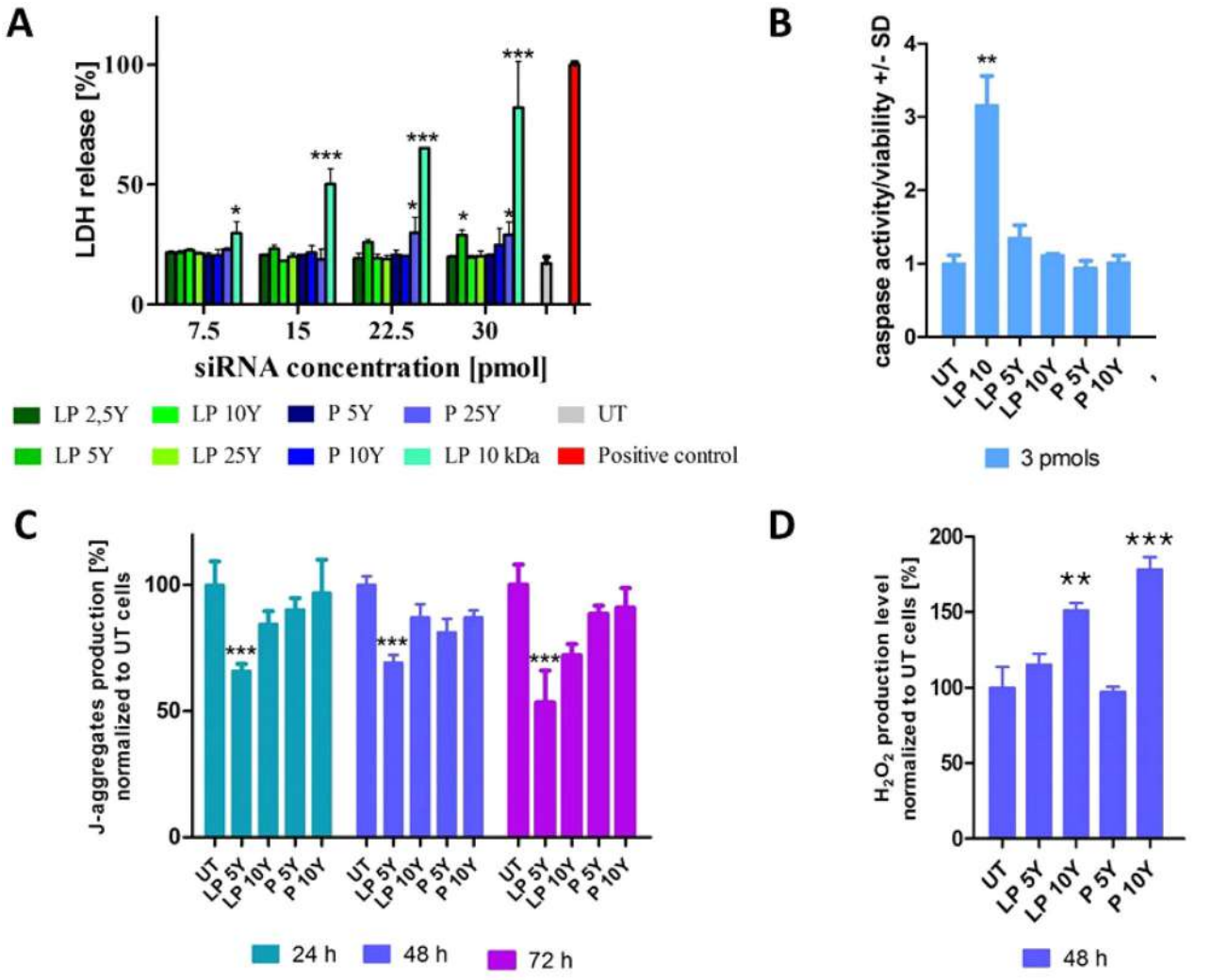

Figure 4. (A) LDH release in HCT 116 cells after 24 h incubation with complexes. Polymer/siRNA mass ratio was always 2.5, with different complex amounts applied onto the cells, as indicated by the siRNA amounts. UT, untreated cells cultured in medium only. Cells treated with 10% Triton X-100 served as positive control. Medium alone was treated as a blank sample and LDH release from medium was subtracted from the results. (B) J-aggregate production in healthy mitochondria in HCT 116 cells after 24/48 h incubation with polymer/siRNA complexes (mass ratio 2.5, 6 pmol siRNA). (C) Caspase 3/7 induction, as shown by caspase activity normalized for viable cell numbers, in HCT 116 cells after 24 h incubation with polymer/siRNA complexes (mass ratio 2.5, 3 pmol siRNA). (D) H_2O_2 production in HCT 116 cells after 48 h incubation with polymer/siRNA complexes (mass ratio 2.5, 6 pmol siRNA). UT, untreated cells as 100% control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; $n = 3$.

knockdown efficacy, this model resembles more closely the *in vivo* situation and GAPDH downregulation was overall less profound. Most efficient in this *ex vivo* model were complexes based on LP10Y, with an almost 50% reduction of GAPDH levels, with a lesser ∼ 15–30% knockdown in the case of the other complexes (Figure 5(B)).

For analyzing knockdown on the protein level, flow cytometry and confocal microscopy were used to evaluate the expression of EGFP as a target gene. HCT 116-Luc3/EGFP expressing cell lines were incubated for 72 h with polymer/siEGFP complexes containing 30 pmol siRNA (mass ratio 2.5). Flow cytometry histograms showed a shift to the left, indicating reduced EGFP emission (Figure 5(A)). The quantitation revealed knockdown efficacies of 80% or above (Figure 5(B)). Flow cytometry data were also confirmed by confocal microscopy imaging, revealing that complexes were able to decrease the level of EGFP expression after 72 h incubation (Figure 6).

To monitor the dependence of knockdown efficacies on siRNA amounts, luciferase knockdown in HCT 116-Luc3/EGFP expressing cell lines was analyzed by using the different polymer/siRNA complexes at a broader range of different amounts

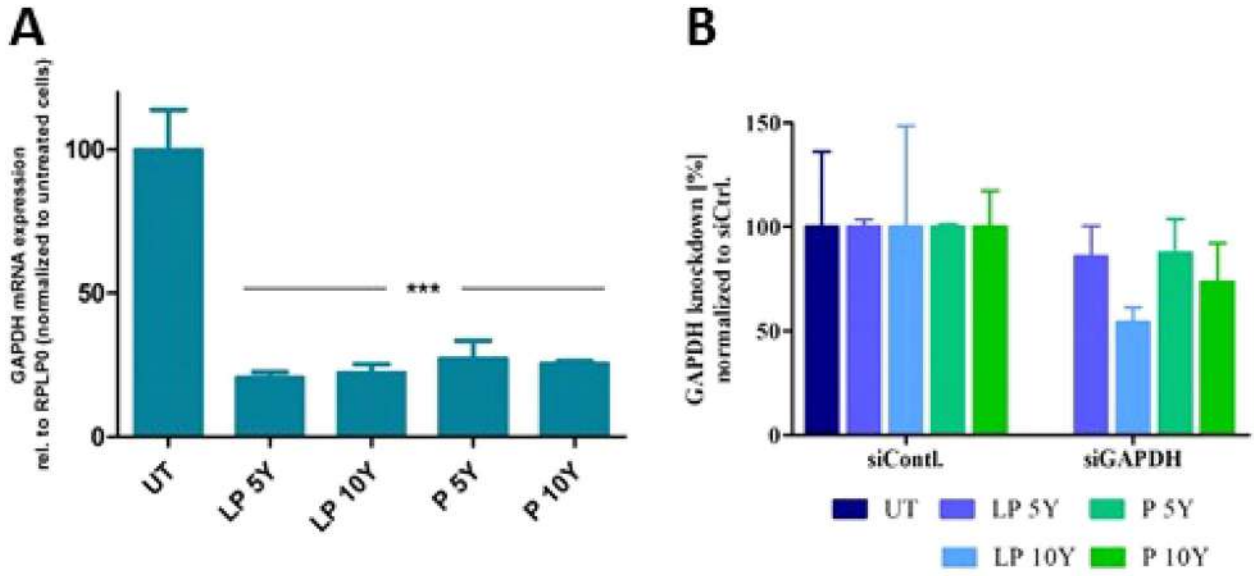

Figure 5. (A) Relative mRNA expression of GAPDH in HCT 116 cells after 72 h incubation with polymer/siRNA complexes (ratio 2.5, 30 pmol siRNA) normalized to untreated cells (UT). RPLP0 was used as housekeeping gene for normalization. (B) -. Knockdown of GAPDH expression in an A549 tissue slice model after 72 h incubation with polymer/siRNA complexes (2.5, 30 pmol siRNA). Results are presented as relative values, normalized to siCtrl, with RPLP0 serving as housekeeping gene.

(Figure 7). Luciferase intensity was measured after 72 h incubation with complexes containing 7.5–30 pmol siLuc3 as compared to siCtrl. The tyrosine modified linear polymers performed well even at the lowest concentration of siRNA. This was also seen for complexes based on P5Y, while P10Y and P25Y lagged behind and required somewhat larger siRNA amounts for gene knockdown. Complexes based on the non-modified LP 10 kDa did not deliver siLuc3 efficiently when lower amounts of siRNA were used, while knockdown at higher concentrations of siRNA was again associated with cytotoxicity.

Discussion

Polyethylenimines are considered as one of the most promising non-viral nanocarriers for nucleic acid delivery. In this study, we compared the properties of linear and branched tyrosine-modified PEIs for siRNA delivery. Linear non-modified PEIs are generally preferred for nucleic acid delivery, due to their chemical properties and higher biocompatibility (Kwok and Hart 2011; Lungu et al. 2016). Nevertheless, their biological activity for delivering double-stranded, comparatively small siRNA is rather poor. A poor polymer interaction with siRNA can be caused by the short length of nucleic acid, what affect electrostatic interaction with cationic, soluble PEI. Such complexes exhibited a very low stability in contact with polyanions located on the cell surface. Tyrosine modification increase aggregating properties due to the presence of hydrophobic domains was introduced in order to make polymers more self-assembly (Creusat and Zuber 2008). In order to improve complexation efficacy and biological activity of these polymers, tyrosine was introduced in their structure (Ewe et al. 2016; Karimov et al. 2021b). Biophysical properties of PEI/siRNA complexes were tested by several methods. DLS and zeta potential measurements are a standard technique used for complex characterization. It was demonstrated that the ionic strength of the complexation medium is a factor affecting diameter and charge of the complexes. This is also in line with previous studies (Curtis et al. 2016; Karimov, Schulz, et al. 2021; Sang et al. 2015). Our data revealed that stable PEI/siRNA complexes were generated under both buffer conditions. Complexes formed in HN buffer (HEPES/NaCl) were generally larger than those formed in HT/HN buffer. Ionic conditions may affect also the potential of the complexes. Generally, all complexes displayed positive values which is advantageous for cellular uptake. Nanoparticles complexed in HT/HN buffer were stable and showed less variability of zeta potential values between polymer/siRNA mass ratios 2.5 and 5.

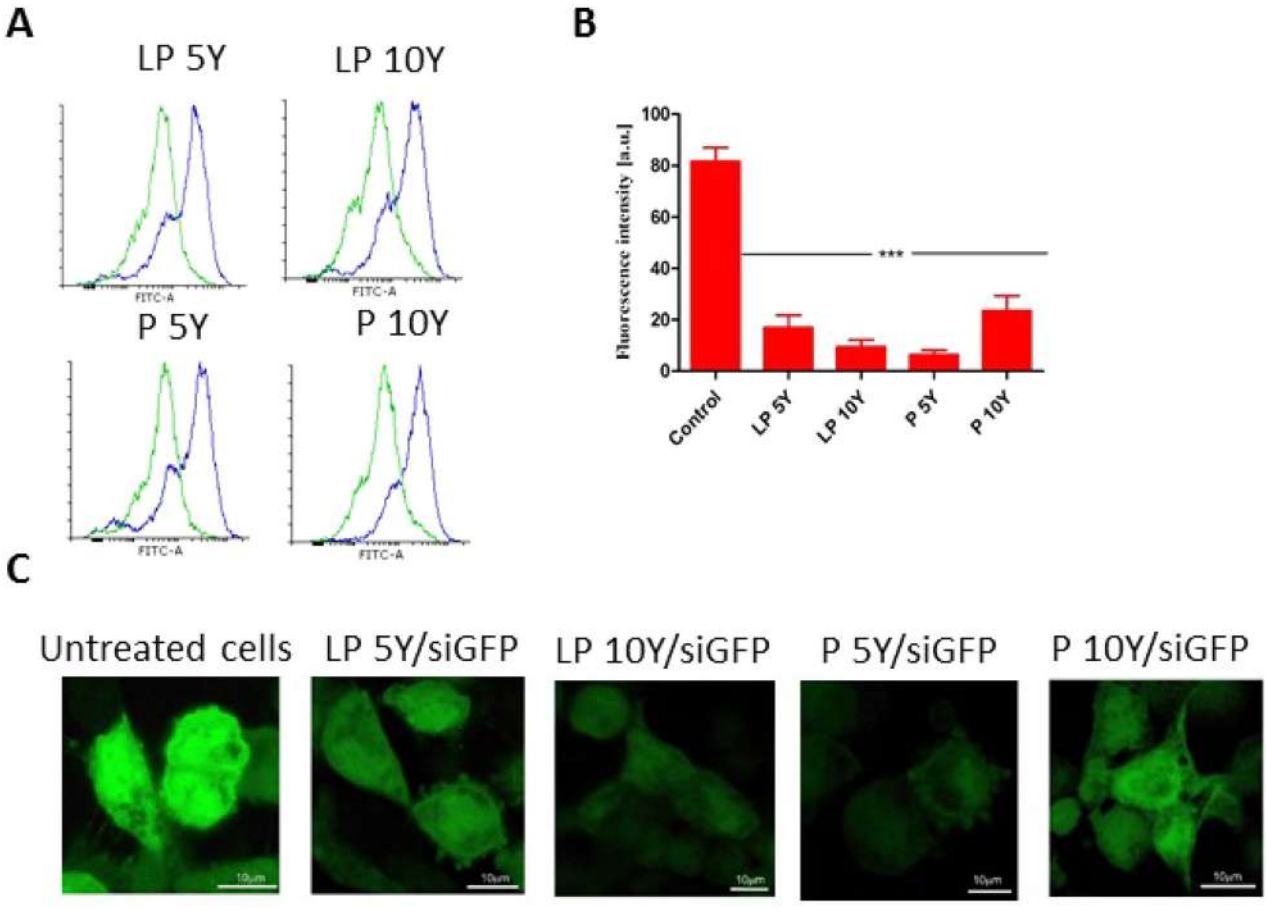

Figure 6. (A) Histograms (siCtrl – dark line, siGFP – bright line) from flow cytometry analysis of EGFP expression levels in HCT 116-Luc3/EGFP reporter cells after 72 h incubation with polymer/siEGFP complexes (mass ratio 2.5, 30 pmol siRNA). (B) Determination of mean fluorescence intensities from EGFP expression knockdown from confocal microscopy studies (***, $p < 0.001$, $n = 3$). (C) Confocal microscopy of EGFP-expressing cells upon transfection vs. untransfected.

It seems that the addition of trehalose prevented aggregation. This sugar is known as a protein stabilizer and as a component of inhalable drugs, providing stability (Jain and Roy 2009; Keil et al. 2019). Latest data suggests that complexes generated in trehalose buffer performed better *in vivo*. For *in vitro* studies, the complexes from HEPES/NaCl buffer are more efficient (Karimov, Schulz et al. 2021), probably due to their more efficient sedimentation on adherently growing cells.

Tyrosine modified polymer/siRNA complex formation was evaluated by gel electrophoresis and circular dichroism. It was shown previously that tyrosine modified polymers could complex siRNA already at a comparably low mass ratio 2.5 (Ewe et al. 2016; Karimov, Schulz, et al. 2021). Polymers exhibited protective effects from nuclease digestion. Due to the stability of complexes, heparin could only partially release siRNA from the complex. Complex stability and the protective effect of nanovectors are essential for siRNA delivery (Ionov et al. 2015; Michlewska et al. 2018; Rodríguez-Prieto et al. 2021; Sanz del Olmo et al. 2020). PEI polymers are known for their proton sponge effect, which may affect both, endosomal rupture for complex release and siRNA liberation from the complex (Creusat et al. 2010).

Changes in the secondary structure of siRNA can be followed by circular dichroism. A standard spectrum of siRNA has two characteristic peaks around 210 and 260 nm. Complexation efficacy can be observed by analysis of the changes in siRNA CD spectra upon addition of nanocarrier (Ferenc et al. 2013; Ionov et al. 2015; Sanz del Olmo et al. 2020). The decrease in ellipticity to around 0 was observed for all polymers at mass ratio 2.5, suggesting that

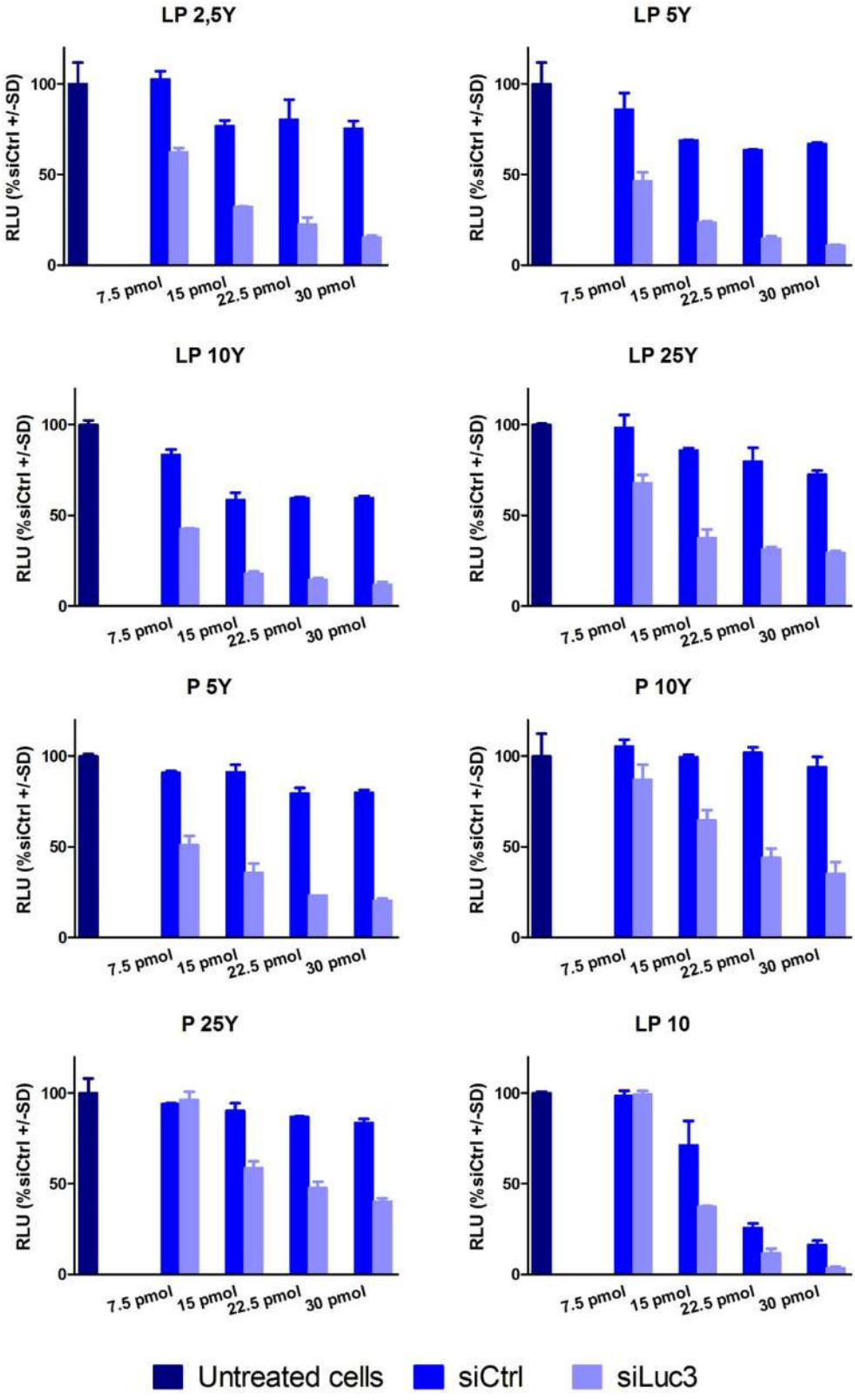

Figure 7. Luciferase knockdown in PC 3 cell line after 72 h incubation with polymer/siLuc3 and polymer/siCtrl (siLuc2) complexes (mass ratio 2.5). Results are presented as average luciferase intensity, normalized to untreated cells (UT).

this is sufficient for full siRNA complexation by the polymers. Changes in the ellipticity values can be explained by the decrease in the absorbance of nucleotides complexed by polymers (Law et al. 2008). The registered red shift of CD spectra peaks at $\lambda = 260$ nm in the presence of increasing

amounts of the PEIs indicate significant structural changes in the siRNAs upon interaction with the polymers.

Complex toxicity is an important parameter in the assessment of novel nanocarriers. It was demonstrated that PEI toxicity increases with molecular mass (Gholami et al. 2014; Moghimi et al. 2005; Monnery et al. 2017). In our study, the tyrosine-modified polymers proved to be rather nontoxic nanovectors. Acute cell damage monitored by LDH release showed good biocompatibility, with values comparable to untreated cells. In turn, non-modified polymer LP 10 kDa in complex with siRNA caused serious cell damage. Reactive oxygen species levels and mitochondrial membrane potential serve as early events in the apoptosis process. Nanoparticles are considered as inducers of the intrinsic apoptosis pathway which involves mitochondria (Ceremuga et al. 2020; Wigner et al. 2021). Notably, the complexes tested here mostly did not affect mitochondria. The level of J-aggregates is proportional to the healthy state of these organelles (Elefantova et al. 2018). Only LP5Y/siRNA and, to a lower extent, LP10Y/siRNA complexes led to some decrease in J-aggregate production. Complexes based on LP5Y also led to a very slight activation of caspase 3/7, whereas branched polymers did not elevate caspase levels. It has been previously observed that linear PEIs are more toxic than their branched counterparts. The grafting of tyrosine remarkably reduced the toxicity of the polymers, with some dose-dependent toxic effects a large amounts still being observed (Kubczak et al. 2022).

Studies on complex activity *in vitro* showed that tyrosine-modified polymers have a great potential for siRNA delivery. Linear, non-modified polymers are practically unable to bind siRNA, as opposed to branched PEIs which are also known as nanovectors for plasmid DNA (Kwok and Hart 2011). Addition of tyrosine allowed PEIs to complex smaller, more rigid nucleic acids like siRNA. Biologically active complexes can be formed even at low polymer/siRNA ratios. This allows for efficient gene knockdown with little toxic effects. Knockdown efficacy mainly relies on complex stability, charge and hydrophobicity. Tyrosine-modified polymers form complexes that are more stable compared to their non-modified counterparts, which can be explained by the tyrosines' contribution to electrostatic and π-π interactions (Dougherty 1996; Huang et al. 2015; Plyte and Kneale 1991). Notably, complexes based on LP10Y also mediated quite efficient GADPH gene knockdown in the *ex vivo* tissue slice model, indicating penetration of the LP10Y/siGAPDH complexes into deeper cell layers of the tissue slices. This indicates that results obtained in traditional 2 D cell culture are not always confirmed in more advanced culture systems (Karimov, Appelhans et al. 2021; Merz et al. 2017). LP10Y seems to form complexes which are readily taken up by the cells, while simultaneously also being able to reach deeper parts of the tissue.

Conclusions

In conclusion, this paper reports directly compares linear and branched, tyrosine-modified low molecular weight polyethylenimines as siRNA delivery vectors. The chemically modified PEIs are able to form stable complexes with siRNA and protect them against degradation in the presence of RNAses, as one important requirement for effective siRNA delivery. The complexes can be efficiently internalized into the cells, with the tyrosine modification in the PEIs also reducing their cytotoxicity.

Acknowledgments

The authors are grateful to Magdalena Gapińska for expert confocal microscope maintenance. The authors are also grateful for funding by grants from the project "EUROPARTNER" of the Polish National Agency for Academic Exchange (NAWA).

Disclosure statement

The authors declare no conflict of interest.

Funding

This work was supported by the grant from the Nacional Science Center of Poland 'BEETHOVEN LIFE 1' program, project no: 2018/31/F/NZ5/03454 and Deutsche Forschungsgemeinschaft [DFG; AI 24/21-1; AI 24/24-1].

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Colloids and Surfaces B: Biointerfaces 227 (2023) 113359

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

The effect of novel tyrosine-modified polyethyleneimines on human albumin structure – Thermodynamic and spectroscopic study

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ARTICLE INFO

Keywords:
Human serum albumin
Tyrosine modified polyethyleneimines
Circular dichroism
Thermodynamics

ABSTRACT

The interaction of proteins with nanoparticle components are crucial for the evaluation of nanoparticle function, toxicity and biodistribution. Polyethyleneimines (PEIs) with defined tyrosine modifications are a class of novel polymers designed for improved siRNA delivery. Their interactions with biomacromolecules are still poorly described. This paper analyzes the interaction of different tyrosine-modified PEIs with human serum albumin as the most abundant serum protein. The ability of tyrosine modified, linear or branched PEIs to bind human serum albumin (HSA) was analyzed and further characterized. The interaction with hydrophobic parts of protein were studied using 1- nilinonaphthalene-8-sulfonic acid (ANS) and changes in the HSA secondary structure were evaluated using circular dichroism (CD). Complex formation and sizes were studied by transmission electron microscopy (TEM) and dynamic light scattering methods (DLS). We demonstrate that tyrosine modified PEIs are able to bind human serum albumin. Based on thermodynamic studies, van der Waals interaction, H-bonding and hydrophobic interactions are determined as main molecular forces involved in complex formation. Analysis of secondary structures revealed that the polymers decreased α-helix content, while increasing levels of randomly folded structures. Complex formation was confirmed by TEM and DLS. These findings are crucial for understanding polymer-protein interactions and the properties of nanoparticles.

1. Introduction

Almost all routes of administration for nanoparticles (NPs) are associated with the contact between blood components and the administrated material [1]. Blood proteins immediately cover nanoparticles what can lead to the changes in pharmacokinetics, biodistribution and metabolism [2–5]. So called protein corona on the NPs surface dynamically changes over time, although human serum albumin (HSA), fibrinogen and immunoglobulins seem ubiquitously found components of tightly-bound hard protein corona [6].

Among all blood proteins, albumins represents the most abundant group [7]. They are responsible for transport and delivery of endogenous and exogenous substances, also drugs and nanoparticles [8,9]. Human serum albumin is a single peptide chain of 585 amino acids and molecular mass around 67 000 Da. HSA consists of three α-helical domains and is stabilized by 17 disulfide bonds [10]. The structure of HSA is similar to heart-shaped form with length and height around 8 nm and 3 nm, respectively [11]. Due to the presence of hydrophobic cavity HSA plays an important role in drug delivery [12]. Albumin is able to bind to a wide range of compounds, like fatty acids, hemin, bilirubin and hormones. Many of commonly used drugs bind to HSA, to the primary sites in subdomains IIA and IIIA [8,13–15].

Adsorption of proteins onto nanoparticles surface may lead to the conformational changes and alteration in protein function [16–21]. Interaction between proteins and nanoparticles depend on NPs surface properties and the nature of the protein [3,6]. Conformational changes include unfolding, aggregation, loss of activity, presence of intermediate states and denaturation [11,16,22,23]. Some studies demonstrated that

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https://doi.org/10.1016/j.colsurfb.2023.113359
Received 4 April 2023; Received in revised form 10 May 2023; Accepted 16 May 2023
Available online 16 May 2023

interaction led to the fibrillation of proteins [24].

Tyrosine-modified PEIs are a class of cationic nanoparticles, designed for nucleic acid delivery. In comparison to a non-modified PEIs, they have shown reduced toxicity and a higher efficiency in gene delivery (especially smaller nucleic acids like siRNA, miRNA) [25–27]. Due to the combination of positive charge and proton sponge effect in endolysosomes, PEIs are considered as one of the most promising vehicles [28]. Little is known about their interaction with biomacromolecules what constitute a drawback in the understanding of PEIs mechanism of toxicity [29]. To be useful in bioapplications, polymers should not cause adverse effects on the structure of human serum albumin [26].

The main goal of this work was to investigate interactions between tyrosine-modified PEIs, linear and branched, with different molecular weight and human serum albumin. Alterations in protein structure, type of interactions, thermodynamic parameters were calculated.

2. Materials and methods

2.1. Materials

Linear, non-modified PEIs were obtained from Sigma-Aldrich, Taufkirchen, Germany. Branched 5 kDa PEI was a kind gift from BASF, Ludwigshafen, Germany. 10 kDa PEI (Cat. 25414–2) and uranyl acetate (Cat. 10417) was obtained from Polysciences, Eppelheim, Germany. Tyrosine modification was introduced to the polymer structure according to the previously published protocols [26,27,30,31]. L-tryptophan (Cat.T0254), human serum albumin (Cat. A3782, >99% purity), 1- nilinonaphthalene-8-sulfonic acid ANS (Cat. 10417) were obtained from Merck, Darmstadt, Germany. Dimethyl sulfoxide DMSO (Cat. 113635509), disodium hydrophosphate (Cat. 117992300) and sodium dihydrophosphate (Cat. 117992004) were purchased from Chempur, Piekary Śląskie, Poland.

2.2. Fluorescence spectroscopy

Fresh solutions of HSA were prepared. Albumin was dissolved in 10 mmol/L phosphate buffer in a stock solution at 10 mg/mL, with a subsequent dilution to 50 μg/mL (0.075 μM). Fluorescence intensity measurements (Perkin-Elmer LS-55B, UK) were performed to analyze interactions between HSA and polymers. Fluorescence spectra were registered at 305–460 nm. Scan speed, excitation and emission slits were set at 50 nm/min and 5.0 nm, respectively. Tryptophan fluorescence was excited at λexc = 295 nm. Measurements were conducted in 30, 33 and 37 °C. Small volumes of polymers were used to make sure that dilution effect is negligible. As polymers did not absorbed UV light in the wavelengths of choice the inner filter effect correction was not introduced [32,33].

Interactions between polymers and hydrophobic parts of albumin were analyzed using 1-anilinonaphthalene-8-sulfonic acid (ANS). ANS was dissolved in DMSO at a stock solution of 10 mmol/L, with a final concentration of 5 μmol/L. Fluorescence spectra were read at 37 °C, between 400 and 600 nm (scanning speed 50 nm/min., λexc. = 370 nm, excitation slit 5 nm, emission slit 2.5 nm).

L-Tryptophan was dissolved in 10 mmol/L phosphate buffer at a concentration of 0.5 μM. The excitation wavelength of 295 nm was used and the emission spectra were recorded from 300 to 440 nm at 37 °C. The excitation and emission slit width were 5.0, respectively. Next, increasing concentrations of polymers were added and fluorescence spectra were recorded.

2.3. Circular dichroism (CD)

The CD spectra of HSA/polymer complexes were measured with a Jasco J-815 CD spectrometer (Oklahoma, Japan). The recording parameters were as follows: scan speed 50 nm/min, step resolution 1 nm, response time 2 s, bandwidth 1.0 nm, slit auto. Spectra have shown the average of a minimum of 3 independent scans. CD spectra were corrected against the baseline where the buffer with maximal concentration of polymers was measured, without HSA. The mean residue ellipticity, Θ, expressed as [cm^2 $dmol^{-1}$], was calculated using software provided by Jasco. Percentage changes in secondary structure of HSA on the basis of CD spectra were determined using CDNN software (by Dr. Gerald Böhm, Institute of Biotechnology, Martin-Luther University of Halle-Wittenberg).

2.4. Z-Size measurements

Hydrodynamic diameters of complexes were measured by the dynamic light scattering technique (DLS) using a zetasizer Nano ZS, Malvern, UK. Measurements were made with a pure protein 5 μg/mL and protein/polymer complexes formed in the presence of increasing concentration of polymers in 10 mM phosphate buffer, pH 7.4, at 37 °C. Particle size was measured from the average 7–9 cycles. The laser light was set at 633 nm. Malvern software was used to analyze the data.

2.5. Transmission electron microscopy (TEM)

The effect of polymers on morphology of HSA was examined by transmission electron microscopy (TEM). Images were obtained with a JEOL-10 (JEOL Ltd., Tokyo, Japan) microscope. To form the complexes, polymers were added to HSA dissolved in 10 mmol/L phosphate buffer. The complexes were placed on a carbon surface of 200-mesh copper grid (Ted Pella, Inc) and incubated for 10 min before being drained with blotting paper. The grids were negatively stained with 2% (w/v) uranyl acetate for 2 min. Images were taken at a magnification of from 50,000 to 100,000.

2.6. Statistical analysis

All results were obtained from at least 3 independent experiments and were presented as mean ± SD. Statistical analyses were performed using one-way ANOVA (Friedman ANOVA and Kruskall Walis test with post-hoc analysis) and two-way ANOVA. Significance levels were defined as * $p < 0.05$; * * $p < 0.01$ and * ** $p < 0.001$.

3. Results

3.1. Fluorescence spectroscopy

Binding parameters and the nature of HSA-polymer interactions were determined by measuring the intrinsic fluorescence of HSA in the presence of various polymer concentrations. The fluorescence emission spectra of HSA (10 mmol/L in phosphate buffer, pH 7.4) at different molar ratios of tyrosine-modified polymers are shown in (Fig S1, S2). It was observed that linear PEIs showed generally higher affinities towards HSA molecules ($> 10^5$ M^{-1}) than branched PEIs ($> 10^4$ M^{-1}) (Table 1). A decrease in Stern Volmer quenching constant values was observed with increasing temperature. In order to evaluate the differences in the interactions between albumin and polymers in different temperatures statistical analysis was performed. LP10Y interaction with HSA was significantly stronger than interactions of other polymers in all investigated temperatures ($p < 0.001$). LP 5Y compared with P 5Y exhibited significant difference in 303 K ($p < 0.001$), In 306 and 310 K the differences were still significant, however p value was higher ($p < 0.05$). LP 5Y interactions were also significantly different from P 10Y interactions in 303 K ($p < 0.01$) and 306 K ($p < 0.05$). In 310 K, the differences were seen, but not significant. Comparison between P 5Y and P 10Y has shown no statistically significant differences between these groups in 303, 306 and 310 K.

In order to evaluate the mechanism of fluorescence quenching, the fluorescent data were analyzed by using the Stern-Volmer equation [34,

Table 1
Stern Volmer quenching constant (K_{sv}) and bimolecular quenching constant (k_q) values of HSA interactions with different polymers, i.e., linear (LP) or branched (P), tyrosine-modified (Y) 5 or 10 kDa PEIs, at different temperatures. R^2 - correlation coefficient. Thermodynamic parameters for HSA-polymer interactions and the nature of predominant interactions predicted from these parameters. Results presented as mean + /- SD from three independent exepriments.

Polymer	Temp. [K]	Ksv [M^{-1}]	kq [M^{-1} s^{-1}]	R^2	ΔH^0 [kJ/mol]	ΔS^0 [kJ/mol*K]	ΔG° [kJ/mol]	R^2	Predominant interaction
LP 5Y	303	3.5 ± 0.61 ×105	7.0 ± 0.77 ×1013	0.93	-50.3 ± 9.78	-0.076 ± 0.03	-32.1 ± 0.46	0.90	Van der Waals and H-bonding
	306	2.5 ± 0.66 ×105	5.0 ± 0.49 ×1013	0.97			-31.8 ± 0.38		
	310	2.2 ± 0.57 ×105	4.5 ± 0.54 ×1013	0.96			-31.6 ± 0.27		
LP 10Y	303	7.4 ± 0.29 ×105	1.5 ± 0.29 ×1014	0.91	-7.7 ± 3.04	0.086 ± 0.01	-33.8 ± 0.76	0.97	Hydrophobic and H- bonding
	306	7.1 ± 0.33 ×105	1.4 ± 0.33 ×1014	0.90			-34.1 ± 0.79		
	310	6.9 ± 0.32 ×105	1.4 ± 0.33 ×1014	0.88			-34.4 ± 0.82		
P 5Y	303	3.2 ± 0.96 ×104	6.4 ± 0.36 ×1012	0.97	-36.4 ± 15.0	-0.16 ± 0.048	-26.1 ± 0.34	0.99	Van der Waals and H-bonding
	306	2.7 ± 0.55 ×104	5.4 ± 0.36 ×1012	0.99			-26.0 ± 0.19		
	310	2.3 ± 0.59 ×104	4.6 ± 0.59 ×1012	0.96			-25.9 ± 0.02		
P 10Y	303	5.9 ± 0.61 ×104	1.2 ± 0.13 ×1013	0.98	-32.1 ± 9.2	-0.043 ± 0.023	-27.7 ± 0.14	0.99	Van der Waals and H-bonding
	306	5.0 ± 0.90 ×104	1.0 ± 0.09 ×1013	0.97			-27.6 ± 0.15		
	310	4.4 ± 0.56 ×104	8.9 ± 2.45 ×1012	0.93			-27.5 ± 0.21		

35]. The plots of F_0/F vs. Q were linear for all polymers at all temperatures, indicating that static or dynamic quenching may occur (Fig. 1**A**). The K_{sv} values were obtained from the slope of the linear regression of Stern-Volmer plots. Quenching rate constants were obtained from the Equation 1:

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = k_q \tau_0$$

with F_0, fluorescence of the protein without the quencher; F, fluorescence of the protein in the presence of the quencher; K_{SV}, quenching constant, Q, concentration of the quencher and k_q bimolecular quenching constant. The value of an average lifetime (τ_0) of the protein fluorescence was 10^{-8} s. All k_q values were greater than the maximum of diffusion-controlled quenching mechanism (10^{10} $M^{-1}s^{-1}$), suggesting that the mechanism of quenching was static. Static quenching was also confirmed by temperature variations. In the case of a static quenching mechanism, k_q values decrease with increasing temperature. This decrease in k_q values was seen for all polymers, additionally confirming the static mechanism of quenching.

Thermodynamic analysis of the polymer-HSA complexes were performed in order to describe intermolecular forces involved in complex formation. The standard enthalpy (ΔH^0), standard entropy (ΔS^0) and Gibbs free energy (ΔG^0) were obtained by plotting the logarithm of the binding constants (ln K_{sv}) vs. the reciprocal temperature 1/K (Fig. 1**B**). Thermodynamic parameters were calculated according to the equations:

$$\ln K = \frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R}$$

$$\Delta G^0 = \Delta H^0 - T\Delta S^0$$

The results revealed that enthalpy values for all polymer-HSA complexes were negative and entropy values were negative as well, except for polymer LP10Y where entropy was positive. Gibbs free energy values were negative (Table 1).

1-anilinonaphthalene-8-sulfonic acid (ANS) added to HSA was used in order to evaluate polymer-induced alterations in the albumin hydrophobic pocket. Indeed, fluorescence intensity of ANS decreased with increasing polymer concentrations (Fig. 2). Furthermore, a slight "red shift" of the fluorescence maximum was observed when polymers were added to the ANS-HSA complex.

Next, we investigated polymer binding affinities to L-tryptophan, which is an amino acid abundantly found in most proteins, including albumin. In all cases, a decrease in tryptophan fluorescence intensity was detected upon mixing with the different tyrosine-modified polymers (Fig. S3,**S4**). The respective Stern Volmer quenching constant was calculated from the Stern-Volmer equation (Fig. 3) with F_0, fluorescence of the protein without the quencher; F, fluorescence of the protein in the presence of the quencher; K_{sv}, quenching constant and Q, concentration of the quencher. The quenching constants were higher for LP 10Y (4.8×10^5 M^{-1}) and P 10Y (3.6×10^5 M^{-1}) than for LP 5Y (2.2×10^5 M^{-1}) and P 5Y (1.9×10^5 M^{-1}). The differences in the interactions between polymers and albumin were not statistically significant, except LP 10Y which exhibited significant difference from P 5Y ($p < 0.001$).

3.2. CD measurements

Far UV CD spectra revealed alterations in the secondary structure of HSA upon addition of the polymers (Figs. 4–5). The HSA spectrum is characterized by two negative bands around 210 and 222 nm and indicates that mostly α-helix domains are present [36]. When linear or branched polymers were added to the protein, a loss of α-helix structure was observed. These changes were more profound when branched polymers were used. In parallel, a slight increase in the percentages of β-turn and random coil structures was observed. α-helix loss was statistically significant for LP 5Y complexes ($p < 0.05$ for 1:5 ratio and $P < 0.01$ for 1:10 ratio). Due to more profound changes in case of branched polymers the changes in α-helix and random coil content were considered significant. The loss of α-helix was considered significant at 1:10 ratio ($p < 0.01$)for both P 5Y and P 10Y. Random coil content was significant at 1:10 ratio in P 5Y and P 10Y ($p < 0.05$). No statistically significant changes in the protein structure were seen when LP 10Y was tested.

3.3. Size measurements

The ability of tyrosine modified polymers to form complexes with HSA was confirmed by size measurements (Fig. 6**A, D**). Particle sizes of human serum albumin in phosphate buffer were around 6 – 7 nm. When the polymers were added, the formation of larger particles was observed (Fig. 6**B, C, E, F**). Depending on the type of polymer, the size of these complexes varied from 100 to 200 nm for linear polymers and 500 – 800 nm for branched polymers. Based on the PDI values for the HSA/polymer complexes determined at < 0.5, the solution of HSA and polymers was considered as monodisperse. Statistical analysis revealed that all polymers created structures significantly larger than human serum albumin molecule, starting from 1:1 ratio.

3.4. TEM analysis

The impact of PEIs on the morphology of HSA was also examined by transmission electron microscopy (TEM). As shown in Fig. 7, HSA was visible as thin fibrils. This protein morphology, however, was markedly altered in the presence of the polymers. Addition of PEIs to the HSA solution caused the formation of different electron-dense structures. Complexes formed by branched PEIs and HSA were visible as more electron dense structures than those created by linear polymers. TEM

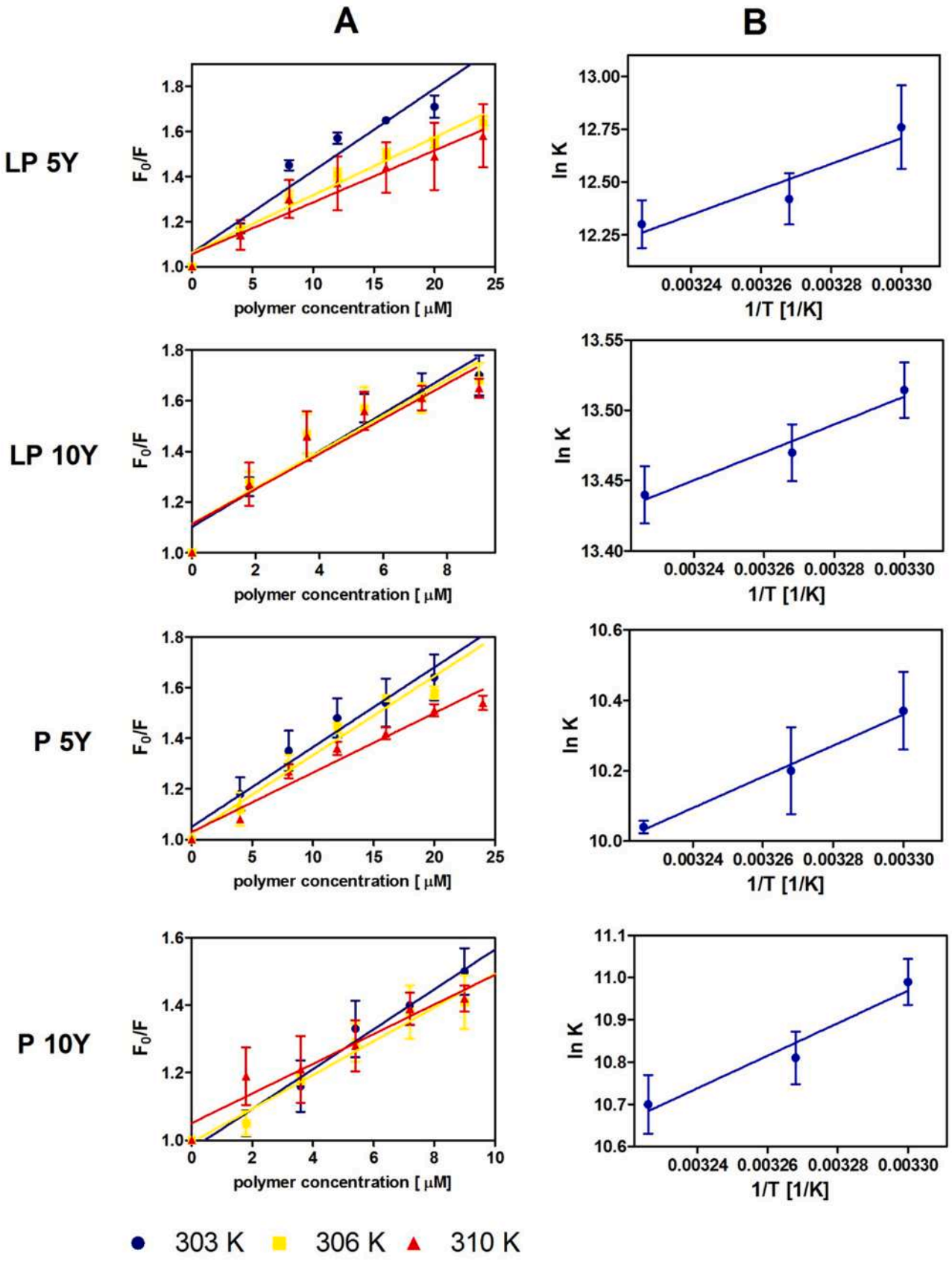


Fig. 1. (A) Stern-Volmer plots of HSA interactions with different linear or branched, tyrosine-modified PEIs, at different temperatures. (B) Van't Hoff plots for HSA-polymer interactions at 303, 306 and 310 K. Results presented as mean + /- SD from three independent experiments.

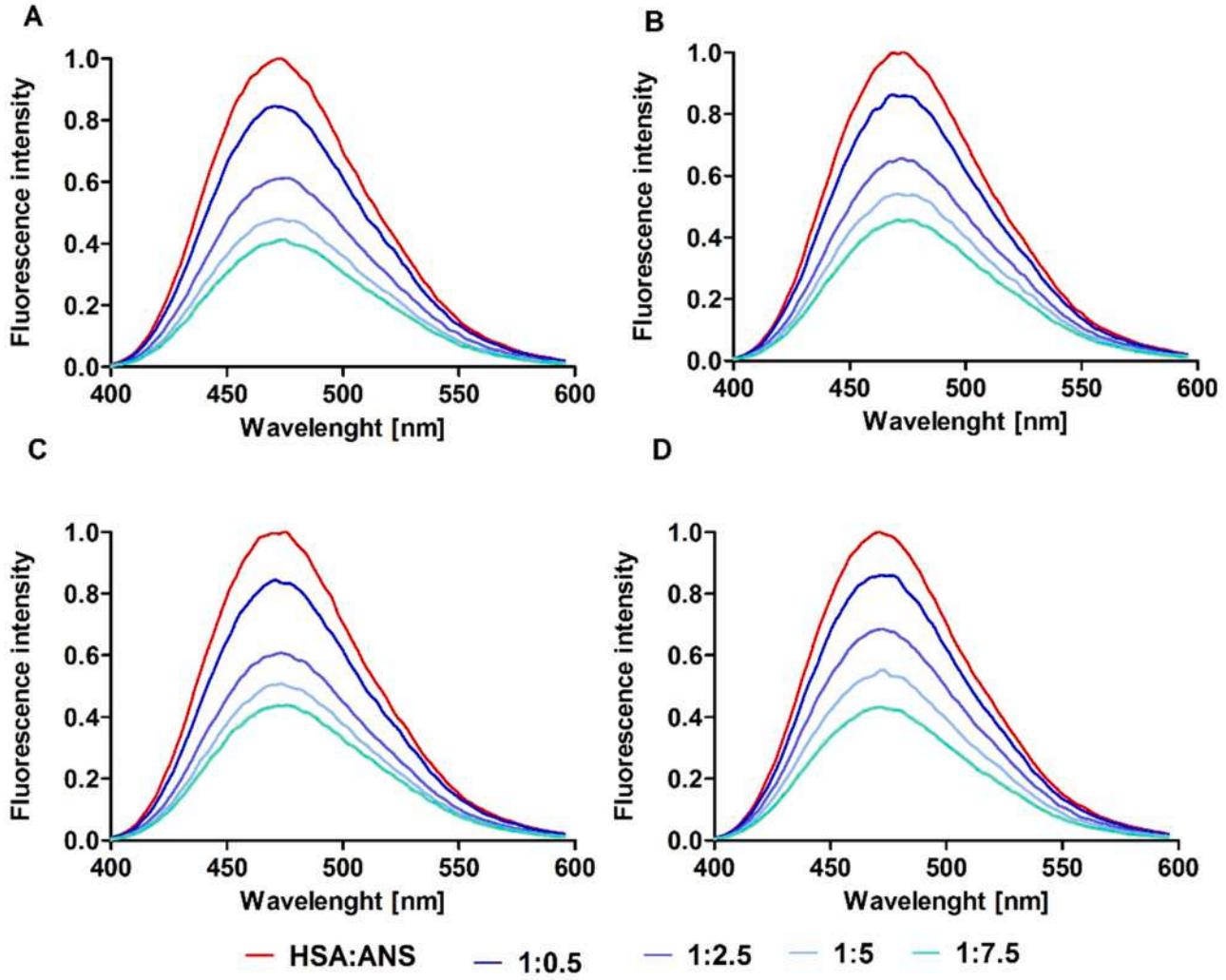
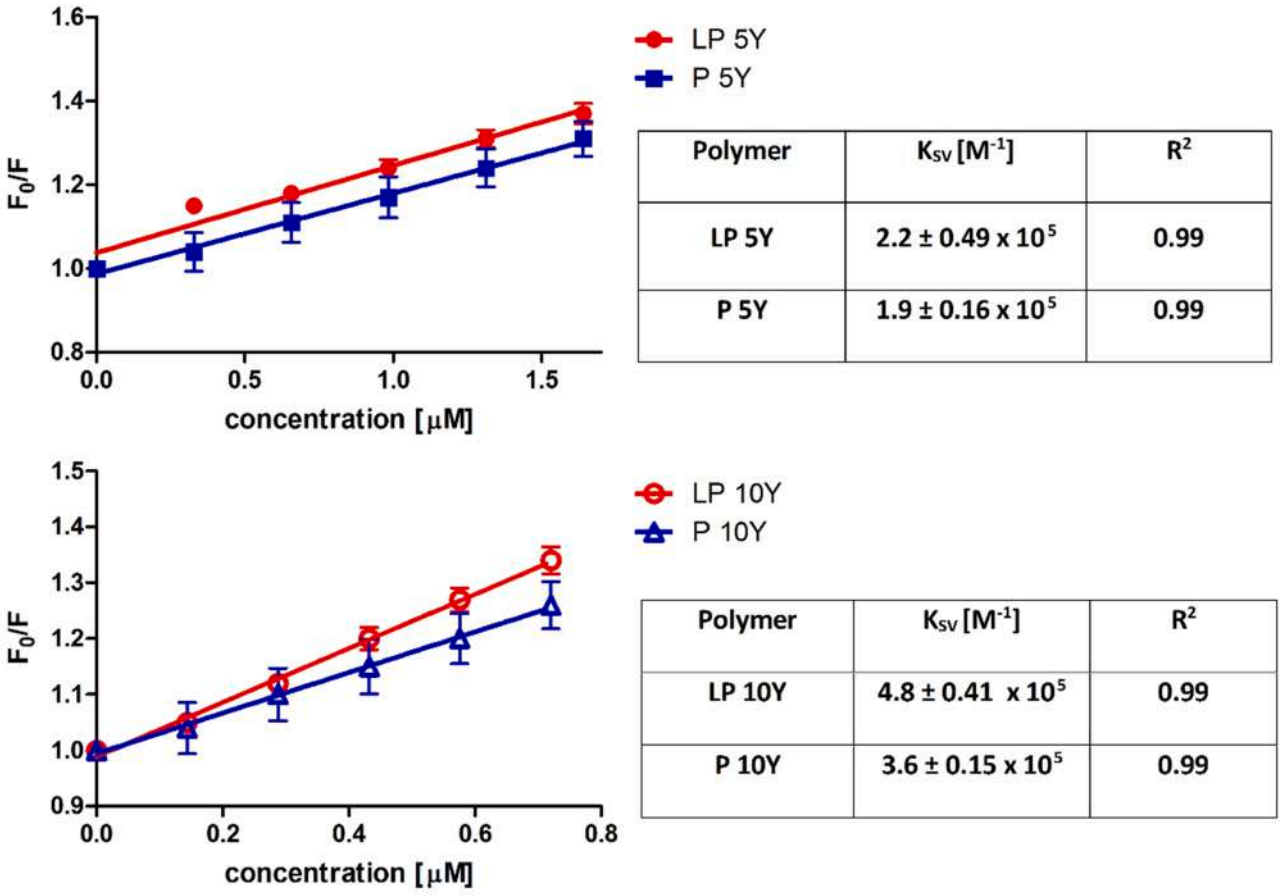


Fig. 2. Alterations in ANS fluorescence intensity in HSA-ANS solution (red line) upon addition of the indicated polymers (A) LP 5Y, (B) LP 10Y, (C) P 5Y, (D) P 10Y at different HSA+ANS:polymer molar ratios.

results indicate that HSA can bind PEI molecules and form complexes.

4. Discussion

All compounds administered into the bloodstream are almost immediately covered by blood components, including proteins. The binding and composition of proteins will also affect the fate of polymeric nanoparticles after their administration, thus protein-polymer interactions must be well studied and described. Here we present the physicochemical analysis of the interaction of human serum albumin with tyrosine-modified polyethyleneimines. HSA is the most abundant protein in the bloodstream and plays a pivotal role in binding low molecular weight compounds as well as in the formation of a protein corona around nanoparticles. It contains one Trp residue (Trp-214), which is located in the IIA domain, in the hydrophobic pocket [37]. Thus, the measurement of differences in the tryptophan fluorescence intensity provide information on the type of interaction and alterations in the protein structure. Indeed, binding affinities of tyrosine-modified PEIs with L-tryptophan revealed profound interaction and a concomitant decrease in fluorescence intensity. The Stern Volmer quenching constants indicated that polymers with higher molecular mass showed higher binding affinities. Previously, tryptophan interactions with PAMAM dendrimers had been studied [8,38] and, in contrast to our results, described an increase in fluorescence intensity and “red-shift”. This was explained as a possible effect of electrostatic interactions between L-tryptophan and PAMAM dendrimers [8] and highlights the differences between PAMAM and our polymers. This was also seen when studying HSA. Fluorescence spectroscopy studies revealed that tyrosine modified PEIs are able to interact with albumin. Stern Volmer constant inform us about the accessibility of human serum albumin to the tyrosine-modified polymers. Plots exhibited a good linearity over the whole tested concentration range what suggests that of only one type of quenching mechanism (dynamic or static). Linear plot suggests the presence of only a single class of fluorophores that are equally accessible to the quencher Differences between linear and branched PEIs binding affinity were observed, indicating that the structure of polymers plays a role in the protein-polymer interaction. LP 10Y and P 10Y binding affinities were found to be higher compared to LP 5Y and P 5Y. Similar results were also obtained by Guo et al. for non-modified PEIs, where binding affinities to albumin increased with molecular mass of the polymer [39]. Stern-Volmer constant for all polymers were in range 10^4 – 10^5 what is in line with Stern-Volmer constant values for HSA/drug interaction in vivo [35]. As long as a Stern-Volmer plot exhibits a linear correlation with increasing quencher concentrations, the mechanism of quenching can be evaluated [10,40]. Two main mechanisms of fluorescence quenching have been described: a dynamic and a static mechanism of quenching [41]. When a collision process between the quencher and the fluorophore occurs, fluorescence is quenched through



Polymer	K_{sv} [M^{-1}]	R^2
LP 5Y	2.2 ± 0.49 x 10^5	0.99
P 5Y	1.9 ± 0.16 x 10^5	0.99

Polymer	K_{sv} [M^{-1}]	R^2
LP 10Y	4.8 ± 0.41 x 10^5	0.99
P 10Y	3.6 ± 0.15 x 10^5	0.99

Fig. 3. Stern-Volmer plots for free L-tryptophan with increasing concentrations of tyrosine modified PEIs in 10 mmol/L phosphate buffer, at 37 °C. Stern Volmer quenching constant values for L-tryptophan-polymer adducts. Results presented as mean + /- SD from three independent experiments.

a dynamic mechanism [42,43]. Dynamic quenching is defined by the Stern-Volmer constant increasing with temperature. On the contrary, when K_{sv} decreases with increasing temperatures, static quenching takes place. Static quenching is caused by a complex formation and K_{sv} decreases because of reduced complex stabilities with increasing temperature [40,44]. In our study, all tyrosine-modified polymers quenched the fluorescence intensity by a static mechanism. Bimolecular quenching constants were above 2×10^{10} M^{-1} s^{-1} which is considered as a maximum value for a diffusion-controlled collisional quenching mechanism [10]. This type of quenching was previously seen for other polymers, including non-modified PEIs [23,45–47].

The fundamental molecular interactions in protein binding include hydrogen bonding, van der Waals, hydrophobic and electrostatic interactions. Thermodynamic parameters (ΔH^0, ΔS^0, ΔG^0) provide information on the molecular forces involved in complex formation [48]. According to the K_{sv} values obtained in different temperatures, ΔS^0, ΔG^0 can be calculated using the van't Hoff equation [49]. Depending on the obtained values, the different types of interaction can be distinguished. When $\Delta H^0 < 0$ and $\Delta S^0 < 0$, the main forces engaged in complex formation are van der Waals and hydrogen bonding; when $\Delta H^0 < 0$ and $\Delta S^0 > 0$, hydrophobic forces and hydrogen bonding are predominant forces. Finally, $\Delta H^0 > 0$ and $\Delta S^0 < 0$ indicate electrostatic interactions as main force [40,48,50]. Negative values of enthalpy changes, ΔH^0, and Gibbs free energy, ΔG^0, were obtained for all HSA/polymer adducts, indicating that the complexation process was exothermic and spontaneous [10,41]. ΔS^0 is a measure of the disorder in the system. In ligand-protein interaction, entropy measurements are based on two processes: on the one hand, ligand binding to the protein leading to positive entropy values due to the increased freedom of complex, and on the other hand, decrease of freedom and negative entropy values when the protein getting into the proximity of the ligand [40]. Positive entropy values provide one basis for lowering the standard Gibbs free energy. In our study, only LP 10Y showed alterations in Gibbs free energy based on increased entropy (positive value), in combination with negative enthalpy values. In the case of all other polymers, negative enthalpy values alone led to lower ΔG^0, rather than higher ΔS^0, which were in fact negative for LP 5Y, P 5Y and P 10Y.

Circular dichroism analysis is an analytical method which allows to estimate structural changes in the protein structure [51,52]. Human serum albumin has a characteristic profile with two minimal bands around 222 (n – π* transition) nm and 208 nm (π – π* transition) [49]. Alterations in albumin secondary structure are very common and have been seen previously with nanoparticles and other compounds [10,16,49,53,54]. In this work, alterations in the HSA secondary structure were observed as well. The changes in the ellipticity are often related to the changes in protein functionality. We observed the decrease in HSA ellipticity upon addition of polymers with a subsequent loss of helical structure. It can be attributed to the loss of protein stability and unfolding. These were more profound in the case of the branched polymers and consisted of a loss of α-helix in parallel with an increase in β-turn and random coil structures. At higher concentrations, branched polymers clearly affect secondary and probably tertiary structures by denaturation and unfolding of the protein [50]. Linear polymers also caused some minor changes in the α-helix percentage, with a minimal increase in β-turn and random coil structures, which may suggest unfolding processes. Previous studies with non-modified PEIs indicated that they change albumin structure in a minimal way with practically no changes in helical structure [39,46]. Our control experiments with non-modified PEI confirmed these results: in contrast to their tyrosine modified counterparts used in this study, they did not affect secondary

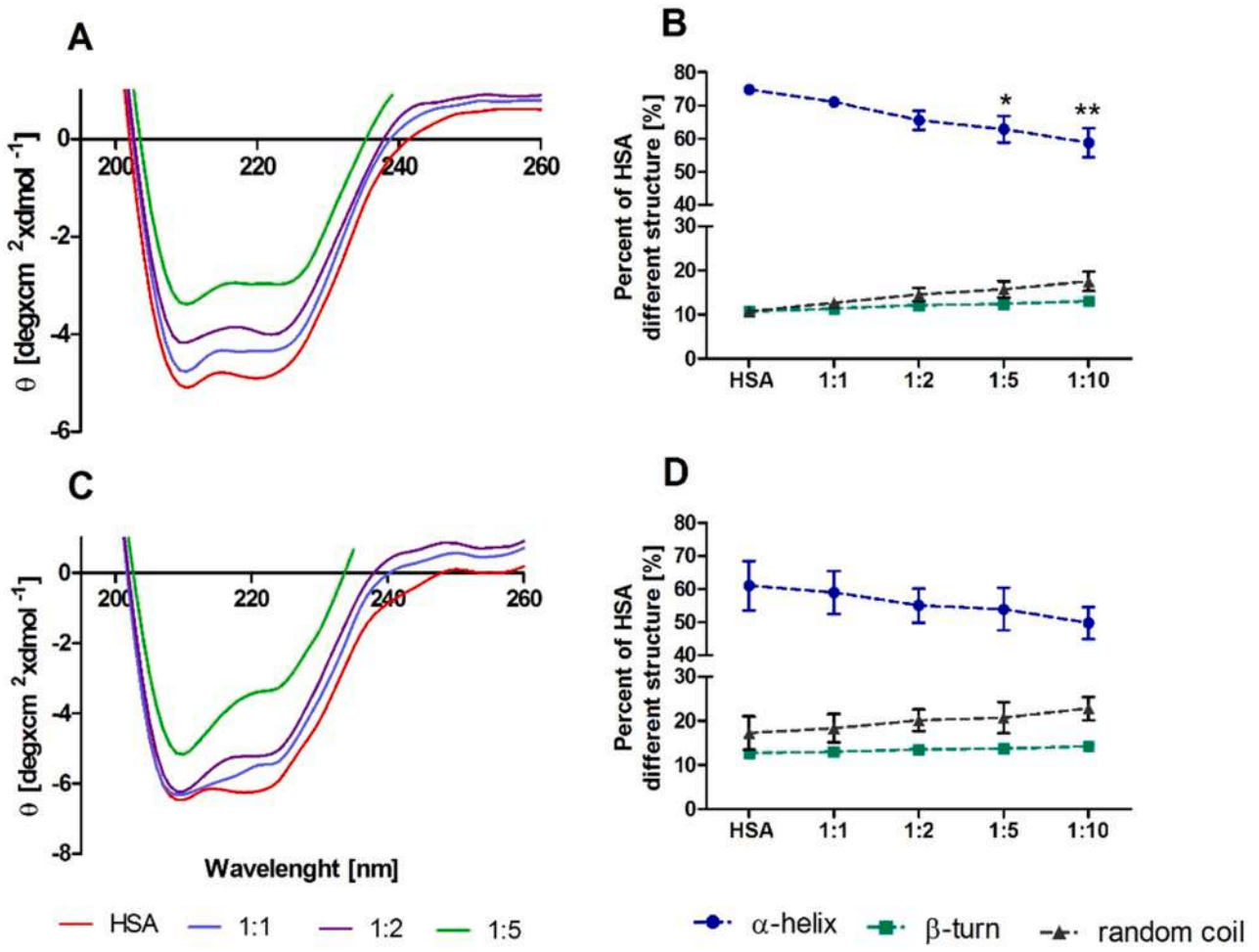


Fig. 4. Alterations in the α-helix structure of HSA (10 mmol/L phosphate buffer) upon addition of the linear, tyrosine-modified polymers LP 5Y (A) or LP 10Y (C) at different HSA:polymer molar ratios. Quantitation of these differences in α-helix, β-turn and random coil percentage in HSA in the presence of LP 5Y (B) or LP 10Y (D). Results presented as mean + /- SD from three independent exepriments. * *- $p < 0.01$, * - $p < 0.05$.

structure near 210 nm and did not change α-helix content in albumin, even in higher concentrations (Fig. S5 – S8). This suggests that the tyrosine-modification of PEI may lead to enhanced interactions with proteins.

ANS is a well-known fluorescent dye which can be used for investigating conformational changes related to the translocation of hydrophobic pockets [32]. In aqueous solution, ANS fluorescence is very low with a z maximum intensity around 540 nm. In the presence of HSA, ANS fluorescence intensity increases with an observed blue shift to a maximum intensity around 475 nm [38]. The addition of polymers, however, led to a decrease in ANS fluorescence intensity suggesting that the polymers caused a displacement of ANS from an HSA hydrophobic pocket [14]. The decrease in the fluorescence intensity indicates that the environment around ANS has been changed to more hydrophilic. One of the explanation for this is the translocation of the hydrophobic pocket where ANS binds to albumin. Hydrophobic pocket could be relocated due to polymer addition. Based on the decrease, which can be seen on the graph and circular dichroism data we assume that location of the hydrophobic pocket changes with increasing concentration of polymers. The translocation of the hydrophobic pocket may be attributed to the HSA unfolding process. These findings suggest hydrophobic forces to play a role in the interaction of tyrosine modified PEIs with HSA. A slight "red shift" observed during the experiments further indicates that a translocation of hydrophobic pockets to the protein surface with a more polar environment cannot be excluded [32,55,56]. The vast majority of ligands bind reversibly to the HSA subdomains IIA and IIIA, with binding affinities ranging from 10^4 to 10^6 M^{-1} [14]. ANS was found to bind to subdomains IIA and IIIA with a binding affinities of 7.9×10^4 M^{-1} and 8.7×10^5 M^{-1}, respectively. Thus, values are similar to the binding constants for tyrosine modified PEIs. Subdomain IIIA binding properties are less specific than those of subdomain IIA. The tryptophan residue in HSA is located in the subdomain IIA and is well described for studying van der Waals interactions at that site [14]. Our results indicate that tyrosine modified polymers can bind to the sites where usually ANS binds in HSA.

Z-size measurements and TEM analyses were performed in order to further evaluate the complex formation between human serum albumin and the polymers. We confirmed that pure HSA in solution possesses a mean diameter of around 7 – 12 nm and in dry state is observed as thin fibrils in TEM. These findings were consistent with a literature [49]. Upon addition of polymers, the HSA peak around 10 nm disappeared and larger structures were found, suggesting complex formation based on interactions between the polymers and HSA. However, it should be noted that tyrosine-modified PEIs are also able to form self-aggregates in the absence of HSA (Suppl. Fig. 9–10), which, of note, can be distinguished from the HSA-polymer complexes by differences in PDI and in size. More specifically, LP 5Y and P 10Y formed complexes with HSA with sizes similar to the LP 5Y and P 10Y self-aggregates, but higher PDI values. This may be caused by the kinetics of the complex formation process and poor stability of the LP5Y/HSA and P10Y/HSA complexes. To the contrary, in the case of LP 10Y self-aggregates presented with a high PDI, suggesting a broad range of size populations. In complex with

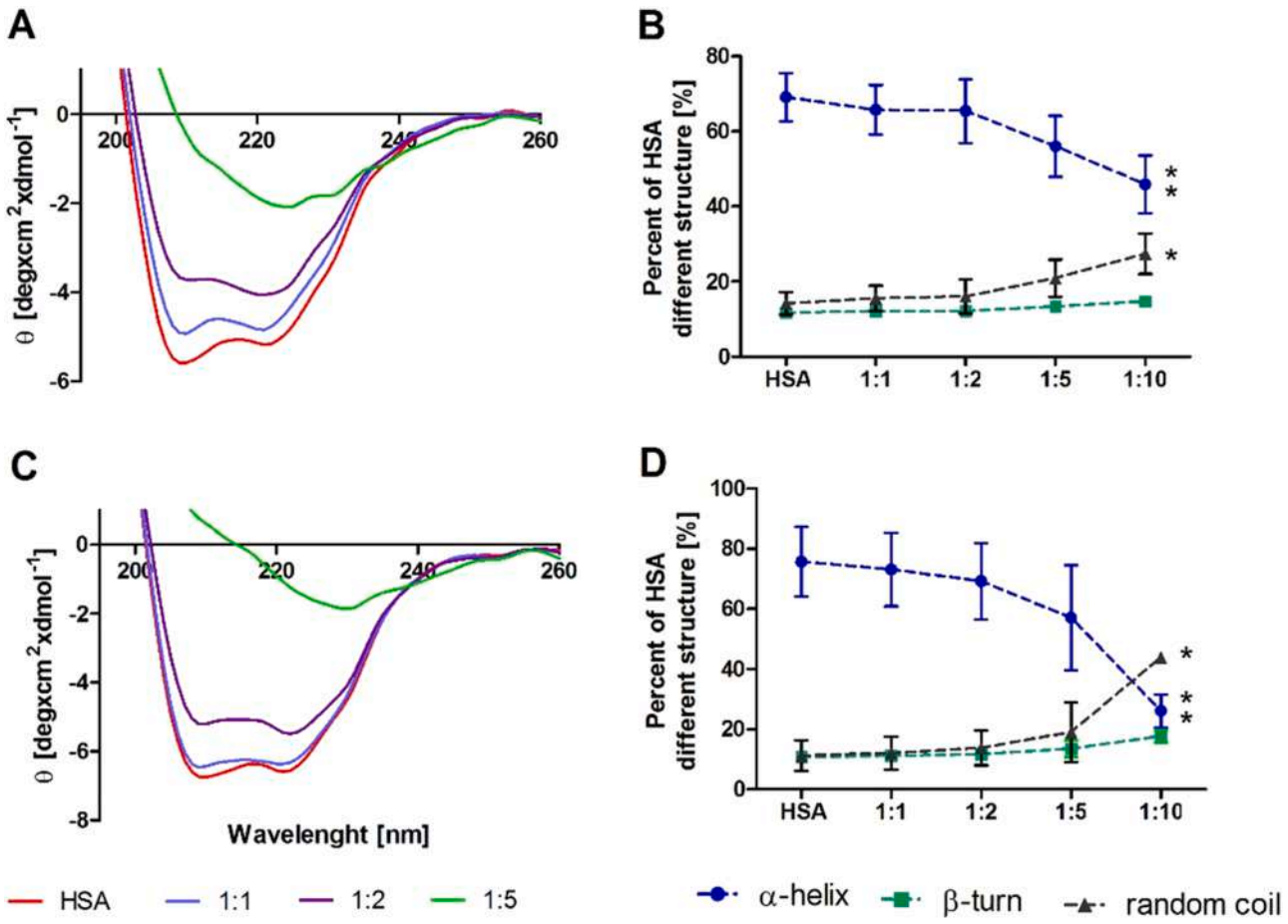


Fig. 5. Alterations in the α-helix structure of HSA (10 mmol/L phosphate buffer) upon addition of the branched, tyrosine-modified polymers P 5Y (A) or P 10Y (C) at different HSA:polymer molar ratios. Quantitation of these differences in α-helix, β-turn and random coil percentage in HSA in the presence of P 5Y (B) or P 10Y (D). Results presented as mean + /- SD from three independent exepriments. * *- $p < 0.01$, * - $p < 0.05$.

HSA, the size values were similar to LP 10Y self-aggregates, but with a lower PDI corresponding to more homogenous particle sizes. This may be attributed to a higher binding affinity of LP 10Y to human serum albumin, compared to other polymers. Finally, complexes of P 5Y with HSA were larger in size than the P 5Y self-aggregates, with a similar range of PDI values. It seems that P 5Y forms a heterogeneous size population, alone as well as in combination with HSA. Another important issue related to size distribution and values are buffer conditions. Ionic strength and pH strongly affect nanoparticles/complex formation [27,57].

Finally, TEM analyses confirmed alterations in the structure of HSA in the presence of PEIs, and thus the interaction between both the protein and the polymer. This is in line with previous studies on other protein and dendrimers [58–60] and also indicates the relevance of these interactions on nanoparticles based on these polymers. It is agreed with results obtained for complexes formed by thrombin and dendrimers.

5. Conclusion

In the present study our aim was to describe the interaction between human serum albumin and tyrosine-modified polymers. Human serum albumin is the most abundant protein in the bloodstream and is responsible for binding and transport of many compounds, including nanoparticles. Protein: polymer complexes were formed, with sizes smaller for linear PEIs than for branched PEIs. Polymers were able to bind to L-tryptophan residues as well as to HSA. Spectrofluoroscopy studies at different temperatures revealed that complexes were spontaneously formed, mainly due to van der Waals interaction and hydrogen bonding. Hydrophobic interactions were involved in complex formation as well, based on the polymers' ability to interact with hydrophobic pockets of HSA. A decrease of α-helix content in HSA was observed upon addition of any of the investigated polymers, however, this effect was more profound in the case of branched PEIs. The results from this work would be helpful for better understanding of the polymer:protein interactions and in the evaluation of the pharmacokinetic profile of the tyrosine-modified complexes in the bloodstream. However, some further studies with a mixture of proteins (human plasma) should be performed in order to describe protein corona effect.

CRediT authorship contribution statement

Małgorzata Kubczak: Conceptualization, Methodology / Study design, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review and editing, Visualization, Data curation. **Marika Grodzicka**: Methodology / Study design, Investigation, Data curation. **Sylwia Michlewska**: Methodology / Study design, Investigation, Visualization, Data curation. **Michael Karimov**: Investigation. **Alexander Ewe**: Investigation, Supervision. **Achim Aigner**: Supervision, Project administration, Funding acquisition. **Maria Bryszewska**: Resources, Supervision, Project administration, Funding acquisition. **Maksim Ionov**: Conceptualization, Methodology / Study design, Validation, Resources, Supervision, Project administration, Funding acquisition.

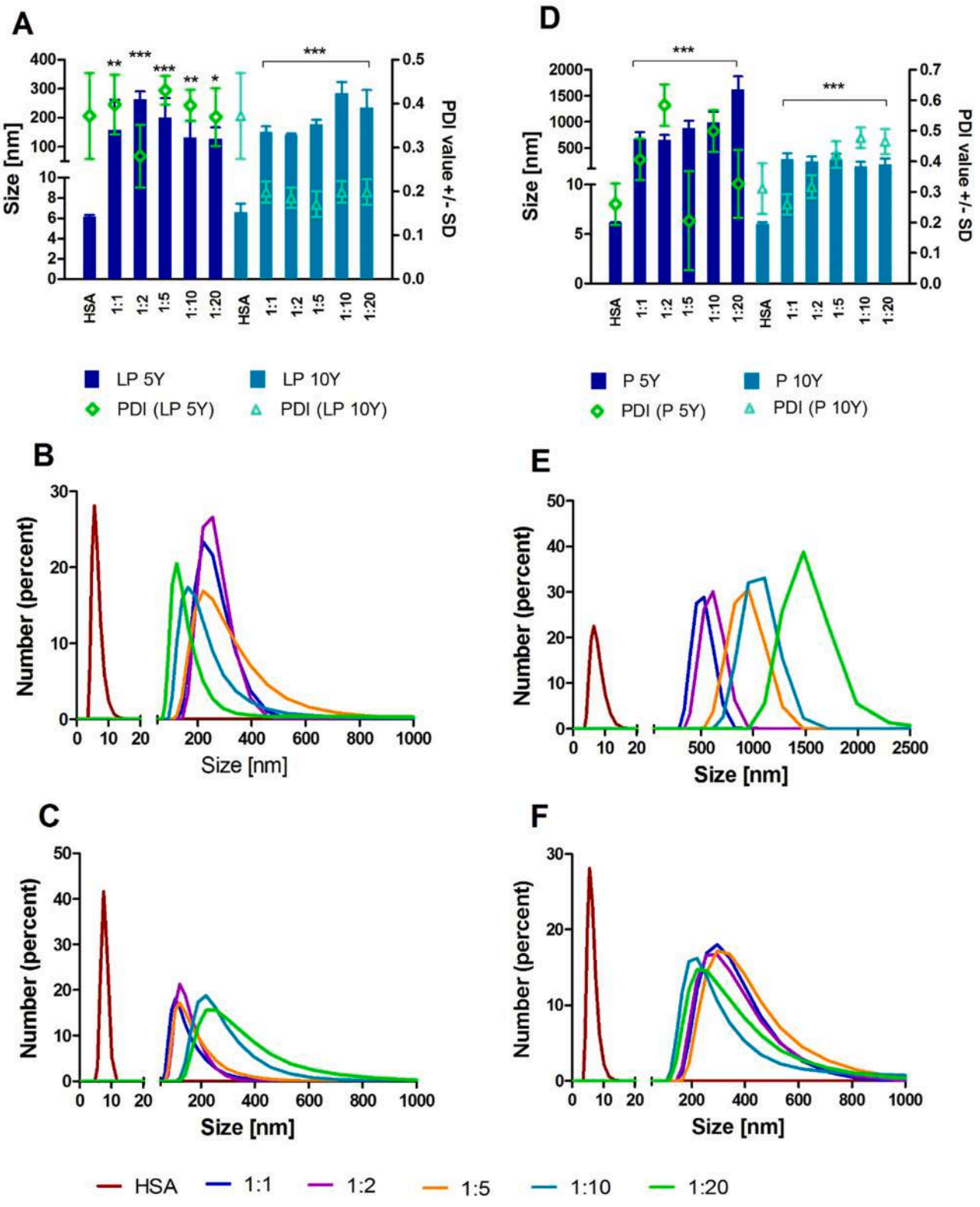


Fig. 6. A - Size values (bars) and polydispersity index (PDI; open symbols) of HSA- linear polymer complexes in 10 mmol/L phosphate buffer. Size peaks obtained for HSA and HSA/polymer complexes containing LP 5Y (B) and LP 10Y (C) at different HSA:polymer molar ratios in 10 mmol/L phosphate buffer. D - Size values (bars) and polydispersity index (PDI; open symbols) of HSA- branched polymer complexes. Size peaks obtained for HSA and HSA/polymer complexes containing P 5Y (E) and P 10Y (F) at different HSA:polymer molar ratios in 10 mmol/L phosphate buffer. Results presented as mean + /- SD from three independent experiments. * ** - $p < 0001$, * *- $p < 0.01$, * - $p < 0.05$.

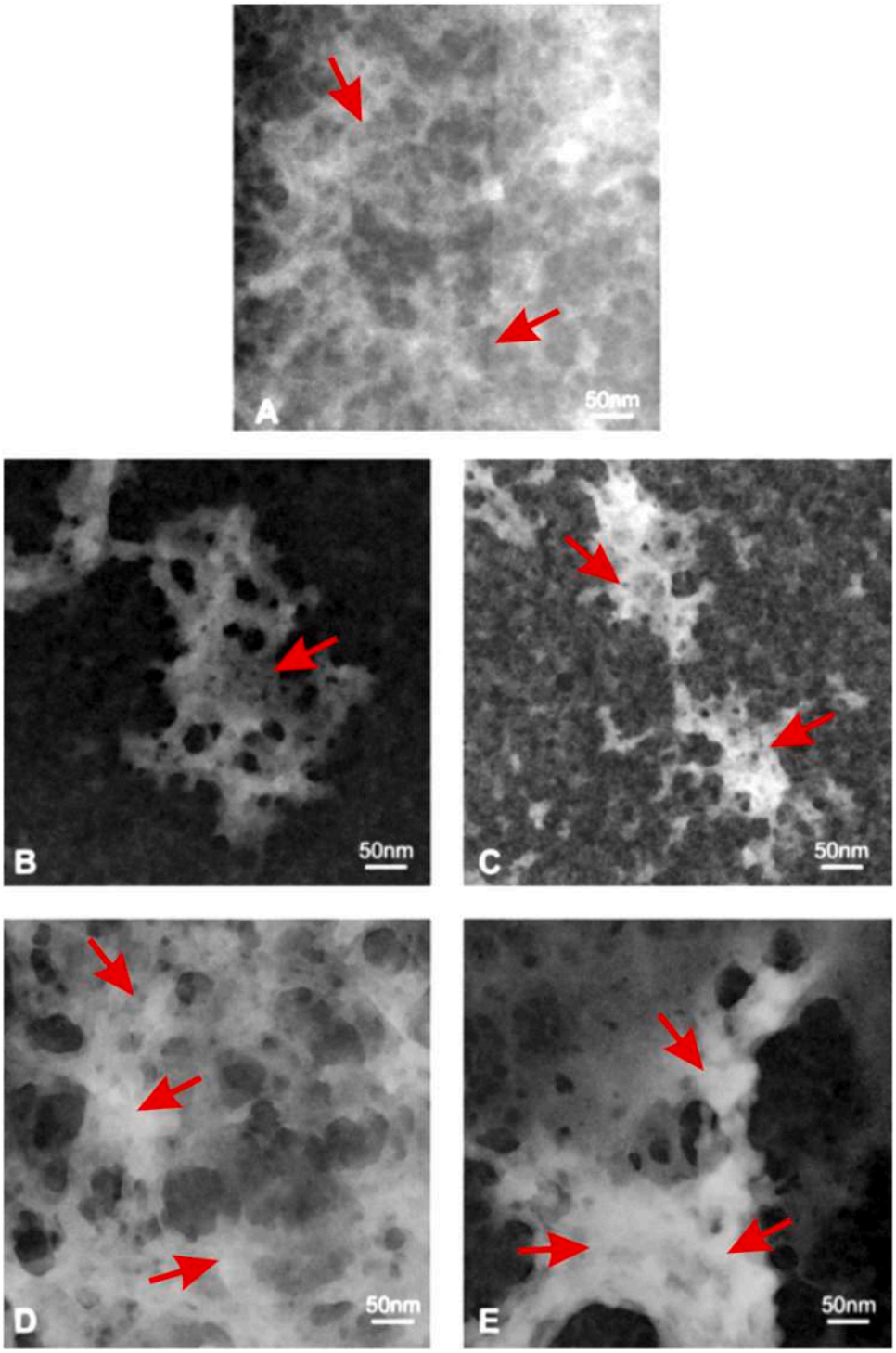


Fig. 7. Transmission electron microscopy images of HSA alone (A) or upon addition of tyrosine modified PEI P 5Y (B), P 10Y (C), LP 5Y (D) or LP 10Y (E). Red arrows point to the complexes (seen as white structures). Molar PEI/protein ratios were set at 1:1 in 10 mmol/L phosphate buffer, pH 7.4. For improving the contrast, the colours of the microphotographs have been inverted. Bar = 50 nm.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Maria Bryszewska reports financial support was provided by National Science Centre Poland. Achim Aigner reports financial support was provided by German Research Foundation.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by a joint grant from the National Science Centre of Poland, project no: 2018/31/F/NZ5/03454 to M.B., and the Deutsche Forschungsgemeinschaft (DFG; grant number AI 24/24–1 to A.

A.) within the "BEETHOVEN LIFE 1″ program, and the grant AI 24/21–1 to A.A. The authors are also grateful for funding by grants to M.B. and A. A. from the project "EUROPARTNER" of the Polish National Agency for Academic Exchange (NAWA).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.colsurfb.2023.113359.

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Supplementary Materials

The effect of novel tyrosine-modified polyethyleneimines on human albumin structure – thermodynamic and spectroscopic study.

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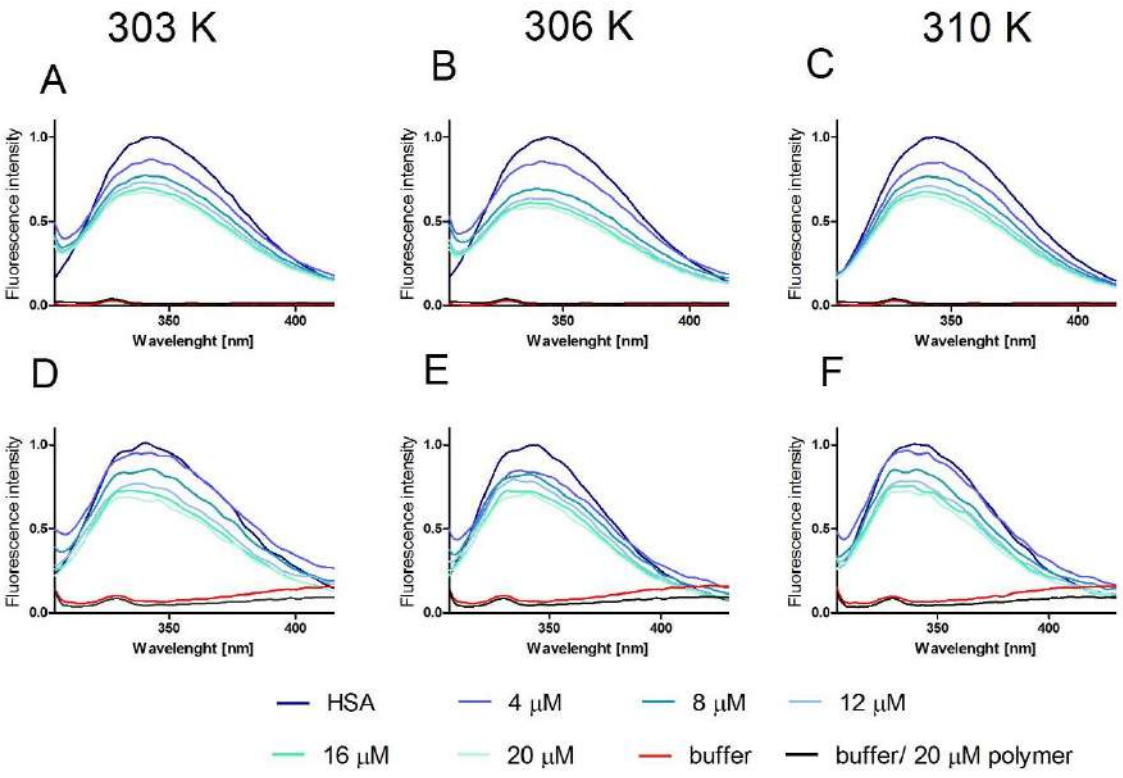

Figure S1. Fluorescence intensity spectra of HSA (0.075 μM) upon addition of increasing concentrations of LP 5Y (A – C) and P 5Y (D – F) at different temperatures. Fluorescence intensities of buffer and polymer used in the highest concentration are shown by the black and red lines, respectively.

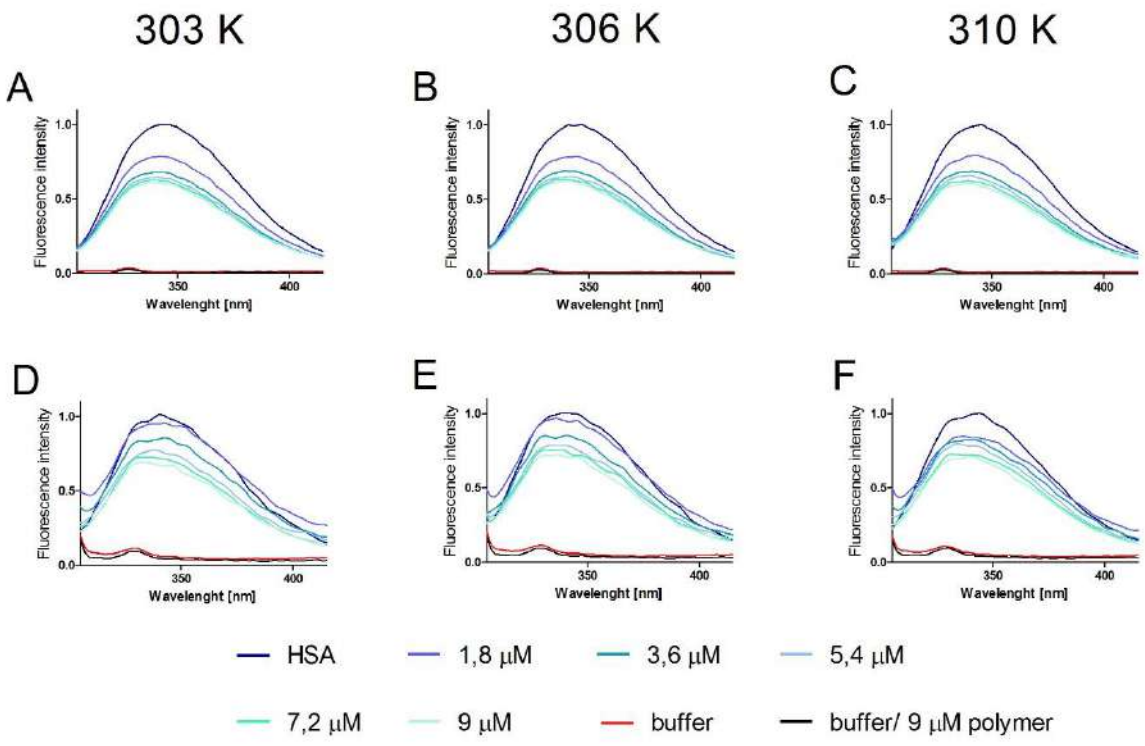

Figure S2. Fluorescence intensity spectra of HSA (0.075 μM) upon addition of increasing concentrations of LP 10Y (A – C) and P 10Y (D – F) at different temperatures. Fluorescence intensities of buffer and polymer used in the highest concentration are shown by the black and red lines, respectively.

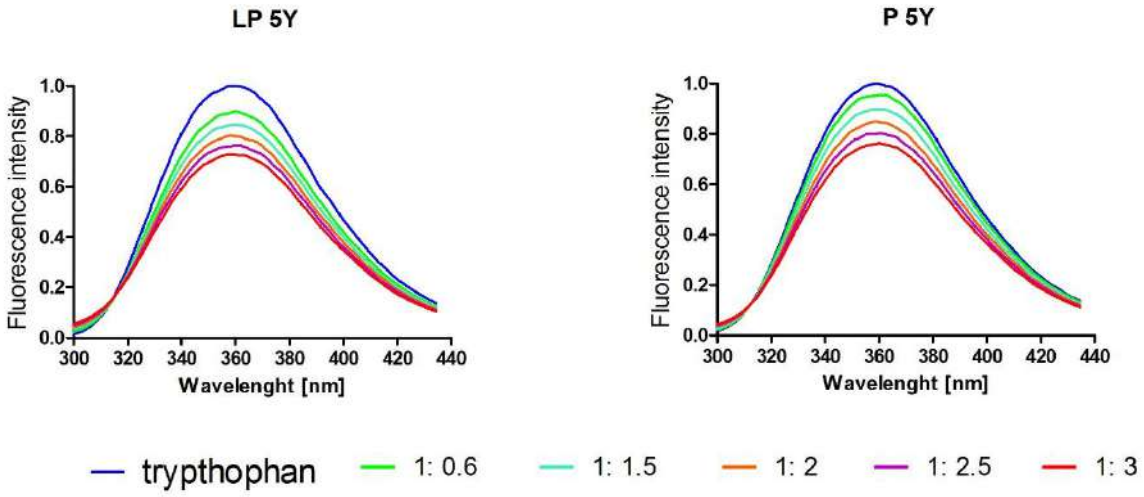

Figure S3. Fluorescence intensity spectra of L-tryptophan (0.5 μM) with increasing HSA:polymer molar ratios, Na-phosphate buffer 10 mmol/L, pH 7.4.

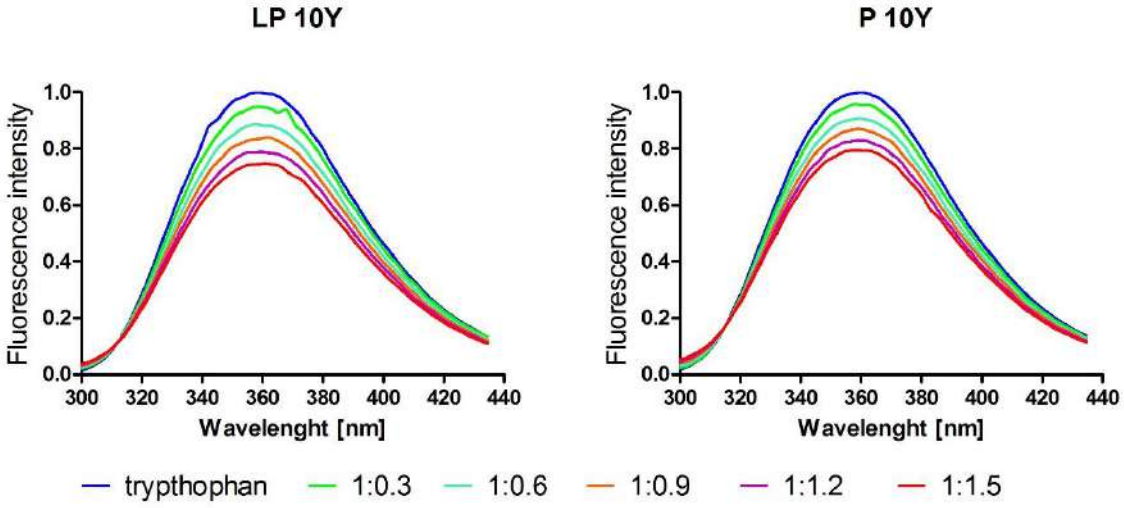

Figure S4. Fluorescence intensity spectra of L-tryptophan (0.5 µM) with increasing HSA:polymer molar ratios, Na-phosphate buffer 10 mmol/L, pH 7.4.

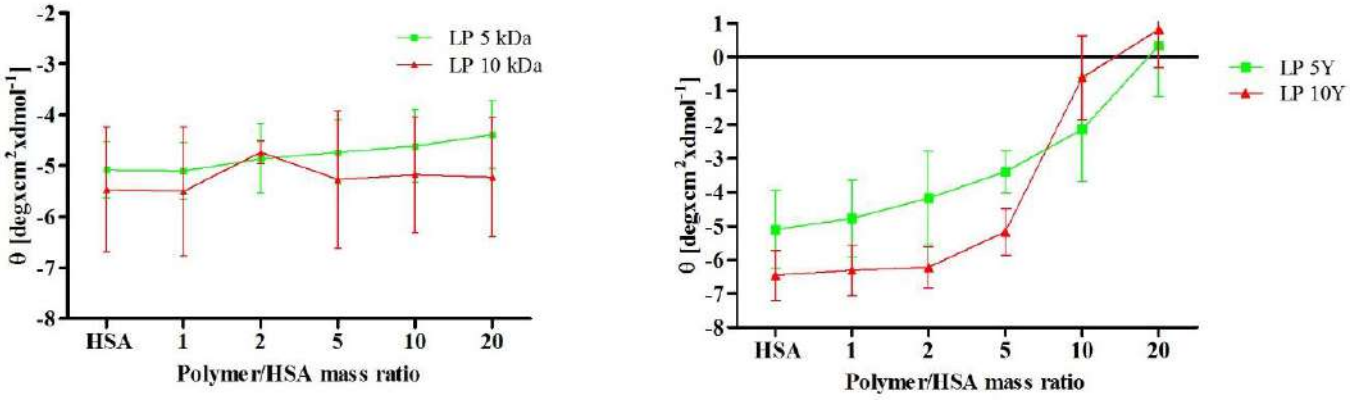

Figure S5. Changes in mean residue ellipticity of HSA, at λ=210 nm in the presence of linear polymers. Results are mean ± standard deviation (SD), with n = 3. HSA concentration 5 µg/mL, wavelength 195–260 nm, scan speed 50 nm/min, bandwidth 1.0 nm, Na-phosphate buffer 10 mmol/L, pH 7.4.

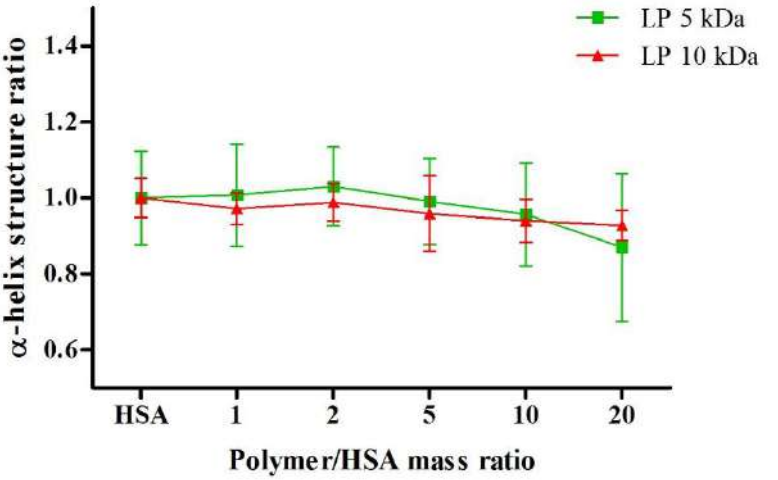

Figure S6. Changes in α-helix structure ratio in HSA in the presence of linear, non-modified polymers. Results are mean ± standard deviation (SD), with n = 3. HSA concentration 5 µg/mL, wavelength 195–260 nm, scan speed 50 nm/min, bandwidth 1.0 nm, Na-phosphate buffer 10 mmol/L, pH 7.4.

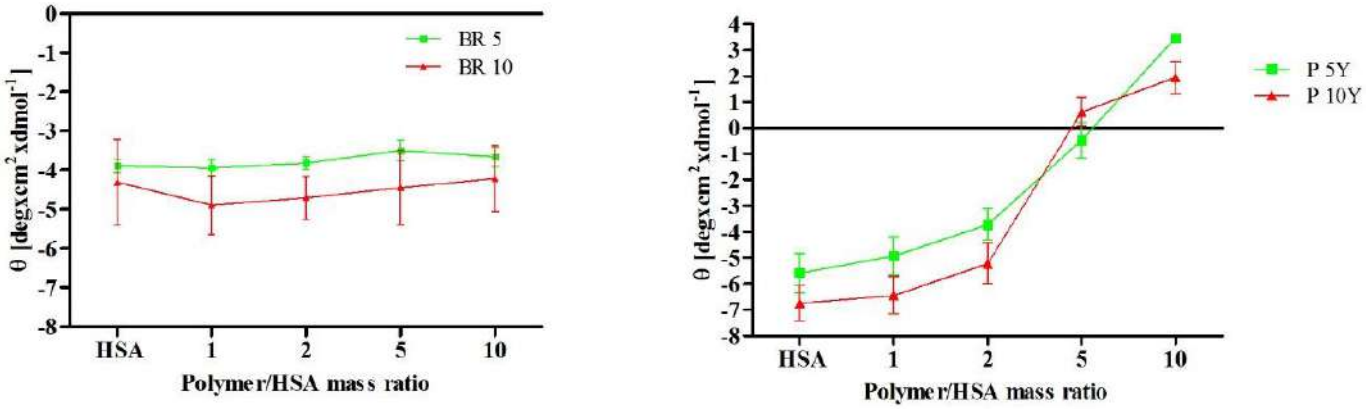

Figure S7. Changes in mean residue ellipticity of HSA, at λ=210 nm in the presence of branched polymers. Results are mean ± standard deviation (SD), n = 3. HSA concentration 5 µg/mL, wavelength 195–260 nm, scan speed 50 nm/min, bandwidth 1.0 nm, Na-phosphate buffer 10 mmol/L, pH 7.4.

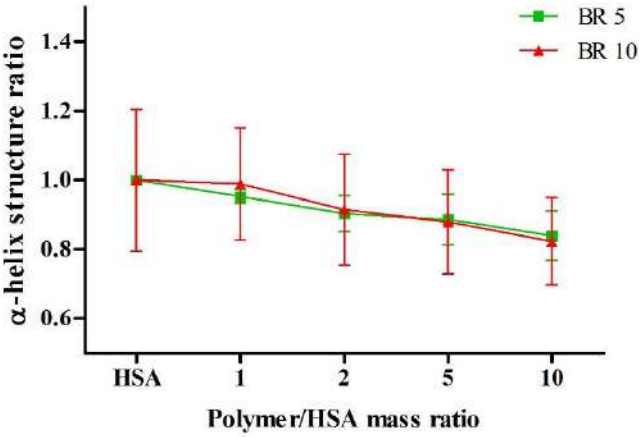

Figure S8. Changes in α-helix structure ratio in HSA in the presence of branched, non-modified polymers. Results are mean ± standard deviation (SD), with n = 3. HSA concentration 5 µg/mL, wavelength 195–260 nm, scan speed 50 nm/min, bandwidth 1.0 nm, Na-phosphate buffer 10 mmol/L, pH 7.4.

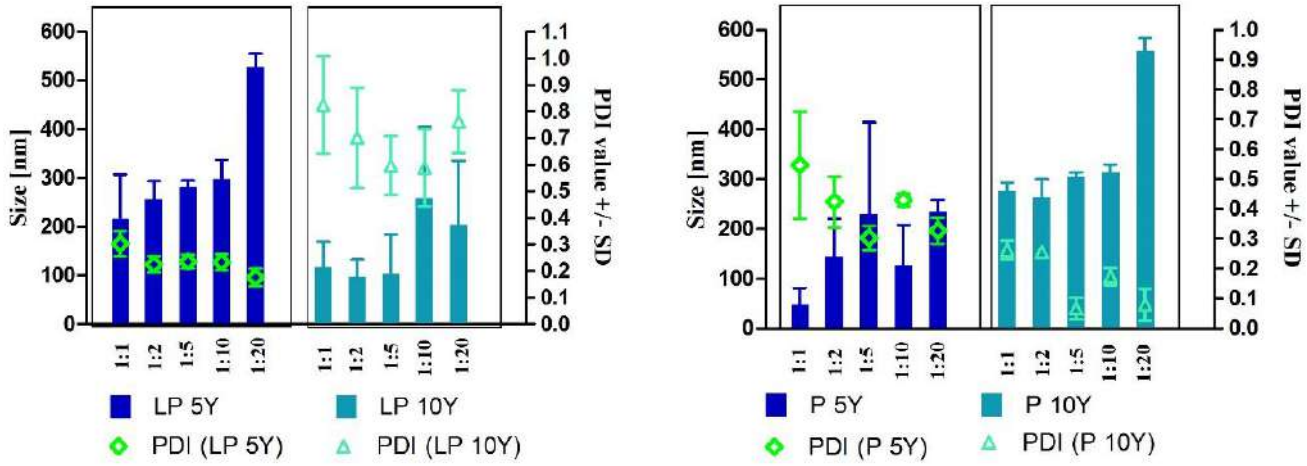

Figure S9. Size values (bars) and polydispersity index (PDI; open symbols) of tyrosine-modified polymers in 10 mmol/L phosphate buffer, in increasing HSA:polymer molar ratios. Polymer concentrations were identical as used for HSA:polymer complex preparation.

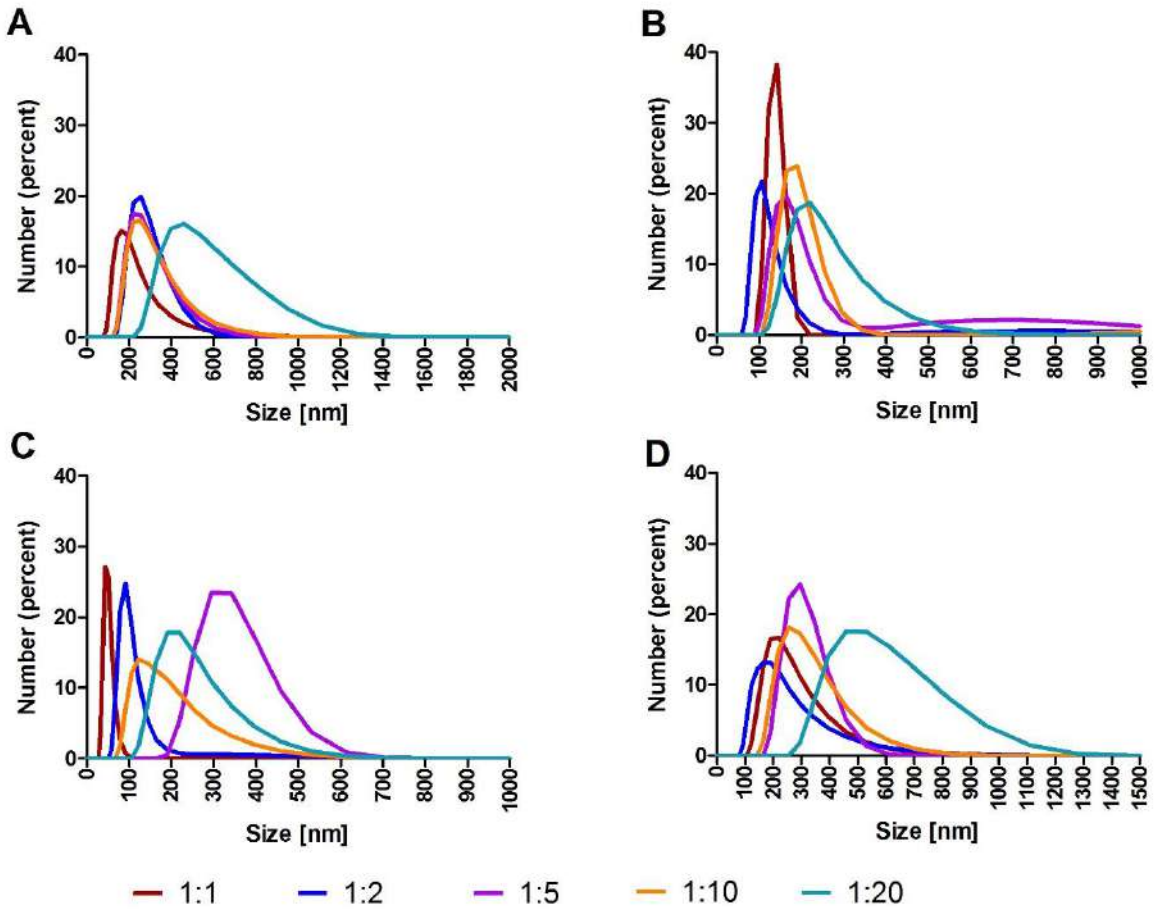

Figure S10. Size peaks obtained for tyrosine modified PEI complexes LP 5Y (A), LP 10Y (B), P 5Y (C) or P 10Y (D) at different HSA:polymer molar ratios in 10 mmol/L phosphate buffer.

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I declare that in the publication "Unmodified and tyrosine-modified polyethylenimines as potential carriers for siRNA: biophysical characterization and toxicity" **Kubczak, M**, Michlewska, S, Karimov, M, Ewe, A, Noske, S, Aigner, A, Bryszewska, M, Ionov, M, International Journal of Pharmaceutics, 2022, 121468, 0378-5173 my contribution is 65 % and includes: performing zeta potential and size measurements, membrane fluidity assay, MTT, LDH, JC-1 assays, gel electrophoresis, florescence polarization, comet assay and luciferase assay, statistical analysis, preparation of manuscript, graphs and figures, submission to the journal as a corresponding author, correspondence with the Editor, preparation of answers for reviewers.

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Statement

Hereby we declare, that author contribution statement signed by Dr Michael Karimov is in agreement with author willingness.

A scan version of Dr Michael Karimov's author contribution statement has been included to the PhD doctoral thesis due to the delay in obtaining the original versions.

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