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Incorporation of Indole Significantly Improves the Transfection Efficiency of Guanidinium-Containing Poly(Methacrylamide)s

Ceren Cokca, Leon Zartner, Ilja Tabujew, Dagmar Fischer, and Kalina Peneva**

A highly efficient transfection agent is reported that is based on terpolymer consisting of *N*-(2-hydroxypropyl)methacrylamide (HPMA), *N*-(3-guanidinopropyl) methacrylamide (GPMA), and *N*-(2-indolethyl)methacrylamide monomers (IEMA) by analogy to the amphipathic cell-penetrating peptides containing tryptophan and arginine residues. The incorporation of the indole-bearing monomer leads to successful plasmid DNA condensation even at a nitrogen-to-phosphate (N/P) ratio of 1. The hydrodynamic diameter of polyplexes is determined to be below 200 nm for all N/P ratios. The transfection studies demonstrate a 200-fold increase of the transgene expression in comparison to P(HPMA-co-GPMA) with the same guanidinium content. This study reveals the strong potential of the indole group as a side-chain pendant group that can increase the cellular uptake of polymers and the transfection efficiency of the respective polyplexes.

Viruses often use the interplay of charge and polarity to efficiently cross biological barriers like the cell membrane.[1] The same principle has been widely applied to enhance the internalization of cell-penetrating peptides (CPPs), for example, by the incorporation of hydrophobic residues such as phenylalanine or tyrosine.[2,3] Ever since the first report of gene therapy by Merril et al. in 1971, one of the pressing challenges we still face is the invention of an efficient non-viral delivery vector that can transport genetic materials inside living cells.[4] The design of such synthetic vectors is often inspired by nature and it is frequently based on cationic macromolecules with well-defined molecular weight, dispersity, and functionality.[5,6] However, positive charge is only one important fragment that is needed to ensure efficient binding to the negatively charged nucleic acids.[7,8] The second crucial step that determines the efficiency of many gene delivery agents is the release of the intact genetic material inside the cell. At this stage of intracellular transport, a rather subtle balance between charge and hydrophobicity has proven to be vitally important. For example, the translocation of CPP such as Pep-1 and MPG could be improved by the incorporation of hydrophobic residues.[9] Similar design principle was applied to polymeric siRNA carriers where the incorporation of segregated hydrophobic monomers demonstrated that a hydrophobic block is required to reach an improved internalization.[10,11]

Recently, we demonstrated that guanidinium-containing methacrylamides with guanidinium monomer content equal to or higher than 60% can efficiently bind plasmid DNA (pDNA), regardless of whether gradient or statistical copolymers were prepared.[12] However, a high percentage of guanidinium monomers in the copolymers shows lower transfection efficiency compared to linear poly(ethylene imine) polymers with similar molar mass due to delayed release and poor translocation through the endosomal membrane. To address this challenge, we incorporated *N*-(2-indolethyl)methacrylamide (IEMA) and copolymerized it with methacrylamide monomers. Inspired by the amino acid tryptophan, we report the first terpolymer that contains *N*-(2-hydroxypropyl)methacrylamide (HPMA), *N*-(3-guanidinopropyl)methacrylamide (GPMA), and their indole counterpart IEMA. Transfection studies using pDNA demonstrated a 200-fold increase of the transgene expression in comparison to a P(HPMA-co-GPMA) copolymer with comparable guanidinium content. Probing the effect of the insertion of indole monomers could pave the way to better design of non-viral delivery agents.

The synthesis of HPMA, GPMA, and IEMA monomers was performed in a one-step process (Schemes S1, S2, and S3, Supporting Information). To be able to determine the influence of the indole group over the binding and transfection of pDNA, we selected a high-performing P(HPMA-co-GPMA) copolymer from our previous study and conducted a direct comparison with the terpolymer counterpart. Their most important physicochemical

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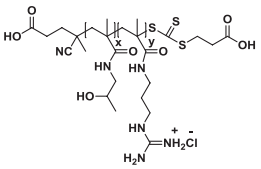
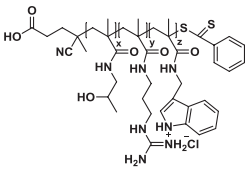
Table 1. Monomer contents, molar masses, and dispersity indices of copolymer and terpolymer samples.

Polymer samples	Experimental [mol%]			$Mn_{experimental}$ [g mol^{-1}]	$Đ$
	HPMA	IEMA	GPMA		
 P(HPMA-co-GPMA)[a]	47	–	53	18 000	1.09
 P(HPMA-co-GPMA-co-IEMA)[b]	26	16	58	19 000	1.13

characteristics are summarized in **Table 1**. The polymers were synthesized via aqueous reversible addition–fragmentation chain transfer polymerization with some modification of previously reported polymerization procedures (Scheme S4, Supporting Information).[12–14] Two different chain transfer agents were utilized during the synthesis of the copolymer and the terpolymer due to the difference in solubility and reactivity of the indole monomer. The guanidinium content was kept around 60 mol% for both copolymers, whereas HPMA monomer, used as spacer unit, was lower in content due to the IEMA monomer inclusion (16 mol%) in the terpolymer structure. Our attempts to increase the IEMA content higher than 20% led to coagulation and precipitation during the polymerization reaction, which in turn increased the dispersity of the resulting terpolymers. All polymer samples showed molar masses ≈20 000 g mol^{-1} as well as a low dispersity index ($Đ$) < 1.2. P(HPMA-co-GPMA) and P(HPMA-co-GPMA-co-IEMA) were compared with respect to their biocompatibility, toxicity, and performance as gene carriers side by side, to understand to what extent the addition of the indole comonomer can improve the transfection efficiency of guanidinium-containing polymers.

Cationic polymers are known to cause toxic effects on biological membranes in different ways, for example, nanoscale pore formation, loss of membrane integrity, impairment of the metabolic activity, or change in the phospholipid composition of the lipid bilayer.[15] To assess the cell viability, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed on L-929 mouse fibroblasts compared to the negative control (untreated cells, 100%) and the positive control (0.2% thiomersal, <3% viability) (**Figure 1**A). Increased toxicity was observed from the terpolymer at concentrations

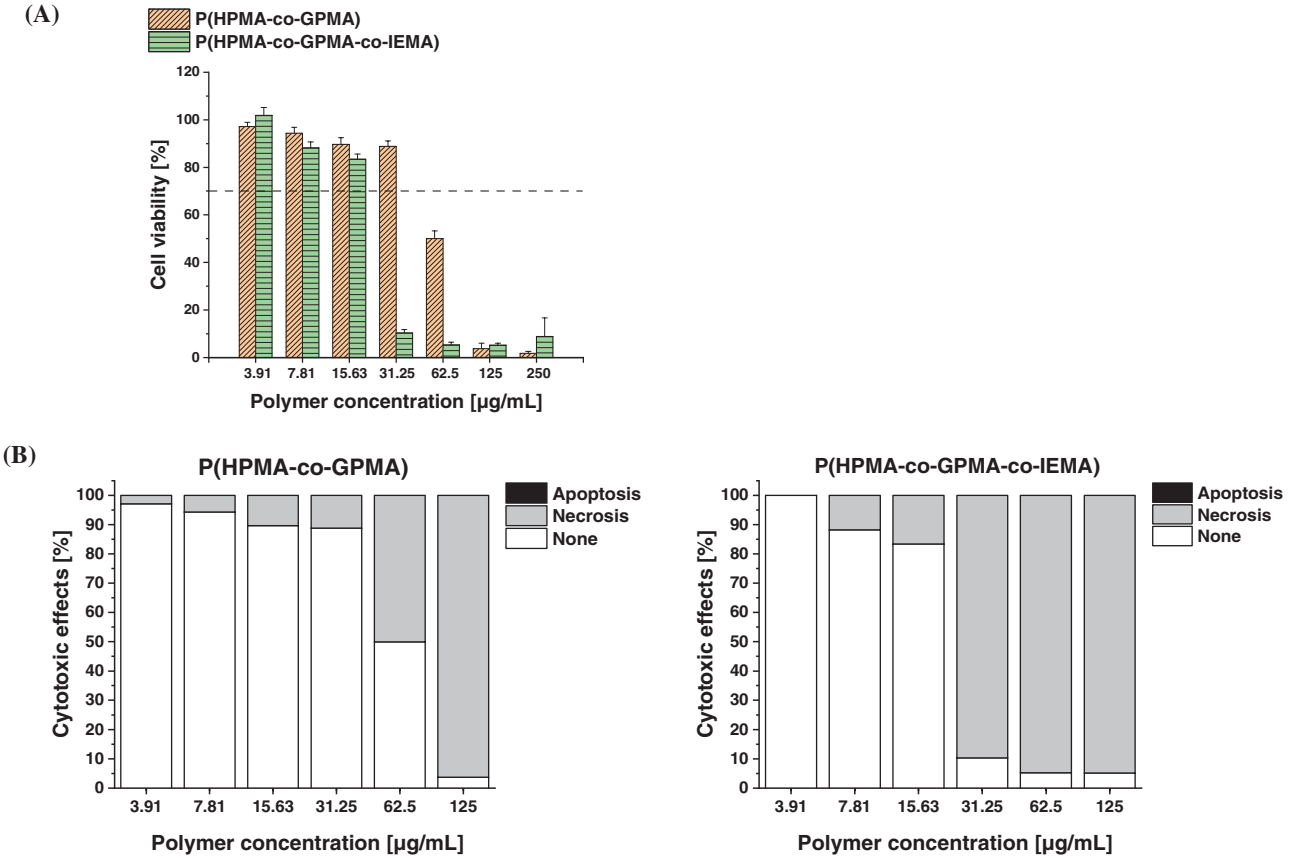


Figure 1. A) Cell viability assay performed on L-929 mouse fibroblasts with increasing polymer concentrations ($n = 7 \pm$ SD). B) Apoptosis determined by caspase activity Caspase-Glo 3/7 assay($n = 3 \pm$ SD) versus necrosis and viability.

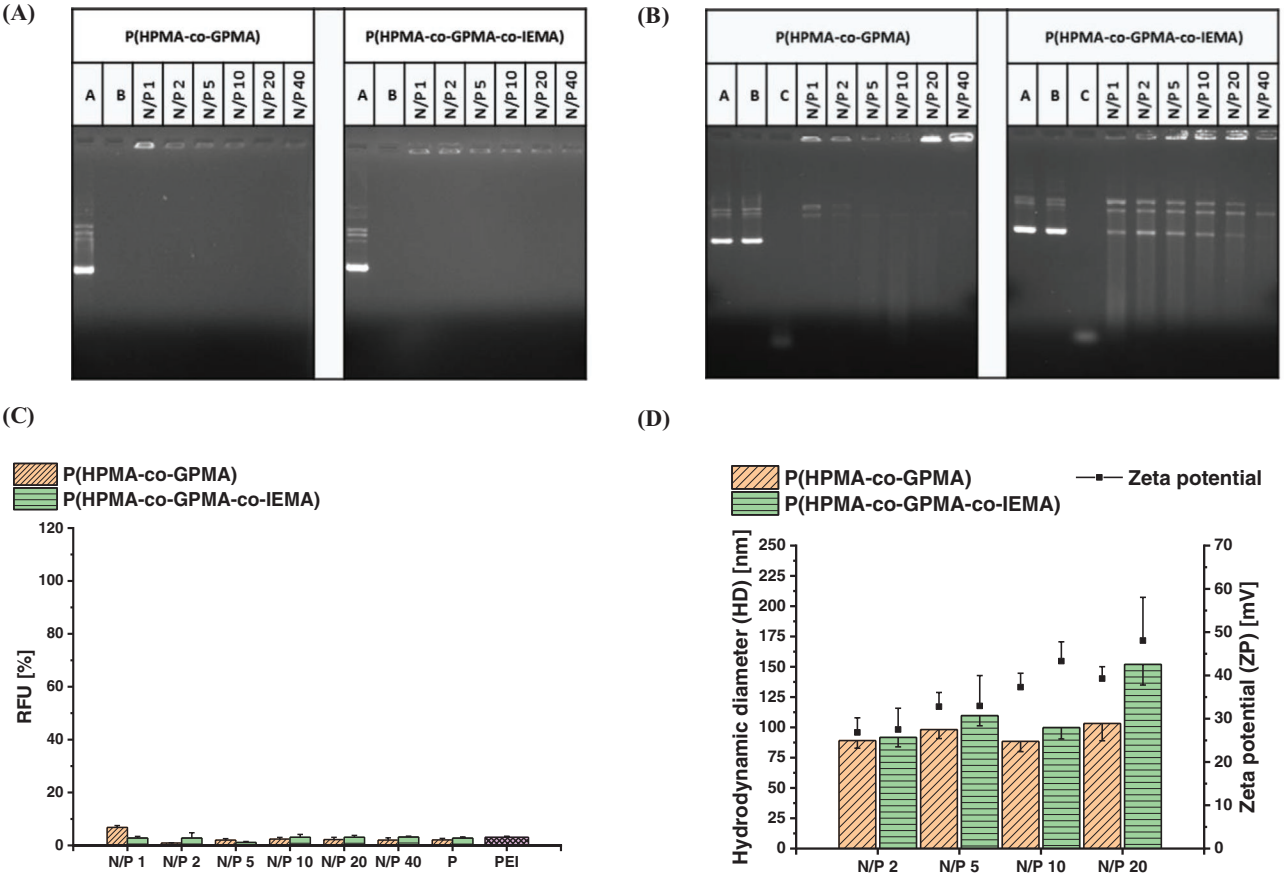


Figure 2. Physicochemical characterization of the polyplexes: A) pDNA binding of P(HPMA-co-GPMA) and P(HPMA-co-GPMA-co-IEMA) determined by horizontal agarose gel electrophoresis with pDNA (Lane A) and polymer (Lane B) controls. B) Protection from enzymatic degradation and polyplex dissociation visualized by horizontal agarose gel electrophoresis with untreated pDNA (Lane A), heat-treated pDNA without DNAse I (Lane B), and pDNA treated with DNAse I (Lane C). C) Fluorophore exclusion assay with the polymers (P) and lPEI 2.5 kDa/pDNA N/P 20 (PEI) controls ($n = 4 \pm$ SD). D) Hydrodynamic diameters (HD) and zeta potentials (ZP) of P(HPMA-co-GPMA) and P(HPMA-co-GPMA-co-IEMA) polyplexes with a cumulative analysis of HD and ZP ($n = 4 \pm$ SD).

higher than 31.25 μg mL^{-1}. This was in accordance with the literature, where increased cell toxicity of indole containing peptides has been reported earlier.[16] For instance, Jobin et al. in 2015 synthesized arginine-rich seven peptide analogues where they changed phenylalanine residue into tryptophan to systematically explore cellular uptake. In their study, all indole-bearing peptides showed higher cytotoxicity.[17] To gain better insights into the cell death mechanism, cellular caspase 3/7 activity assay following the polymer treatment was performed as a measure for apoptosis (Figure 1B).[18] Both polymers did not show any apoptotic events measured by the caspase 3/7 activity assay, which suggested a non-apoptotic cell death pathway could be responsible for the cell death.

The binding ability of the polymers to pDNA was assessed qualitatively by horizontal gel electrophoresis and quantitatively by the AccuBlue dye exclusion assay. In agarose gel electrophoresis (**Figure 2**A), naked pDNA displayed the typical band pattern of nicked circular, linear, and supercoiled plasmid conformations while the polymer alone did not exhibit any signal. Upon polyplex formation, the migration of the negatively charged plasmid toward the anode was impeded due to the charge compensation (complexation) and/or the formation of large complexes of several hundred nanometers unable to migrate through the gel.[19–21] The electrophoresis assay demonstrated efficient pDNA binding at all tested N/P ratios with complete retardation of the plasmid for both P(HPMA-co-GPMA) and P(HPMA-co-GPMA-co-IEMA). In addition to the immobilization of pDNA, an N/P ratio-dependent fluorescence quenching was observed for both polymers, which was interpreted as molecular collapse of the plasmid (condensation) and displacement of the nucleic acid stain from the double helix. The AccuBlue assay supported the successful pDNA condensation quantitatively (Figure 2C). Even at the lowest tested N/P ratio of 1, for both polymers, more than 90% pDNA were no longer accessible for the intercalation of the fluorescent dye. Consequently, the polymers reached binding affinities comparable to the pDNA polyplexes (N/P 20) with 2.5 KDa PEI under optimized conditions.

To understand whether the two polymers can protect pDNA against enzymatic degradation, a stability assay was performed using the enzyme DNAse I, followed by removal of pDNA from the polyplexes with heparin and separation with agarose gel electrophoresis. Although untreated intact pDNA as well as pDNA treated without enzyme revealed the typical band pattern excluding any unspecific degradation effect, after 45 min treatment with DNAse I, all bands disappeared, indicating the

degradation to smaller fragments with lower molar masses (Figure 2B). Polyplexes of P(HPMA-co-GPMA) and P(HPMA-co-GPMA-co-IEMA) represented intact bands at all tested N/P ratios from 1 to 40 as an evidence of protected pDNA payload from enzymatic degradation. However, displacement of pDNA payload from the polyplexes by heparin decreased with increasing N/P ratios. Additionally, the release of pDNA from the polyplexes was found to be more efficient for the P(HPMA-co-GPMA-co-IEMA) than for the P(HPMA-co-GPMA) where most of the pDNA was still located at the beginning of the gel, starting at N/P 10. Significantly higher heparin affinity was observed in several amphipathic peptides which can explain the easier pDNA release from the P(HPMA-co-GPMA-co-IEMA)/pDNA polyplexes.[22]

The hydrodynamic diameter (HD) and zeta potential (ZP) of polyplexes can impact their uptake and intracellular distribution. For this reason, HD and ZP of the polyplexes were measured through dynamic light scattering and laser Doppler anemometry (Figure 2D). The sizes of polyplexes at N/P ratios 2–20 were between 75 and 200 nm independent from the polymer type with a slight trend to agglomeration for p(HPMA-co-GPMA-co-IEMA) at N/P 20. ZP measurements indicated an increase of the ZP values from about 30 to 60 mV with the increase in N/P ratio comparable for both polymers.

Transfection studies of the P(HPMA-co-GPMA)/pDNA and P(HPMA-co-GPMA-co-IEMA)/pDNA polyplexes containing 4 µg pGL3 pDNA, which encodes for the luciferase gene under a SV40 promotor, were conducted on CHO-K1 cells (**Figure 3**A,B). Reporter gene expression was presented as relative light units quantified by bioluminescence measurements. Naked pDNA served as control and failed the production of detectable expression signals. Both polymer/pDNA polyplexes transfected CHO-K1 cells and induced an N/P-dependent

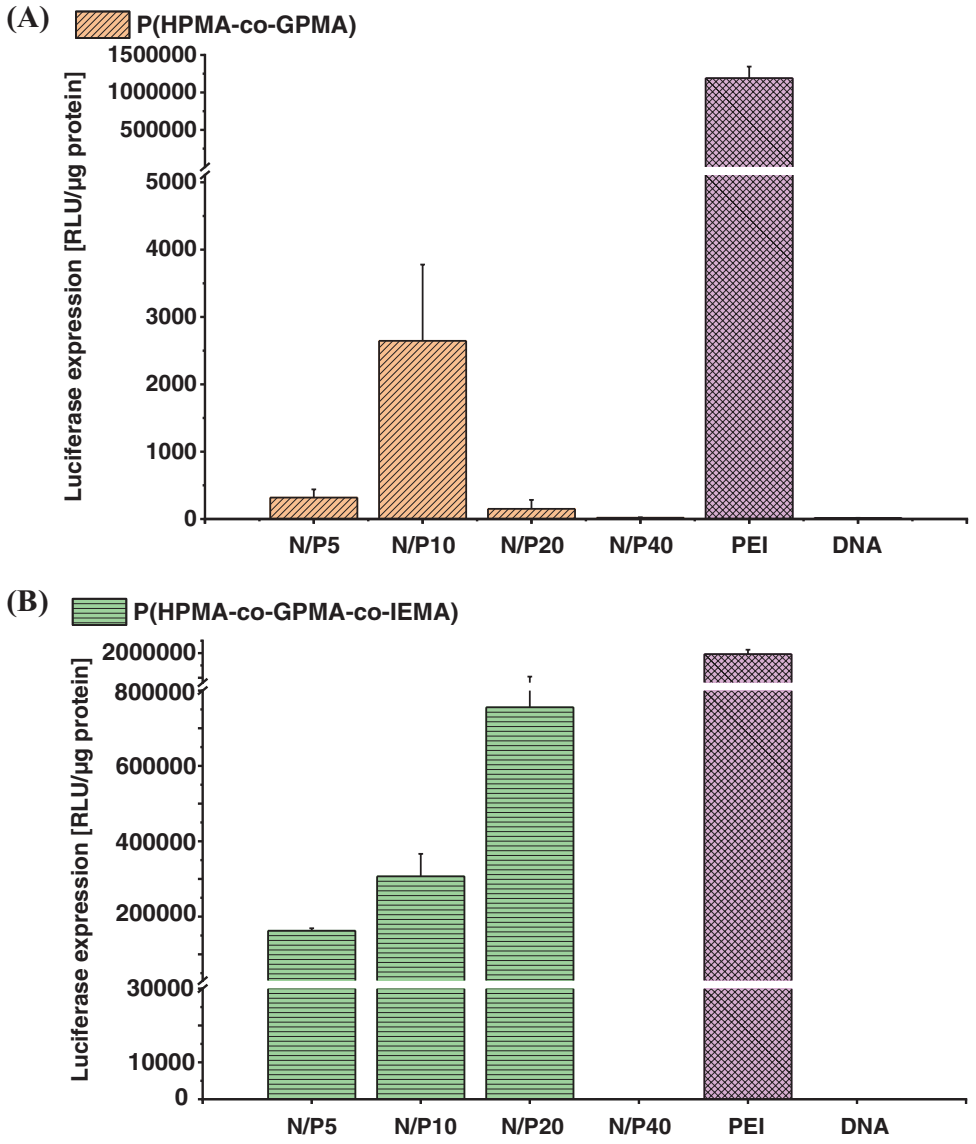


Figure 3. Transfection studies of A) P(HPMA-co-GPMA) and B) P(HPMA-co-GPMA-co-IEMA) on CHO-K1 cells (DNA: 4 µg pDNA, PEI: 2.5 kDa linear PEI at N/P 20).

luciferase transgene expression, although they did not reach the values of the positive control PEI/pDNA polyplexes under the chosen conditions. As a major difference, the P(HPMA-co-GPMA-co-IEMA) polyplexes induced over 200 times higher transgene expression than P(HPMA-co-GPMA) polyplexes. In both cases, N/P > 20 did not increase the transfection ability either because of high toxicity or too strong binding of the pDNA. The higher transfection efficacy might be related on the one hand to the better release of pDNA by the indole-containing polymer as shown above in the electrophoresis experiments. On the other hand, guanidinium has already proven its cell-penetrating efficiency due to its ability to form bidentate hydrogen bonds with anionic groups or self-aggregation for effective translocation in the cell membrane.[23] However, it has been shown that hydrophobic residues in vectors containing also guanidinium groups improved cell translocation dramatically due to the interaction with the cell membrane.[7] Especially, the indole group of tryptophan has the ability of π–π stacking with aromatic parts of membrane proteins.[24,25] It should be mentioned that the polypelexes were able to transfect cells even in the presence of serum. As a result, the positive charges of the polyplexes can be shielded further and membrane translocation can occur more efficiently.

In summary, we report the first example of a water-soluble terpolymer containing HPMA, GPMA, and IEMA monomers and its successful application in gene delivery. Although the presence of indole group increased the toxicity after a certain N/P ratio, the synergy between indole and guanidinium group proved to significantly increase the transfection efficiency. This study reveals the strong potential of the indole group as side-chain pending group that increases the cellular uptake of polymers and the transfection efficiency of the respective polyplexes. Further investigations in more complex biological models as well as in vivo experiments will follow.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

gene delivery, guanidinium, indole, methacrylamide

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[1] M. S. Ravindran, P. Bagchi, C. N. Cunningham, B. Tsai, *Nat. Rev. Microbiol.* **2016**, *14*, 407.
[2] E. Wexselblatt, J. D. Esko, Y. Tor, *J. Org. Chem.* **2014**, *79*, 6766.
[3] Y. Li, Y. Li, X. Wang, R. J. Lee, L. Teng, *Int. J. Pharm.* **2015**, *495*, 527.
[4] C. R. Merril, M. R. Geier, J. C. Petricciani, *Nature* **1971**, *233*, 398.
[5] I.-K. Park, K. Singha, R. B. Arote, Y.-J. Choi, W. J. Kim, C.-S. Cho, *Macromol. Rapid Commun.* **2010**, *31*, 1122.
[6] K. Klinker, K. Klinker, M. Barz, *Macromol. Rapid Commun.* **2015**, *36*, 1943.
[7] I. Tabujew, M. Lelle, K. Peneva, *BioNanomaterials* **2015**, *16*, 59.
[8] I. Tabujew, C. Freidel, B. Krieg, M. Helm, K. Koynov, K. Mullen, K. Peneva, *Macromol. Rapid Commun.* **2014**, *35*, 1191.
[9] M. C. Morris, S. Deshayes, F. Heitz, G. Divita, *Biol. Cell* **2008**, *100*, 201.
[10] C. Buerkli, S. H. Lee, E. Moroz, M. C. Stuparu, J.-C. Leroux, A. Khan, *Biomacromolecules* **2014**, *15*, 1707.
[11] C. Wang, L. Du, J. Zhou, L. Meng, Q. Cheng, C. Wang, X. Wang, D. Zhao, Y. Huang, S. Zheng, H. Cao, J. Zhang, L. Deng, Z. Liang, A. Dong, *ACS Appl. Mater. Interfaces* **2017**, *9*, 32463.
[12] I. Tabujew, C. Cokca, L. Zartner, U. S. Schubert, I. Nischang, D. Fischer, K. Peneva, *J. Mater. Chem. B* **2019**, *7*, 5920.
[13] N. J. Treat, D. Smith, C. Teng, J. D. Flores, B. A. Abel, A. W. York, F. Huang, C. L. McCormick, *ACS Macro Lett.* **2012**, *1*, 100.
[14] I. Tabujew, C. Freidel, B. Krieg, M. Helm, K. Koynov, K. Muellen, K. Peneva, *Macromol. Rapid Commun.* **2014**, *35*, 1191.
[15] A. Hall, U. Lächelt, J. Bartek, E. Wagner, S. M. Moghimi, *Mol. Ther.* **2017**, *25*, 1476.
[16] H. L. Aamand, H. A. Rydberg, L. H. Fornander, P. Lincoln, B. Norden, E. K. Esbjoerner, *Biochim. Biophys. Acta, Biomembr.* **2012**, *1818*, 2669.
[17] 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.
[18] D. Fischer, Y. X. Li, B. Ahlemeyer, J. Krieglstein, T. Kissel, *Biomaterials* **2003**, *24*, 1121.
[19] D. Fischer, H. Dautzenberg, K. Kunath, T. Kissel, *Int. J. Pharm.* **2004**, *280*, 253.
[20] J. Narayanan, J.-Y. Xiong, X.-Y. Liu, *J. Phys.: Conf. Ser.* **2006**, *28*, 83.
[21] F. Schlenk, S. Grund, D. Fischer, *Ther. Delivery* **2013**, *4*, 95.
[22] Ü. Langel, in *CPP, Cell-Penetrating Peptides* (Ed: Ü. Langel), Springer, Singapore **2019**, p. 359.
[23] C. L. Hannon, E. V. Anslyn, in *Bioorganic Chemistry Frontiers* (Eds: H. Dugas, F. P. Schmidtchen), Springer, Berlin **1993**, p. 193.
[24] M. L. Jobin, M. Blanchet, S. Henry, S. Chaignepain, C. Manigand, S. Castano, S. Lecomte, F. Burlina, S. Sagan, I. D. Alves, *Bioch. Biophys. Acta* **2015**, *1848*, 593.
[25] Z. Kang, G. Ding, Z. Meng, Q. Meng, *Peptides* **2019**, *121*, 170149.

