Indole, Phenyl, and Phenol Groups: The Role of the Comonomer on Gene Delivery in Guanidinium Containing Methacrylamide Terpolymers

Franz J. Hack, Ceren Cokca, Sebastian Städter, Juliana Hülsmann, Kalina Peneva, and Dagmar Fischer**

This report highlights the importance of hydrophobic groups mimicking the side chains of aromatic amino acids, which are tryptophan, phenylalanine, and tyrosine, in guanidinium bearing poly(methacrylamide)s for the design of non-viral gene delivery agents. Guanidinium containing methacrylamide terpolymers are prepared by aqueous reversible addition–fragmentation chain transfer (aRAFT) polymerization with different hydrophobic monomers, N-(2-indolethyl)methacrylamide (IEMA), N-phenethylmethacrylamide (PhEMA), or N-(4-hydroxyphenethyl)methacrylamide (PhOHEMA) by aiming similar contents. The well-defined polymers are obtained with a molar mass of ≈15 000 g mol^{-1} and ≈1.1 dispersity. All terpolymers demonstrate almost comparable in vitro cell viability and hemocompatibility profiles independent of the type of side chain. Although they all form positively charged, enzymatically stable polyplexes with plasmid DNA smaller than 200 nm, the incorporation of the IEMA monomer improve these parameters by demonstrating a higher DNA binding affinity and forming nanoassemblies of about 100 nm. These physicochemical characteristics are correlated with increased transfection rates in CHO-K1 cells dependent on the type of the monomer and the nitrogen to phosphate (N/P) ratio of the polyplexes, as determined by luciferase reporter gene assays.

The guanidinium group is composed of three amino groups bound to a central carbon atom and it is a planar Y-shaped quasi aromatic structure with a pKa value of 13.6 in water, which is fully protonated in biological media.[1,2] It acts as a hydrogen bond donor and strongly interacts with water molecules in the plane of guanidinium ion, while its π-faces remain hydrophobic as suggested by neutron diffraction and molecular dynamics simulations.[3,4] Recently, the first experimental observation demonstrated that guanidinium ions form stable dimeric complexes in aqueous solution.[5] These distinctive properties are sometimes referred to as "arginine" magic, in particular with regard to polyarginines or arginine-rich peptides such as HIV-1 transactivator of transcription (TAT) that can cross the cell membrane and have generated a lot of interest in the field of drug delivery.[1–6] The strategy of incorporating guanidinium moieties to favorably influence the properties of a material is not limited to the field of peptide-mediated intracellular delivery, but it has also been exploited for the preparation of polymeric carriers for cell transfection, antimicrobial activity, and cell penetration.[7–11] Many recent reports have shown that the addition of hydrophobic counterions or the incorporation of hydrophobic groups into peptides can improve membrane translocation, cellular uptake, polyplex stability, and mRNA or siRNA delivery.[12,13] The cation-π interactions between the side chains of arginine and tryptophan, tyrosine, or phenylalanine play an important role in these processes.[14,15] Considering the hydrophobic nature of the π-faces in guanidinium ions, such

F. J. Hack, J. Hülsmann, Prof. D. Fischer
Pharmaceutical Technology and Biopharmacy
Institute of Pharmacy
Friedrich Schiller University Jena
Lessingstrasse 8, Jena D-07743, Germany
E-mail: dagmar.fischer@uni-jena.de

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/marc.202000580.

C. Cokca, S. Städter, Prof. K. Peneva
Institute of Organic Chemistry and Macromolecular Chemistry (IOMC)
Friedrich Schiller University Jena
Lessingstrasse 8, Jena D-07743, Germany
E-mail: kalina.peneva@uni-jena.de

Prof. K. Peneva, Prof. D. Fischer
Jena Center for Soft Matter (JCSM)
Friedrich Schiller University Jena
Philosophenweg 7, Jena D-07743, Germany
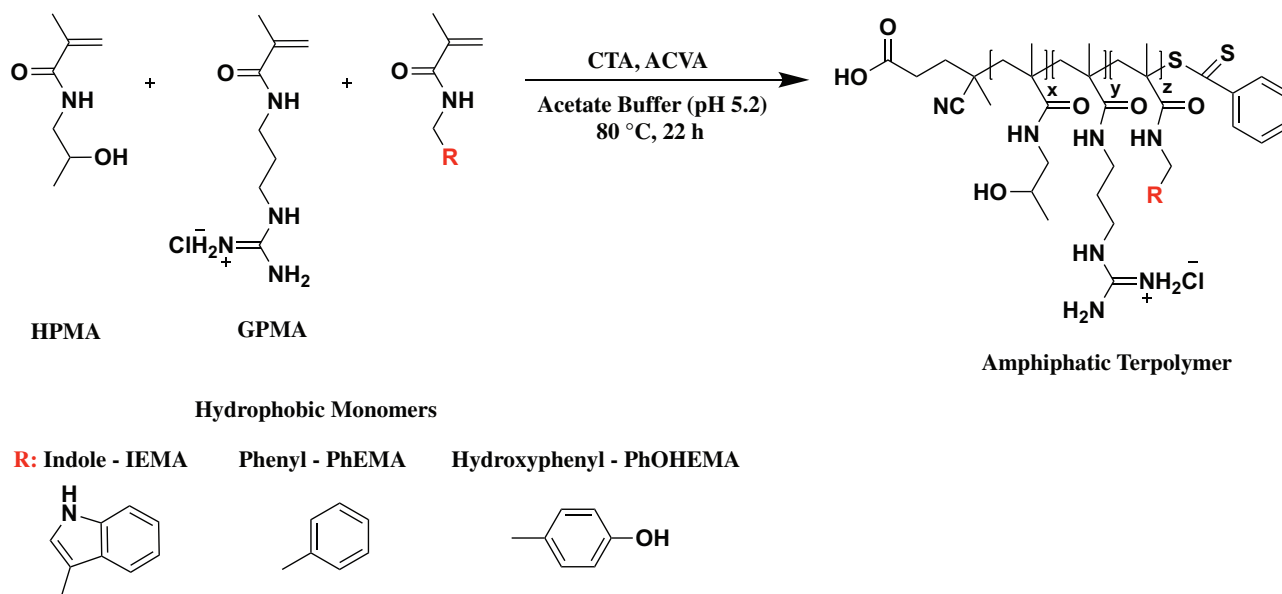
Scheme 1. *a*RAFT polymerization of guanidinium containing terpolymers with different hydrophobic monomers.

stacking against hydrophobic side chain in a protein could reduce the entropic cost of hydrophobic hydration. Jobin et al. showed that not only the number but also the nature and positioning of the hydrophobic residues are important for membrane translocation in arginine-rich cell-penetrating peptides.[16] We recently reported that poly(methacrylamide)s with the content of guanidinium bearing monomers of 60% or higher can efficiently bind plasmid DNA (pDNA).[17] Moreover, we revealed that the incorporation of indole comonomer mimicking the side chain of the amino acid tryptophan, leads to a 200-fold increase of the transgene expression in comparison to a copolymer with comparable guanidinium content.[18] To understand to what extent the origin of the hydrophobic residue influences the internalization, complexation of pDNA, and the ability of the polymers to act as gene carriers, we designed terpolymers that contain side chains mimicking hydrophobic residues found in the side chains of amino acids.
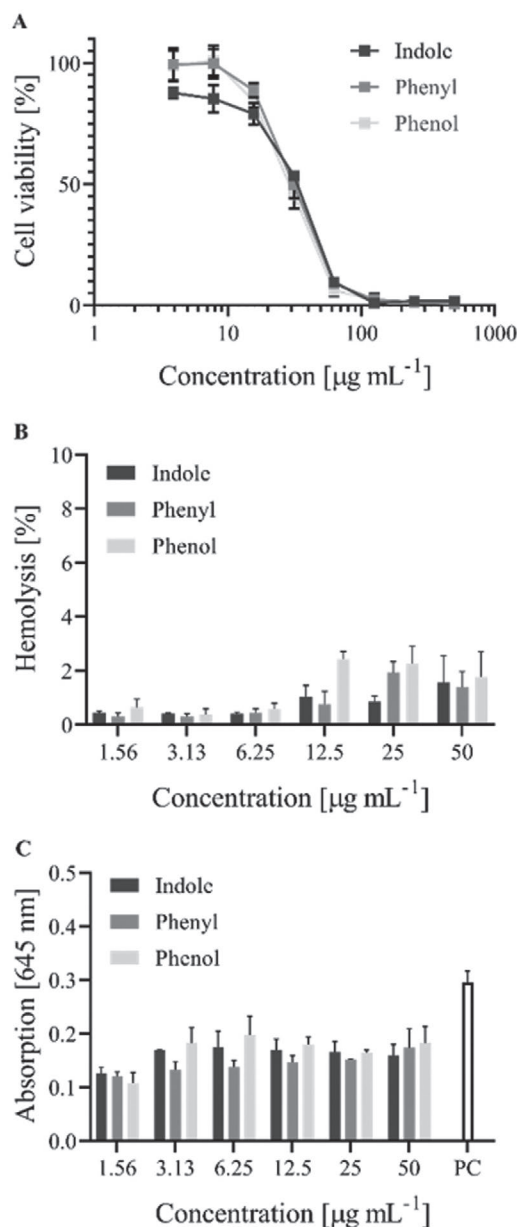
Well-defined guanidinium containing methacrylamide terpolymers were prepared with different hydrophobic monomers, *N*-(2-indolethyl)methacrylamide (IEMA), *N*-Phenethylmethacrylamide (PhEMA) or *N*-(4-hydroxyphenethyl)methacrylamide (PhOHEMA), through aqueous reversible addition–fragmentation chain transfer (*a*RAFT) polymerization while keeping the hydrophobic monomer contents similar (≈4 mol%)

in each polymer structure (**Scheme 1**). The synthesis of monomers, polymers, and their physicochemical characterizations are described in detail in the supporting information. Briefly, predetermined amounts of HPMA and GPMA were placed in a 25 mL Schlenk flask and dissolved in degassed aqueous acetate buffer. The hydrophobic monomer was dissolved in DMF separately and slowly added to the reaction mixture followed by slow addition of 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CTA) and 4,4'-azobis (4-cyano-pentanoic acid) (ACVA) solutions in DMF. The reaction was carried out at ≈80 °C for 22 h under argon. The resulting polymer was purified by dialysis in distilled water (dH$_2$O, pH 4) at 4 °C and dried by lyophilization. The monomer composition of the polymeric product was measured by ^{1}H NMR spectroscopy while the molar mass and dispersity index were determined by size exclusion chromatography (SEC$_{DMAc}$). The data are summarized in **Table 1**.

The safe and biocompatible application of the prepared terpolymers for gene delivery was investigated using the luminescence-based CellTiter-Glo assay on L-929 mouse fibroblasts as a standard cell line for toxicity testing, in concentrations ranging from 3.9 to 500 µg mL^{-1} over 24 h (**Figure 1**A).[19] Untreated cells were set as 100% negative control, whereas 0.02% thiomersal solution acted as the positive control reducing the

Table 1. Monomer contents, molar masses, and dispersity indices of the terpolymer samples.

|	Poly(HPMA-co-GPMA-co-IEMA)	Poly(HPMA-co-GPMA-co-PhEMA)	Poly(HPMA-co-GPMA-co-PhOHEMA)
$M_{n\ Experimental}$ [g mol^{-1}]	23 000	15 000	15 000
Đ	1.07	1.14	1.07
HPMA [mol%]	21	12	12
GPMA [mol%]	75	84	85
IEMA [mol%]	4	–	–
PhEMA [mol%]	–	4	–
PhOHEMA [mol%]	–	–	3

Figure 1. A) Concentration-dependent in vitro cell viability assay of the terpolymers (named according to their side chains) in L-929 mouse fibroblasts after 24 h using the CellTiter-Glo assay ($n = 8$, mean ± SD). B) Dependency of hemolysis after 1 h incubation and C) sheep erythrocyte aggregation after 2 h incubation induced by the terpolymers on polymer concentration compared to 25 000 BPEI (15 µg mL^{-1}) (PC) ($n = 6$, mean ± SD).

metabolic activity of the cells to <1%. The three terpolymers revealed semilogarithmic curves with sigmoidal profiles and almost comparable IC$_{50}$ values of 28.1, 33.0, and 34.7 µg mL^{-1} for poly(HPMA-co-GPMA-co-IEMA), poly(HPMA-co-GPMA-co-PhEMA), and poly(HPMA-co-GPMA-co-PhOHEMA) terpolymers, respectively. The comparison of these data suggested no relevant influence of the type of the hydrophobic side chains.

Since the prepared terpolymers are intended for systemic administration, their compatibility with erythrocytes as the most abundant particular component in the bloodstream was investigated. After 1 h, incubation of sheep red blood cells in the presence of terpolymers in varying concentrations of up to 50 µg mL^{-1}, the spectrophotometric measurements of the hemoglobin release, a common marker for the damage of the erythrocyte membranes due to electrostatic interactions with the cationic polymers, revealed that mean values of hemolysis did not exceed 2% (Figure 1B). This can be categorized as non-hemolytic according to the ASTM F756-08 standard and these results reflect the data previously published for indole derivatives.[18,19] The data were comparable to the HEPES buffered glucose (HBG) solution pH 7.4 which was set as a negative control (0.1%), whereas a Triton X-100 solution was used as a positive control (100%). Moreover, to avoid systemic complications like thrombosis and embolism due to a polycation induced aggregation of erythrocytes, the potential of the terpolymers to aggregate sheep red blood cells was investigated qualitatively by light microscopy including a classification in three stages, as well as quantitatively by UV–vis spectroscopy. As illustrated by light microscopic evaluation (Figure S12, Supporting Information), the negative control (HBG pH 7.4) did not cause any cell cluster formation (stage 1), whereas the positive control (25 000 g mol^{-1} branched poly(ethylene imine), BPEI, 15 µg mL^{-1}) induced the formation of large aggregates (stage 3).[20,21] After a 2 h treatment, none of the terpolymers induced relevant red blood cell aggregating effects as indicated by a stage 1 classification in the light microscopy even at the highest tested concentration of 50 µg mL^{-1}. This quantitative measurement is based on the principle that the diffuse light scattered at the cell membrane of free erythrocytes is reduced with increasing red blood cell aggregation, which leads to a decrease in the UV–vis absorption. Absorption values comparable to the negative control (absorption values of 0.15) were obtained for the terpolymers (Figure 1C). In all cyto- and hemocompatibility tests, the application of polymer controls excluded nonspecific polymer related effects.

To determine the optimal composition of the terpolymer with polyanionic pDNA in the polyplexes, the binding efficacy of the polymers driven by electrostatic interactions was determined by fluorescent dye exclusion measurement using the AccuBlue Quantification kit (**Figure 2**A) as well as by horizontal agarose gel electrophoresis (Figure S13).[22] Naked pDNA demonstrated the highest fluorescence signals serving as 100% control representing the full accessibility of the pDNA for the intercalating dye, whereas polyplexes with linear PEI (LPEI; 2500 g mol^{-1}) at N/P ratio 20 acted as positive control with high binding affinities. The terpolymers in the absence of pDNA revealed no signals excluding non-specific polymer related effects. Although poly(HPMA-co-GPMA-co-IEMA) terpolymer was able to bind pDNA completely already at N/P ratio 1, the other terpolymers demonstrated a full dye exclusion at N/P ratio 2 with an efficacy comparable to the LPEI control. These findings correlate well with the results obtained from the gel electrophoresis experiments. The characteristic band pattern of pDNA with the most prominent open circular and supercoiled form disappeared at N/P ratio 1 for poly(HPMA-co-GPMA-co-IEMA) whereas at N/P ratio 2 for poly(HPMA-co-GPMA-co-PhEMA) and poly(HPMA-co-GPMA-co-PhOHEMA) terpolymers as a result of a completely inhibited movement in the electric
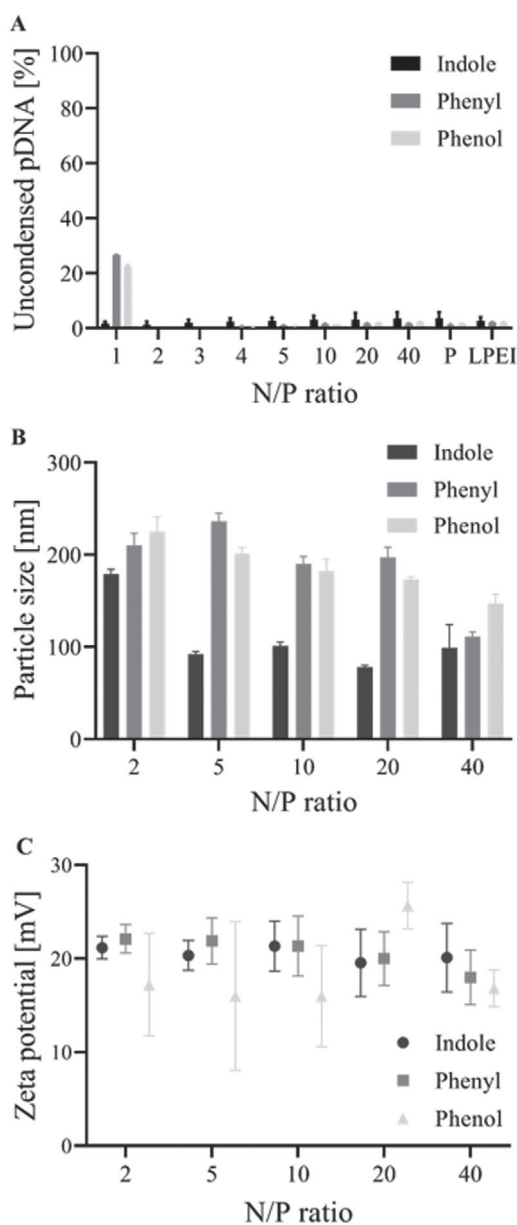
Figure 2. Physicochemical characterization of the polyplexes: A) Binding capacity [%] of the terpolymers (named according to their side chains) for pDNA depending on the N/P ratio determined by the AccuBlue Quantification in comparison to linear PEI (LPEI; 2500 g mol^{-1}, N/P 20) and free polymers (P) ($n = 8$, mean ± SD). B) Particle size measurements of the pDNA/terpolymer polyplexes using nanoparticle tracking analysis (mean ± SD measured in triplicates). C) Zeta potentials of polyplexes formed at different N/P ratios in water (WFI) and measured by laser Doppler anemometry in triplicates (mean ± SD).

field either by the increase in size and/or masking the anionic charge of pDNA due to the formation of polyplexes. The increase in N/P ratio is known to be correlated with more effective binding of genetic materials through cationic polymers.[23] Additionally, the better binding performance of indole containing terpolymer is in good agreement with the reported data that show that indole modified low molecular
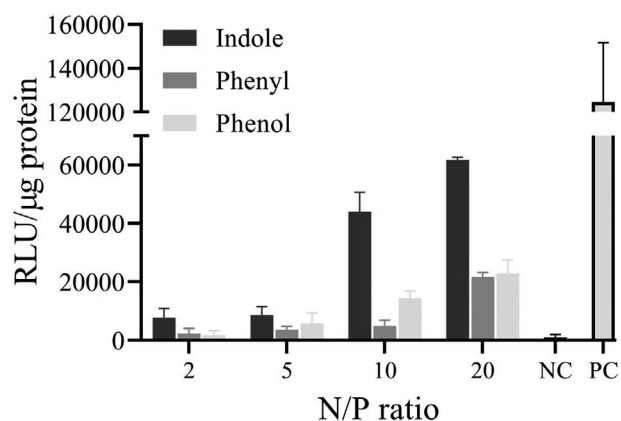
weight PEI has higher pDNA binding ability compared to its phenyl containing counterpart.[24]

These binding characteristics of the terpolymers were also reflected in the reduction of the polyplex size as a function of N/P ratio in the range 2 to 40, as measured by nanoparticle tracking analysis (Figure 2B). The three terpolymers were able to form stable and nano-scaled polyplexes. Under comparable conditions, the indole containing terpolymer was characterized by the smallest polyplexes with mean particle sizes below 100 nm already at N/P 5, whereas the phenyl and phenol bearing terpolymers demonstrated a decrease in their sizes at higher N/P ratios. Conclusively, all terpolymers formed nanoassemblies in suitable size ranges below 200 nm for an efficient cell uptake and an intracellular processing of nanomaterials.[25,26] Up to N/P ratio 10, the phenol bearing terpolymer was characterized by the lowest and highly variable cationic surface charges determined via zeta potential (Figure 2C) in contrast to the indole and phenyl containing terpolymers with zeta potentials around +20 mV independent of the N/P ratio, indicating an efficient electrostatic polyplex repulsion and inhibition in particle agglomeration.

The compact binding of pDNA by the terpolymers is a prerequisite to protect the polyplexes against in vivo enzymatic hydrolysis by the formation of an electrostatic and physical barrier.[27] Using agarose gel electrophoresis, the stability of pDNA complexed with the prepared terpolymers at N/P ratios from 2 to 40 was confirmed after incubation with DNase I and displacement by heparin (Figure S14, Supporting Information). Even though untreated pDNA and pDNA treated without enzyme remained intact excluding unspecific degradation or destruction by the treatment per se, enzyme-treated free pDNA was completely degraded as shown by the disappearance of the characteristic bands. All terpolymers displayed protection of the pDNA against enzymatic hydrolysis already at N/P ratio 2 where a complete binding was shown in the assays described above. At N/P ratios 20 and 40, only a partial pDNA release from the complexes was observed as demonstrated by the fluorescence in the gel slot which is typical for polymers with a very high binding affinity. Changes in the topology of the pDNA are common for such procedures as reported in many other studies.[27–29]

The influence of different side chains on transfection efficacy was determined by a luminescence-based reaction in eukaryotic CHO-K1 cells in the presence of serum using pGL3 pDNA/polyplexes with N/P ratios from 2 to 20 (**Figure 3**). No specific reaction was detected for free pDNA and the solvent 0.9% NaCl which were used as controls. The transfection efficacy increased with higher N/P ratios with poly(HPMA-co-GPMA-co-IEMA) > poly(HPMA-co-GPMA-co-PhOHEMA) > poly(HPMA-co-GPMA-co-PhEMA). By far the highest transfection potential was observed for the indole containing terpolymer corresponding to the highest binding activity and the smallest polyplex size as described above. For comparison, the positive control consisting of 2500 g mol^{-1} linear PEI and pDNA at N/P ratio 20 was used as gold standard for gene transfection[27] and reached a 2-fold higher transfection efficiency compared to the best performing indole-based terpolymer under the chosen conditions. The improved performance of the indole containing terpolymers is in a good agreement with the data reported

**ADVANCED
SCIENCE NEWS**
www.advancedsciencenews.com

**Macromolecular
Rapid Communications**
www.mrc-journal.de

Figure 3. In vitro luciferase reporter gene expression of pGL3 DNA/terpolymer polyplexes at different N/P ratios in CHO-K1 cells compared to the positive control (PC) linear PEI (LPEI; 2500 g mol⁻¹, N/P 20). Experiments are performed in quadruplicates with free pDNA serving as a negative control (NC) (mean ± SD).

previously for tryptophane containing cell-penetrating peptides or indole-functionalized PEI polymers.[30,31] The better transfection induced by the phenol bearing terpolymer compared to its phenyl counterpart can be related to the additional polar interactions (like the formation of counterions) or to the formation of OH-related hydrogen bonds with pDNA.[31] More efficient release from the endosomal/lysosomal compartment as described by Chang et al.[32] could be at play, as well. Further investigation is necessary to support either of these hypotheses.

In summary, well-defined guanidinium containing methacrylamide terpolymers were synthesized by aRAFT polymerization with comparable contents (≈4 mol%) of indole, phenyl, and phenol bearing methacrylamide monomers mimicking the hydrophobic residues of tryptophan, phenylalanine, and tyrosine and used to evaluate to what extent the identity of these hydrophobic groups can influence their pDNA binding, transfection, and polyplex toxicity. For all terpolymers, a marked cyto- and hemocompatibility was demonstrated even at high concentrations. Since all terpolymers gave comparable results in cell viability and hemotoxicity tests, with low content of hydrophobic groups compared to the content of guanidinium groups, the cationic residues appear to have a more pronounced effect on the toxicity than the identity of hydrophobic side chains. However, the transfection data highlighted the importance of the type of the side chain for effective gene transfer with indole bearing terpolymers, which performed the best. The physicochemical characteristics of the polyplexes revealed the formation of polyplexes with important small particle size, high positive surface charge, and the ability of efficient and protective pDNA binding. Conclusively, the careful selection of the type of side chains mimicking hydrophobic residues found in the side chains of tryptophan, phenylalanine, and tyrosine revealed a strong potential for the design of non-viral gene delivery agents. Future studies will focus on more detailed investigations of effects that different residues have on the interactions with cell membranes, endosomal/lysosomal pDNA release, and the intracellular behavior of the polyplexes.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

[1] M. Vazdar, J. Heyda, P. E. Mason, G. Tesei, C. Allolio, M. Lund, P. Jungwirth, *Acc. Chem. Res.* **2018**, *51*, 1455.

[2] B. Xu, M. I. Jacobs, O. Kostko, M. Ahmed, *ChemPhysChem* **2017**, *18*, 1503.

[3] J. B. Rothbard, T. C. Jessop, R. S. Lewis, B. A. Murray, P. A. Wender, *J. Am. Chem. Soc.* **2004**, *126*, 9506.

[4] P. E. Mason, G. W. Neilson, C. E. Dempsey, A. C. Barnes, J. M. Cruickshank, *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 4557.

[5] M. J. Hebert, D. H. Russell, *J. Phys. Chem. Lett.* **2019**, *10*, 1349.

[6] M. Green, P. M. Loewenstein, *Cell* **1988**, *55*, 1179.

[7] K. Koschek, M. Dathe, J. Rademann, *ChemBioChem* **2013**, *14*, 1982.

[8] L. Martin, R. Peltier, A. Kuroki, J. S. Town, S. Perrier, *Biomacromolecules* **2018**, *19*, 3190.

[9] B. M. deRonde, G. N. Tew, *Biopolymers* **2015**, *104*, 265.

[10] J. M. Sarapas, C. M. Backlund, B. M. deRonde, L. M. Minter, G. N. Tew, *Chemistry* **2017**, *23*, 6858.

[11] Z. Tan, Y. Jiang, W. Zhang, L. Karls, T. P. Lodge, T. M. Reineke, *J. Am. Chem. Soc.* **2019**, *141*, 15804.

[12] B. M. deRonde, N. D. Posey, R. Otter, L. M. Caffrey, L. M. Minter, G. N. Tew, *Biomacromolecules* **2016**, *17*, 1969.

[13] C. M. Backlund, L. Parhamifar, L. Minter, G. N. Tew, T. L. Andresen, *Mol. Pharmaceutics* **2019**, *16*, 2462.

[14] H. J. Kim, S. Ogura, T. Otabe, R. Kamegawa, M. Sato, K. Kataoka, K. Miyata, *ACS Cent. Sci.* **2019**, *5*, 1866.

[15] K. Kempe, A. Vollrath, H. W. Schaefer, T. G. Poehlmann, C. Biskup, R. Hoogenboom, S. Hornig, U. S. Schubert, *Macromol. Rapid Commun.* **2010**, *31*, 1869.

[16] M.-L. Jobin, M. Blanchet, S. Henry, S. Chaignepain, C. Manigand, S. Castano, S. Lecomte, F. Burlina, S. Sagan, I. D. Alves, *Biochim. Biophys. Acta, Biomembr.* **2015**, *1848*, 593.

[17] I. Tabujew, C. Cokca, L. Zartner, U. S. Schubert, I. Nischang, D. Fischer, K. Peneva, *J. Mater. Chem. B* **2019**, *7*, 5920.

[18] C. Cokca, L. Zartner, I. Tabujew, D. Fischer, K. Peneva, *Macromol. Rapid Commun.* **2020**, *41*, 1900668.

[19] *ASTM F756, Annual Book of ASTM Standards*, Vol. 13. 01, ASTM, Philadelphia **2013**.

[20] M. Bauer, C. Lautenschlaeger, K. Kempe, L. Tauhardt, U. S. Schubert, D. Fischer, *Macromol. Biosci.* **2012**, *12*, 986.

[21] DIN EN ISO 10993-5:2009-10, Biological evaluation of medical devices - Part5: Test for in vitro cytotoxicity, *European Standard EN ISO 10993–5, Brussels* **2009**.

[22] M. Zink, K. Hotzel, U. S. Schubert, T. Heinze, D. Fischer, *Macromol. Biosci.* **2019**, *19*, 1900085.

[23] D. Fischer, T. Bieber, Y. Li, H.-P. Elsässer, T. Kissel, *Pharm. Res.* **1999**, *16*, 1273.

[24] Q.-Y. Yu, Y.-R. Zhan, J. Zhang, C.-R. Luan, B. Wang, X.-Q. Yu, *Polymers* **2017**, *9*, 362.

[25] Z. Chen, W. Huang, N. Zheng, Y. Bai, P. Chem, *Polym. Chem.* **2020**, *11*, 664.

[26] R. Bhattacharya, B. Osburg, D. Fischer, U. Bickel, *Pharm. Res.* **2008**, *25*, 605.

[27] S. Ochrimenko, A. Vollrath, L. Tauhardt, K. Kempe, S. Schubert, U. S. Schubert, D. Fischer, *Carbohydr. Polym.* **2014**, *113*, 597.

[28] D. Fischer, H. Dautzenberg, K. Kunath, T. Kissel, *Int. J. Pharm.* **2004**, *280*, 253.

[29] C. L. Gebhart, S. Sriadibhatla, S. Vinogradov, P. Lemieux, V. Alakhov, A. V. Kabanov, *Bioconjugate Chem.* **2002**, *13*, 937.

[30] Z. Kang, G. Ding, Z. Meng, Q. Meng, *Peptides* **2019**, *121*, 170149.

[31] K. Kono, H. Akiyama, T. Takahashi, T. Takagishi, A. Harada, *Bioconjugate Chem.* **2005**, *16*, 208.

[32] H. Chang, J. Zhang, H. Wang, J. Lv, Y. Cheng, *Biomacromolecules* **2017**, *18*, 2371.