«pH-sensitive interpolyelectrolyte complexes based on methacrylated chitosan and heparin for intracellular delivery of therapeutic genetic constructs»

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Summary

This project proposed the development and study of cross-linked pH-sensitive interpolyelectrolyte complexes (IPEC) based on methacrylated chitosan (Cs-GMA) and heparin (Hep-AEMA) as a delivery system for therapeutic nucleic acids. Such systems should protect the encapsulated genes from the action of the aggressive environment of the organism before penetration into the target cells, only after which a rapid release of the therapeutic construct should occur. The chemical part of this project was carried out at the Institute of Chemistry, St. Petersburg State University (St. Petersburg, Russia). In the course of this work, crosslinked interpolyelectrolyte nanoparticles based on methacrylated chitosan and heparin were obtained and characterized. A pH-sensitive ketal linker was synthesized. Next, nanoparticles based on methacrylated chitosan and heparin were modified with a pH-sensitive linker. The resulting system was used as a potential therapeutic nucleic acid delivery system. To this end, the following studies were carried out at the Institut für Technische Chemie, Leibniz Universität, Hannover (Germany):

• Evaluation of cytotoxicity for unmodified systems with a pH-sensitive linker and comparison of the results for their cytotoxicity with those for systems modified with a pH-sensitive linker

• Study of the ability of IPEC based on Cs-GMA and Hep-AEMA, cross-linked with a pH-sensitive linker, to penetrate the cell membrane;

• Investigation of the possibility of using the obtained particles for transfection of small interfering RNA, which blocks the synthesis of green fluorescent protein.

Biological experiments were carried out to understand the prospects of the resulting system for biomedical applications.

1. Introduction

Targeted intracellular delivery of therapeutic genetic constructs, be it pDNA, small interfering RNAs, aptamers, antisense or antigenic oligonucleotides, ribozymes or deoxyribozymes, aptamers remains an important task today, since it allows solving the problem of treating hereditary and non-hereditary polygenic diseases caused by certain genetic breakdowns. Free genetic constructs are incapable of penetration through biological membranes, including through the bilipid layer of the cytoplasmic membrane. In addition, getting into the environment of the body, genetic constructs are degraded by enzymes and removed from the body by absorption by phagocytes and renal filtration. Thus, there is a need to create systems for the targeted delivery of therapeutic genetic constructs that can protect the encapsulated drug from intra- and extracellular degradation, as well as efficiently deliver it to the site of action.

Polymer micro- and nanogels, which are interpolyelectrolyte complexes (IPECs), are promising candidates for carriers of drugs based on therapeutic nucleic acids. The advantages of such carriers are the ability to control the properties of the resulting complexes by changing the reaction conditions, as well as the mildness of the encapsulation process itself. Interactions between oppositely charged polyelectrolytes in an aqueous medium lead to the spontaneous formation of IPEC. The structures of the polymers, the ratios between oppositely charged groups and the concentrations of the polyelectrolytes used influence the formation of the IPEC characteristic. Consequently, by varying the conditions for obtaining IPECs, one can change their physicochemical characteristics and, thus, regulate the loading of the resulting IPECs by therapeutic NC molecules. However, the effectiveness of such systems is limited by the lack of release control, kinetic factors, and the stability of the nanoparticles themselves. The solution to the problem is the creation of covalently cross-linked systems that are sensitive to various

environmental factors, capable of releasing loaded substances under the action of appropriate stimuli.

Nucleic acid delivery systems based on interpolyelectrolyte complexes are being intensively developed at the Interdisciplinary Laboratory of Biomedical Chemistry, Institute of Chemistry, St. Petersburg State University. In particular, a number of nanoparticles based on chitosan and heparin, as well as poly-L-lysine and heparin, have been studied for their size, stability, biocompatibility, and rate of biodegradation. The results of the experiments showed that interpolyelectrolyte systems are promising for obtaining nanocarriers of therapeutic genetic constructs.

In the course of previous work, delivery systems were developed based on interpolyelectrolyte complexes of poly-L-lysine (PLL) with heparin, modified by a photosensitive linker, which is destroyed in the presence of near infrared (NIR) radiation with a wavelength center of 980 nm (Photosensitive poly-l-lysine / heparin interpolyelectrolyte complexes for delivery of genetic drugs Korzhikov-Vlakh V., Katernuk I., Pilipenko I., Lavrentieva A., Guryanov I., Sharoyko V., Manshina A.A., Tennikova T.B. (2020) Polymers, 12 (5), art. no. 1077). The formation of nanogels was primarily based on electrostatic interactions with the formation of nanosized hydrogel particles.

This project proposes the design and study of the properties of pH-sensitive systems. Differences in pH are observed in biological systems at both the cellular and systemic levels. These extracellular and intracellular pH gradients can be used to design drug delivery systems that selectively release the transported drug at a specific site of action.

To create such systems, a strategy of crosslinking interpolyelectrolyte complexes based on methacrylated chitosan and methacrylated heparin using thiol-ene click chemistry was developed and implemented. The use of a pH-sensitive ketal linker as part of a cross-linking agent made it possible to obtain systems that are stable in the extracellular environment but destroy and release genetic constructs inside cells, namely at acidic pH. After the destruction of the linker, heparin acquires mobility and begins to compete with the polynucleotide for electrostatic interactions with the polycation. Heparin, being a strong polyelectrolyte, displaces nucleic acid from the complex. The acidic intracellular pH can be used as a stimulus to trigger the release.

The characteristics of the developed nucleic acid delivery system were determined by dynamic and electrophoretic light scattering (hydrodynamic diameter, size distribution, ζ-potential). The pH-dependent kinetics of the release of encapsulated nucleic acids from the resulting systems was also demonstrated.

During the funding period, the biological properties of the resulting systems were evaluated, such as the cytotoxicity of nanoparticles, as well as their ability to penetrate the cell membrane. In addition, the possibility of using the obtained particles for transfection of small interfering RNA, which blocks the synthesis of green fluorescent protein, was demonstrated. The implementation of this project made it possible to identify the main advantages and disadvantages of the developed systems and understand the further stages of work.

2. Methods

Synthesis method of pH-sensitive ketal linker (2,2 '- (propane-2,2-diylbis (-oxy -)) - bis- (ethan-1-amine))

The synthesis of pH-sensitive ketal was carried out in three stages.

At first, ethanolamine protected at the amino group was obtained, which was then converted into a protected ketal in reaction with 2-methoxypropene. Then, to obtain the target diamine, the protecting group was removed under basic conditions.

The structures of the products of all stages were confirmed by 1H NMR spectroscopy.

Synthesis method of crosslinked pH-sensitive interpolyelectrolyte nanoparticles based on methacrylated chitosan (Cs-GMA) and methacrylated heparin (Hep-AEMA).

The synthesis of cross-linked pH-sensitive interpolyelectrolyte nanoparticles includes 5 stages:

The first is the introduction of methacrylate groups into the pH-sensitive ketal linker molecules. The linker was modified using the Schotten-Baumann reaction. Methacryloyl chloride was used as an acylating agent.
At the second step, chitosan was functionalized by a reaction between glycidyl methacrylate (GMA) and the primary OH groups of chitosan.
Third, the introduction of a double bond into the heparin molecule was carried out by interaction between the activated carboxyl groups of heparin and the amino groups of 2-aminoethyl methacrylate.
At the fourth stage, IPEC was formed due to electrostatic interactions of the polycation and polyanions. The procedure for the formation of nanoparticles consisted of alternately adding a solution of a model oligonucleotide and an aqueous solution of Hep-AEMA dropwise to an aqueous solution of Cs-GMA with constant ultrasonic dispersion. As a result, a colloidal suspension of nanoparticles was formed.
At the last step, a light-induced thiol-ene click reaction was used to form a crosslinked hydrogel; dithiothreitol (DTT) was used as a crosslinking agent. The methacrylate groups of the polymers and the linker, as well as the thiol groups of dithiothreitol, were involved in the creation of alkyl sulfide bonds as a result of the light-induced thiol-ene reaction. The I2959 (Irgacure) was used to initiate the formation of radicals participating in the reaction. The reaction mixture was irradiated with ultraviolet light for 20 min with constant stirring.

Preparation and characterization of nanoparticles.
The procedure for preparing crosslinked interpolyelectrolyte nanoparticles consisted of two stages. At the first stage, a solution of a model oligonucleotide and an aqueous solution of Hep-AEMA was added dropwise to an aqueous solution of Hit-GMA under constant ultrasonic dispersion. As a result, a colloidal suspension of nanoparticles was formed. Then, the light-induced thiol-ene click reaction was used to crosslink the hydrogel; dithiothreitol (DTT) was used as the crosslinker. To impart pH-sensitive properties to IPEC during the formation of nanoparticles, a linker (10 wt%) was added to the aqueous solution of the components before the addition of dithiothreitol.
Crosslinked IPECs were characterized by dynamic and electrophoretic light scattering.

Encapsulation efficiency.
To determine the efficiency of the encapsulation of oligothymidine and the kinetics of its release, we used the method of spectrophotometric detection by the characteristic absorption of nucleic acids under irradiation with 260 nm light in the supernatant.
The determination of the amount of oligonucleotide in the supernatant solution was carried out using specialized software, which automatically calculates the concentration of NA from the data of spectrophotometric analysis.
Further, the encapsulation efficiency (EE) was calculated using the following formula:

$$\mathrm{EE} = \frac{W_t - W_f}{W_t} \times 100\ \% \qquad (1)$$

where W_t is the amount of substance taken for encapsulation; W_f - the amount of unbound substance (the amount of substance in the supernatant).

Investigation of the degradation of a pH-sensitive linker
To study the degradation of the linker, it was immobilized on the surface of polystyrene microparticles (PS MP). Exposed to the aqueous phase, the end of the linker was modified with a fluorescent label. By placing the modified particles in different media, the degradation of the linker was quantified by the intensity of the fluorescence of the label in the supernatant.

Investigation of pH-sensitive release of oligothymidine.

The study of the controlled in vitro release of a model nucleic acid - oligothymidine (oligodT), encapsulated in particles based on Cs-GMA, Hep-AEMA, modified with a pH-sensitive linker, was carried out using a spectrophotometer.
To test the effectiveness of the linker, two types of nanoparticles based on Cs-GMA crosslinked with Hep-AEMA were prepared - with and without a linker. OligodT was encapsulated in both types of particles as a model of therapeutic NA. Suspensions of two types of particles were placed in buffer solutions with different pH: acetate (pH 3.8), acetate (pH 4.4), MES (pH 5.6), PBS (pH 7.4).
Suspensions of nanoparticles in various buffer solutions were incubated at 37 ° C for 30 min with stirring at 250 rpm. After a certain period of time, the nanoparticles were precipitated by centrifugation for 3 minutes at 7000 g, and the entire supernatant solution was collected for analysis of the amount of oligodT released. The control of the amount of released oligodT was carried out by determining its concentration in the supernatant liquid spectrophotometrically by the characteristic absorption of nucleic acids under irradiation with light with a wavelength of 260 nm in the supernatant.

The release of oligodT was calculated using the following formula:

$$\text{Release} = \frac{W_t}{W_0} \times 100\ \% \qquad (2)$$

where W_t is the amount of the released substance from the supernatant; W_0 the amount of encapsulated substance.

Cytotoxicity.
Two cell lines were used in the work, namely A549 (human adenocarcinomic alveolar basal epithelial cells) and BEAS 2B (human bronchial epithelial cells). Two types of samples: cross-linked IPEC without a linker, and cross-linked IPEC modified with a linker. 4 × 103 cells were cultured in 200 µl of DMEM (for A549 cells) or LHC-9 (for BEAS 2B cells) in 96-well plates for 24 h in a humidified medium at 37 ° C and 5% CO2. Then the medium was removed and replaced with a fresh one containing different concentrations of the sample (n = 5). The exposure was carried out in an incubator at 37 ° C and 5% CO2. After exposure (24 h and 72 h), the cells were prepared for analysis. The medium was removed, the wells were filled with CTB reagent (10% stock solution in basic medium) and incubated for 1 and 2 hours. The CTB test is based on the ability of living cells to convert resazurin to the fluorescent product resorufin. Then the viability of the cells was determined using a fluoroscan. The amount of detected fluorescence of resorufin (λexc. = 545 nm, λem. = 590 nm) is proportional to the number of viable cells.

GFP knockdown. Flow Cytometry.
Two types of samples carrying Silencer® GFP siRNAs: IPEC without linker and IPEC modified with linker (10%). 6000 NIH 3T3 cells were cultured in 0.1 ml of DMEM medium in 96-well plates for 24 h in a humidified medium at 37 ° C and 5% CO2. Then the medium was removed and replaced with a fresh one containing the sample (constant siRNA concentration of 3 pmol per well). The exposure was carried out for 72 h in an incubator at 37 ° C and 5% CO2. After the expiration of the exposure time, the cells were prepared for analysis. They were detached and centrifuged at 300 rpm for 5 min. Then the medium was removed, the cells were resuspended in 250 µl of PBS and the fluorescence signals were measured by flow cytometry (BD Accuri C6 with argon ion laser).

Cellular penetration and intracellular localization of the oligonucleotide. Microscope visualization.
20×10^3 HEK 293 cells were seeded in an 8-well chamber for fluorescence microscopy. After 24 hours, the medium was removed and a suspension of IPEC nanoparticles with encapsulated FAM-

labeled oligonucleotide in 200 µl of culture medium was added. As a control, the delivery of the so-called "naked" NA was used. The cells were incubated for 24 hours, then the medium was removed and washed with 0.01 M PBS (pH 7.4). Then the cells were fixed with 4% paraformaldehyde (PFA) solution (200 µl per well) for 10 minutes at room temperature, after which the liquid above the cells was removed. The cells were stained with DAPI to indicate the localization of the nuclei. Thereafter, the cells were visualized under a fluorescence microscope. Images were acquired with 20x objectives.

3. Results and Discussion

3.1. Characterization of samples

Hydrodynamic diameter, size distribution, ζ-potential of the obtained IPEC nanoparticles were investigated by dynamic and electrophoretic light-scattering (Fig. 1).

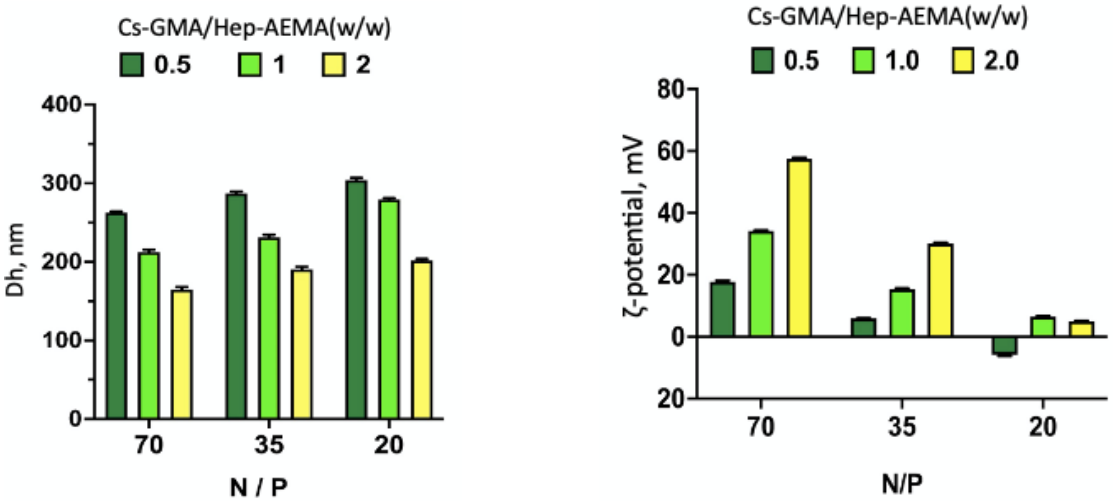

Figure 1. Characteristics of IPEC nanoparticles: Hydrodynamic diameter(left) and ζ-potential (right).
N/P is the ratio of moles of amino groups of the polycation to moles of phosphate groups of NA

3.2. Encapsulation of oligothymidine

Drug loading is important property of a delivery system. The encapsulation efficiency (EE) of oligo-dT was calculated as the ratio of the nucleotide content in the particles to the amount of nucleotide taken to obtain them (see Formula 1). The data for the resulting particles ranged from 93 to 99%. This indicates that the particles are highly efficient in binding the expensive NA-based drug.

3.3. Investigation of the degradation of a pH-sensitive linker

The data obtained show that the degradation of the linker almost does not occur at alkaline pH, but rather intensively proceeds at acidic pH (Fig. 2). At the same time, it was also demonstrated that the destruction of the linker does not occur simultaneously, but develops over time.

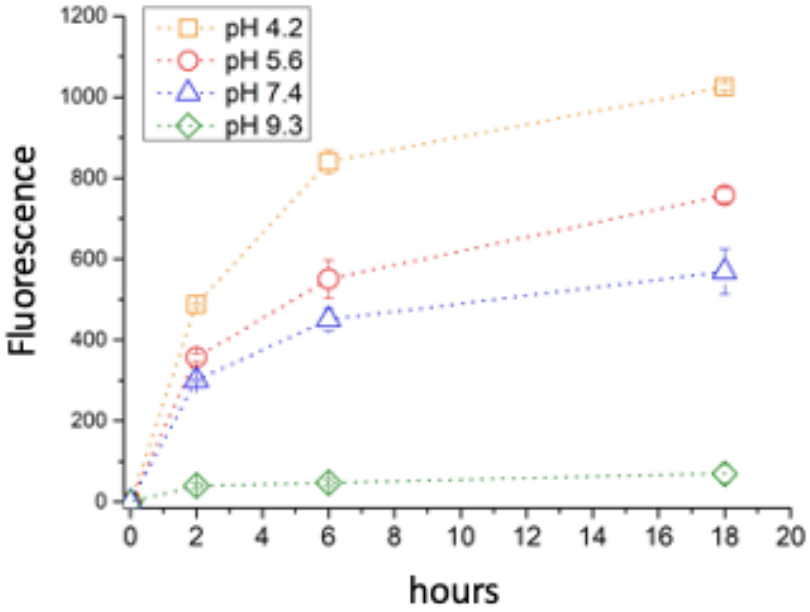

Figure 2. Degradation of the linker on the surface of the PS MP at different pH

3.4 Investigation of pH-sensitive release of oligothymidine.

Analysis of the obtained release profiles (Fig. 3) allows us to conclude that in the absence of a pH-sensitive linker in the composition of the particles, the most pronounced release of oligodT occurs at pH 7.4. This can be explained by the polyelectrolyte effect. Cs-GMA, which is a cationic polymer at pH below 6.5, is deprotonated at neutral pH, as a result of which its interaction with negatively charged oligodT is weakened. Hep-AEMA is an efficient competitive anion with respect to the oligonucleotide. Thus, the most efficient release of oligodT at neutral pH is explained by the deprotonation of Cs-GMA and high competition for the remaining protonated amino groups with Hep-AEMA. Under acidic conditions, with a higher degree of protonation of the Cs-GMA amino groups, better retention of the oligonucleotide in the particles occurs.

The situation changes in the case of nanogels containing a ketal linker. In this case, one can observe (Fig. 3) better retention in a neutral environment, but an increase in the release of encapsulated oligodT in an acidic environment. The observed dependences correlate well with the results of model experiments on the hydrolysis of the ketal crosslinking agent in media with different acidity. Under alkaline conditions, the ketal linker is stable and the nanoparticles remain crosslinked, making it difficult to release the encapsulated substance. In acidic media, the linker degrades, allowing the release of the model oligonucleotide.

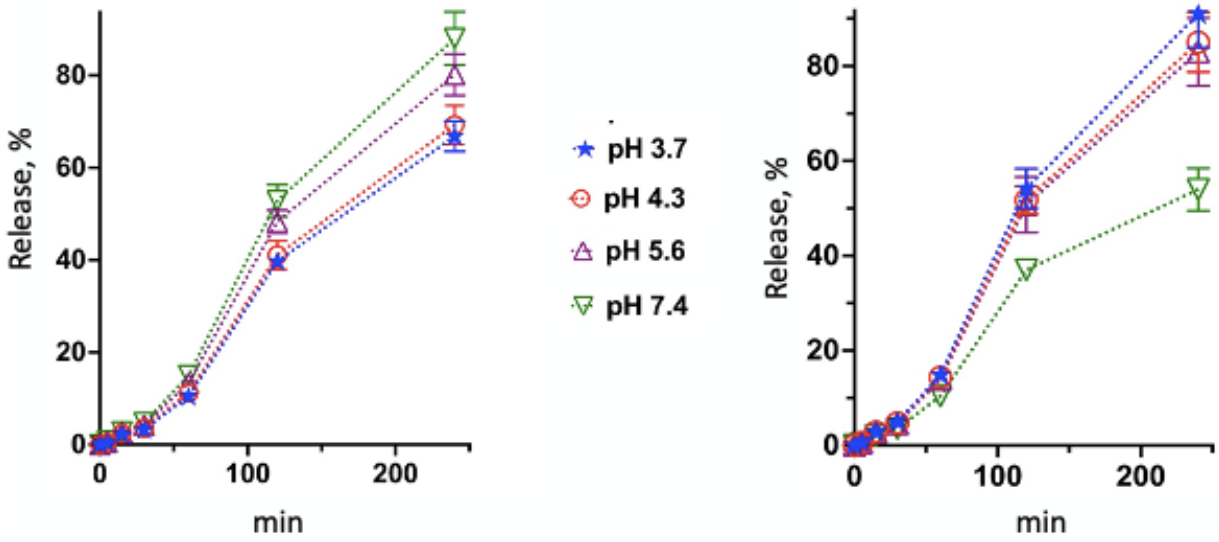

Figure 3. Curves of pH-sensitive release of a model oligonucleotide from IPEC without a linker (left) and from IPEC with a linker (right)

3.5 Cytotoxicity

The cytotoxicity of the in vitro samples was assessed on the A549 and BEAS 2B cell lines. Cell viability was determined at 24 and 72 hours using a CTB assay based on the reduction of resazurin blue to resorufin purple by metabolically active cells.

The data obtained show that both in the case of particles containing a ketal linker and for particles without it in the concentration range from 62.5 to 500 μg/ml, the viability of both types of cells after incubation with particles for 24 and 72 hours was more than 80%, which indicates the non-toxicity of the resulting systems. From this, it can be concluded that particles based on Cs-GMA/Hep-AEMA, containing a pH-sensitive linker in their structure, are promising from the point of view of their further research as delivery systems for genetic constructs.

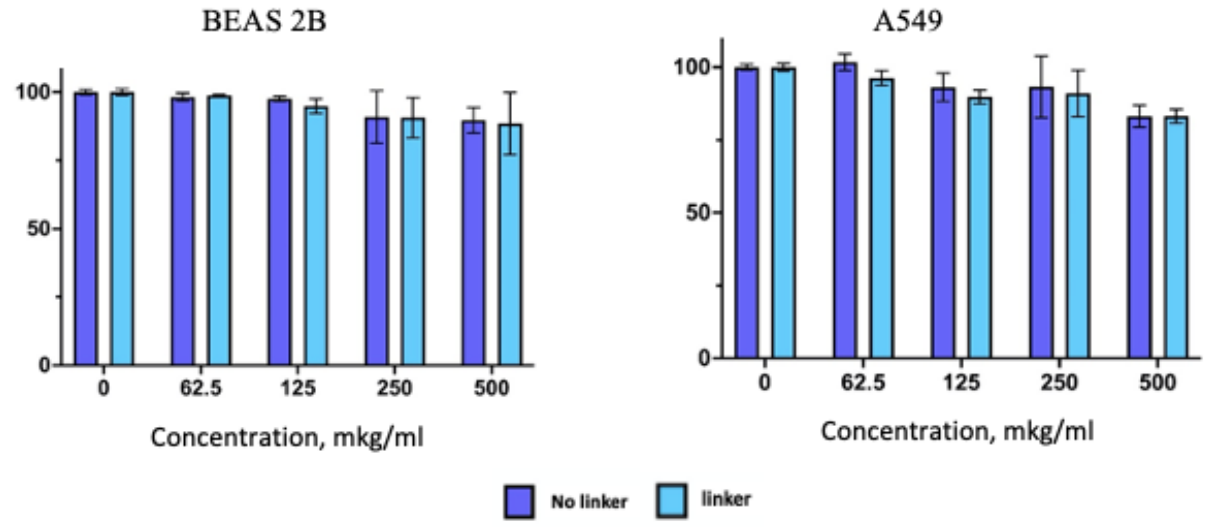

Figure 4. Viability of BEAS 2B(left) and A549(right) cells line incubated with IPEC without a linker, and IPEC modified with a linker for 24 h.

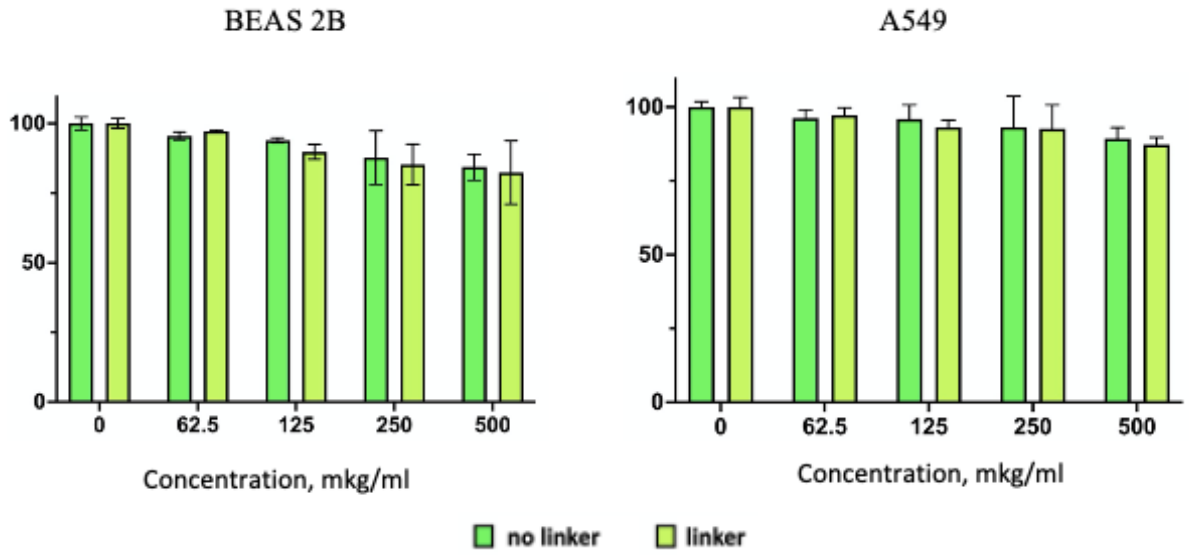

Figure 5. Viability of BEAS 2B(left) and A549(right) cells line incubated with IPEC without a linker, and IPEC modified with a linker for 72 h.

3.6. GFP knockdown. Flow Cytometry.

The efficiency of knockdown of green fluorescent protein GFP by Silencer® GFP siRNA molecules was studied by flow cytometry on mouse fibroblast cells (NIH 3T3) producing green fluorescent protein GFP. Initially, these cells are characterized by intense fluorescence due to the synthesis of the GFP protein, but the introduction of miRNA molecules into them should cause degradation of messenger RNA, which, ultimately, should lead to inhibition of the synthesis of the fluorescent protein. 72 hours after the transfection, the fluorescence intensity of the cells was assessed by flow cytometry.

The obtained histogram shows (Fig. 6) that the inclusion of a linker in the IPEC and an increase in the N / P ratio contribute to an increase in the knockdown efficiency.

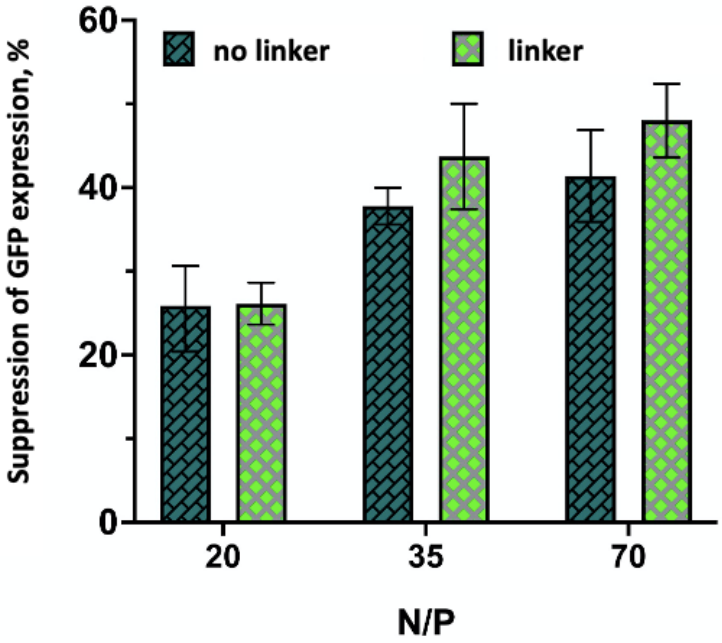

Figure 6. GFP knockdown. Cs-GMA and Hep-AEMA nanoparticles modified and unmodified with a pH-sensitive linker were used as siRNA vectors.

3.5. Cellular penetration and intracellular localization of the oligonucleotide. Microscope visualization.

Qualitative assessment of the efficiency of penetration of nanoparticles through the cell membrane, as well as intracellular localization of the fluorescently labeled FAM oligonucleotide (oligodT-FAM) after delivery into cells using the developed delivery system, mod by fluorescence microscopy.

For this, cells were transfected with model oligodT -FAM using nanoparticles modified with a pH-sensitive linker. The experiment was carried out on the HEK 293 cell line. As a control of the use, the delivery of the so-called "naked" NA.

As you can see from Fig. 7, the oligonucleotide delivered by the designed is distributed evenly within the cytoplasm of the cells. In the case of delivery of "naked" NK, fluorescence of the oligonucleotide in cells was observed, which indicates its inability to independently penetrate into cells.

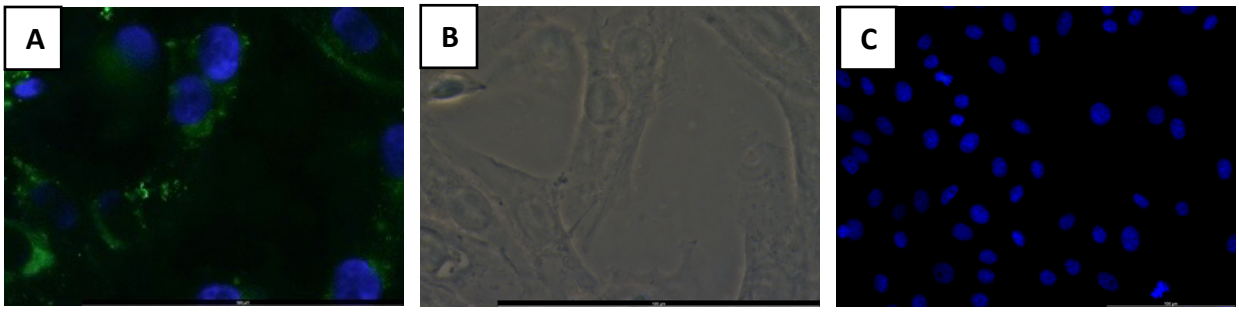

Figure 7. Fluorescent images of HEK 293 cells transfected with FAM-labeled oligonucleotide treated with DAPI dye. (A) Sample in the FITC filter (B) Sample in a bright filter (C) Control in the FITC filter

4. Conclusion

1. It was shown that the synthesized pH-sensitive linker is stable under alkaline conditions and degrades under acidic conditions. The degradation of the linker is prolonged for about a day;

2. A method of nanoparticles based on Cs-GMA and Hep-AEMA, crosslinked with a pH-sensitive linker containing an introduced methacrylate group, has been developed;

3. It was shown that the cross-linking of IPEC based on Cs-GMA and Hep-AEMA with the synthesized pH-sensitive linker leads to a significant increase in the release of the model oligonucleotide upon transition to acidic media. Also, a pH-dependent release kinetics has been established;

4. The absence of cytotoxicity of nanoparticles based on IPEC Cs-GMA and Hep-AEMA, as well as synthesized pH-sensitive-linker, was established;

5. The ability of IPEC based on Cs-GMA and Hep-AEMA, cross-linked with a pH-sensitive linker, to penetrate the cell membrane has been shown;

6. The possibility of using particles for transfection of small interfering RNA, which blocks the synthesis of green fluorescent protein, has been demonstrated.

5. Personal Impressions

This was my first visit to the Institut für Technische Chemie at Leibniz University. I would like to thank G-RISC for making this trip possible. I want to express my gratitude to Professor Thomas Scheper, who supported our project and invited me to his laboratory at such a difficult time. In addition, I am very grateful to Dr. Antonina Lavrentieva, who helped me with all the experiments during my research stay. It is also worth noting the excellent organization and equipment of ITC laboratories.