Peptoidic Amino- and Guanidinium-Carrier Systems: Targeted Drug Delivery into the Cell Cytosol or the Nucleus

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Abstract: Efficient drug delivery is essential for many therapeutic applications. Some cell-penetrating peptides, peptide mimetics, and peptoids express transport function that, however, lack in most cases specific intracellular destination. In this study, carrier-peptoids with either amino or guanidinium side chains, were investigated with regard to their cellular uptake, toxicity, and intracellular localization. Transport specifically to the cytosol or to the nuclei was observed, thus providing a powerful tool for targeted drug delivery.

It has been well-known for several decades that some peptides with basic amino acid residues are taken up rapidly by cells in culture.[1–9] Initial assays suggested that these peptides could directly traverse the plasma membrane by an unknown mechanism, independent of classical receptor-mediated pathways. Some of these peptides were discovered as basic domains responsible for the translocation of naturally transduced proteins and were, therefore, referred to as protein transduction domains (PTDs[a]).[10–12] They can transport into the cell covalently attached cargo molecules of diverse chemical nature (oligonucleotides, proteins, fluorophores, PNAs, and even liposomes or nanoparticles). Many peptides composed of α-amino acids (**1**, Figure 1) with the same properties were discovered or designed since then and are described as a functional group by the term cell-penetrating peptides (CPPs).[10–12]

The most important structural features for cellular uptake efficiency of CPPs appear to be the short size (i), high content of cationic residues (ii), and variable spacing between the charges (iii), while the backbone conformation does not seem to play a critical role.[10–12] However, the bioavailability of CPPs is usually low due to in vivo proteolysis. Therefore, short peptide mimetics with modified backbones, carrying basic functionalities such as amino or guanidinium groups, may serve as a valuable alternative to the CPPs because of their enhanced stability in vivo.

Proteolytically stable β-peptides (**2**, Figure 1), for example, have been under intensive investigation during the past years[13] and were shown to be efficiently internalized by cultured mammalian cells.[3]

Peptoids (oligo-*N*-alkylglycines; **3**, Figure 1) are stable against proteases, like β-peptides, but usually are less prone to aggregation.[3,14,15] In contrast to both α- and β-peptides, the side chains of peptoids are attached to the nitrogen atom instead of the carbon, therefore, they lack hydrogen-bonding potential, which prevents backbone-driven aggregation and thus increases bioavailability.[3,14,15]

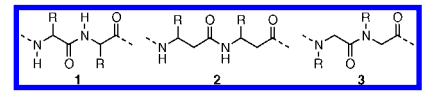

Figure 1. Structures of the backbone of an α-peptide (**1**), β-peptide (**2**), and a peptoid (**3**).

The use of peptoids as effective, water-soluble nontoxic molecular transporters for intracellular drug delivery or as molecular probes for bioconjugation has also been reported.[16–21] Peptoids with guanidinium head groups attached to alkyl chains have also been used to mimic peptide-hormones, antibiotics, and receptor–ligands.[16–20]

The comparison of oligo(poly)-lysine $(Lys)_n$ and oligo(poly)-arginine $(Arg)_n$ peptides has also been under investigation.[3,22–24] It was demonstrated that $(Arg)_n$ shows faster uptake than $(Lys)_n$. Moreover, it seems that major penetration pathways may differ for $(Arg)_n$ and $(Lys)_n$.[23,25] Notably, if CPPs are constructed with a single type of cationic side chain, cellular uptake efficiency usually decreases in the following order: Arg > Lys > Orn > His.

Here we translate those findings to peptoid molecules and compare the carrier potency of peptoids with amino side chains (amino-peptoids) against peptoids with guanidinium side chains (guanidinium-peptoids). Syntheses, toxicity, and cellular uptake are reported. We observe transport to the cytosol or to the nuclei, depending on the choice of the side-chain functionality. To our knowledge, the possibility of determining the intracellular destination without only additional localization signal is reported for the first time, thus providing a powerful tool for targeted drug delivery.

For the experiments, a fluorescently labeled homohexameric peptoid with amino-group carrying side chains was compared with two peptoids carrying guanidinium side chains, assembled as a homopenta- or hexamer.

The building blocks were prepared starting from 1,6-diaminohexane following a slightly modified procedure of Bradley et al.[15a] and Liskamp et al. (for the reaction scheme, see Supporting Information).[18a,21] The protected and functionalized building blocks were assembled on the solid-phase (Scheme 1). The reactions were carried out using Rink amide resin **4** as a result of its stability and ease of the first coupling step. The synthesis of the backbone was completed by coupling a fluorophore. The labels, 5(6)-carboxyfluorescein (Figure 2, **9a**, Fluo, $\lambda_{abs} = 492$ nm, $\lambda_{em} = 517$ nm) or rhodamine-B (Figure 2, **9b**, Rhod, $\lambda_{abs} = 550$ nm, $\lambda_{em} = 580$ nm), were attached to peptoids prior to cleavage from the solid support. A spacer (6-aminohexanoic acid) was used to prevent interactions of the marker with the peptoid moiety (Scheme 1). To isolate the amino-peptoid Fluo-{6,6,6,6,6,6}-NH_2 **10**, it was simultaneously deprotected and cleaved from the resin by using trifluoroacetic acid (TFA)/triisopropyl silane (TIS; Scheme 2). The molecule was precipitated in cold diethylether, filtered off, and dried in vacuo.[21] It was characterized by mass spectrometry, UV/vis, and IR. Before the guanidinium-peptoids Rhod-{6^G,6^G,6^G,6^G,6^G,6^G}-NH_2 **11** and Fluo-{6^G,6^G,6^G,6^G,6^G,6^G}-NH_2 **12** could be cleaved from the solid support, they had to be orthogonally deprotected

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[a] Abbreviations: DAPI, 4′,6-diamidino-2-phenylindol; DBU, 1,8-diazabicyclo[5.4.0]undecen; DIPEA, diisopropyl-ethyl amine; CPPs, cell-penetrating peptides; FACS, fluorescence-assisted cell sorting; PG, protection group; PTDs, protein transduction domains; TFA, trifluoroacetic acid; TIS, triisopropyl silane.

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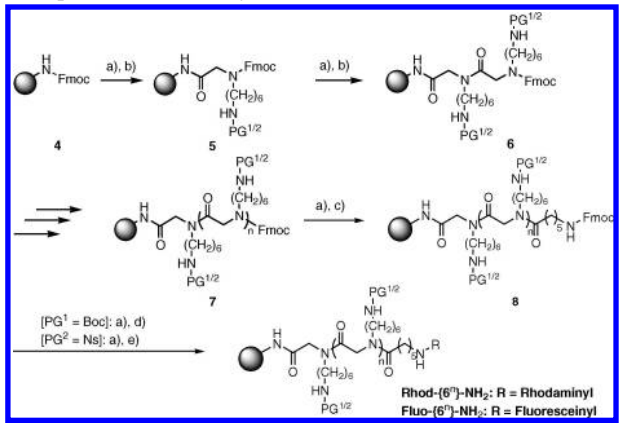
Scheme 1. Solid Phase Synthesis of Peptoids, Attachment of the Spacer (6-Aminohexanoidc Acid), and Labeling with the Fluorophore 5(6)-Carboxyfluorescein **9a** or Rhodamine-B **9b**[a]



[a] Reagents and conditions: (a) 20% piperidine in DMF, 3 × 2 min; (b) 3.00 equiv monomer, 2.00 equiv PyBrOP, 4.00 equiv DIPEA, CH_2Cl_2, rt, 24 h; (c) 3.00 equiv Fmoc-aminohexanoic acid, 2.00 equiv PyBrOP, 4.00 equiv DIPEA in CH_2Cl_2, rt, 24 h; (d) 3.00 equiv 5(6)-carboxyfluorescein, 3.00 equiv HOBt, 3.00 equiv DIC in DMF/CH_2Cl_2 (1:1), rt, 5 h; (e) 3.00 equiv rhodamine-B, CH_2Cl_2, rt, 4 d.[21]

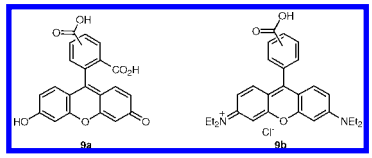
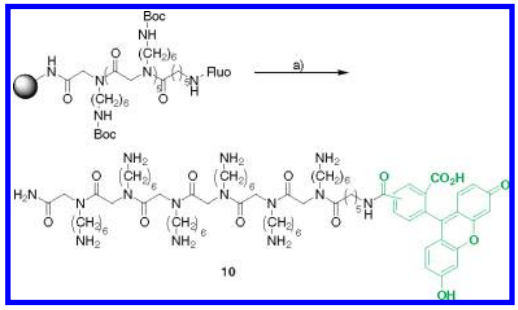


Figure 2. Structures of the fluorophores 5(6)-carboxyfluorescein **9a** and rhodamine-B **9b**.

Scheme 2. Cleavage of Amino-peptoid Fluo-{6,6,6,6,6,6}-NH_2 **10**[a]

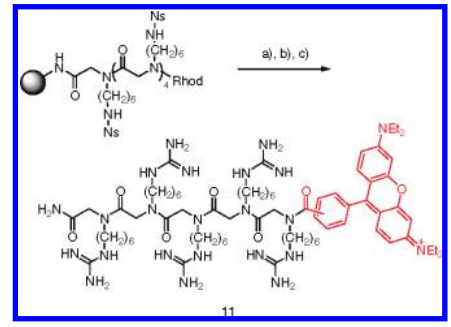


[a] Reagents and conditions: (a) TFA/TIS (95:5), rt, 3 h.

and the free amino side chains were transformed to guanidinium groups. Finally, the guanidinium-peptoids were cleaved from the resin (Schemes 3 and 4), isolated, and characterized following the procedure of **10**. All peptoids were purified by HPLC (purity >95%; for method see SI).

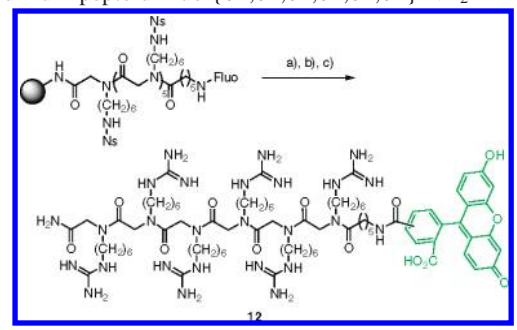
To test the cellular uptake of the peptoids, FACS analysis was performed. The uptake was measured as increase in the number of fluorescent cells. The uptake of the molecules after incubation for 10, 30, and 60 min and at three concentrations (50, 100, 200 μM) was tested on the adherent cell lines from human epithelial lung cancer (A549)[26] and normal human endothelia (ECV304).[27] For the results of the ECV304 cells, see SI. A clear concentration and time-dependent increase in peptoid-associated fluorescence was detected for both cell lines (Figure 4). For the highest concentration of peptoids (200 μM) after 10 min incubation already 90% of the cells could be detected by FACS for **11** and **12**, while only 32% of the cells were detected if incubated with **10**. Thus, uptake rates for guanidinium-peptoids are higher than for the amino-peptoid.

Scheme 3. Deprotection, Formation of the Guanidinium Groups, and Cleavage from the Solid Support of Guanidinium-peptoid Rhod-{6^G,6^G,6^G,6^G,6^G}-NH_2 **11**[a]



[a] Reagents and conditions: (a) 2-mercaptoethanol/DBU (0.3 M in DMF), 3 times 45 min; (b) 10.0 equiv 1*H*-pyrazole-1-carboxamidine hydrochloride, 10.0 equiv DIPEA, DMF, rt, 24 h; (c) TFA/TIS (95:5), rt, 3 h.

Scheme 4. Deprotection, Formation of the Guanidinium Groups, and Cleavage from the Solid Support of the Guanidinium-peptoid Fluo-{6^G,6^G,6^G,6^G,6^G,6^G}-NH_2 **12**[a]



[a] Reaction conditions are identical to those described in Scheme *3*.

Furthermore, as can be seen from Figure 3, the amino-peptoid **10** accumulates preferentially in the cytosol (cytosolic staining), whereas the fluorescence from guanidinium-peptoids **11** and **12** is mainly associated with the cell nuclei and even with the nucleoli (Figure 3 and SI). We did not observe any profound differences in either uptake rate or in intracellular distribution between **11** and **12**. Therefore, in our case, the attached fluorophore had no influence on the diverse destinations inside the cell in contrast to previously reported examples.[3,22–24] Only a slight difference is noted in the intensities due to the lower light extinction coefficient of 5(6)-carboxyfluorescein **9a** (for more information, e.g., the uptake in tabular form, see SI).

Thus, a differential transport to the cytosol (amino-peptoid **10**) and to the nucleus (guanidinium-peptoids **11** and **12**, Figure 3) could be observed. Note that the guanidinium-peptoids do not exclusively accumulate in the nucleous, but preferentially. The results for the uptake efficiency confirm previous findings with $(Arg)_n$ and $(Lys)_n$ peptides[23,24] and can be explained analogously, suggesting an uptake by macropinocytosis for the amino-peptoid and direct membrane transduction for the guanidinium-peptoids.

To verify that the synthesized peptoids could be used for in vivo applications and to check that the faster uptake of carriers **11** and **12** is not due to cell membrane damage or perforation, their toxicity toward mammalian cells was investigated. To do so, the peptoids were tested in different established cytotoxicity assays. For an independent cross-check, two tests based on different detection principles were carried out. The WST-1 test detects only the viable cells by examination of the intracellular reduction potential, whereas the LDH test detects exclusively dead cells.[21,28–30]

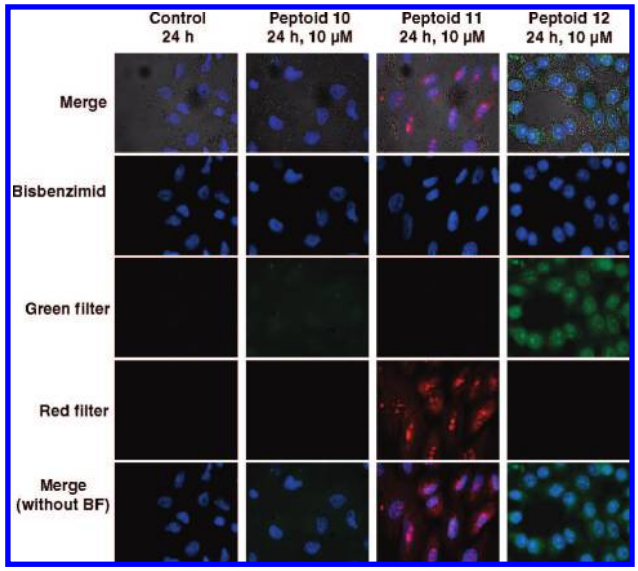


Figure 3. Fluorescence microscopy of the A549 cells incubated with the purified peptoids **10**, **11**, and **12**. Concentration of peptoids, 10 μM; coincubation time, 24 h. The guanidinium-peptoids **11** and **12** show higher uptake rates and accumulate in the cellular nucleoli. For images with other concentrations and incubation times, see SI.

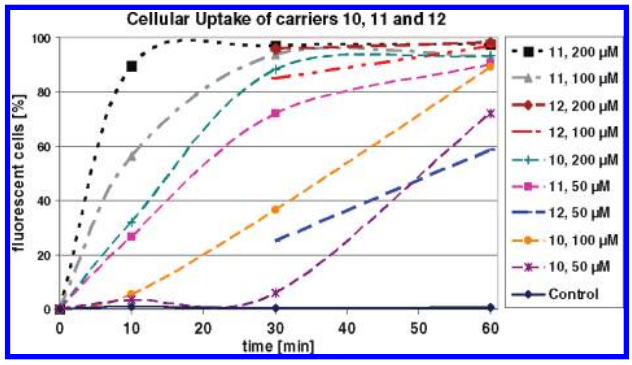


Figure 4. FACS analysis of the uptake of peptoids **10**, **11**, and **12**. The results are given as the amount of fluorescent A549 cells in percent as a function of incubation time for the different concentrations of peptoids.

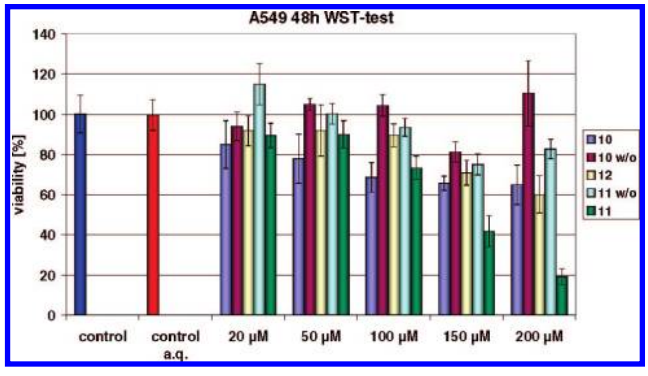


Figure 5. Viability test (WST-1) of A549 cells incubated for 48 h in the presence of fluorescently-labelled peptoids **10**, **11**, and **12** and respective fluorophore-free analogues **10 w/o** and **11 w/o**. The decrease of viability at high concentrations is due to the coupled fluorophore. The results are confirmed by the LDH assay (data shown in SI).

As can be seen from Figure 5, over a prolonged incubation time (48 h), the viability of A549 cells in the presence of the two peptoids **10** and **12** did slightly decrease only for concentrations above 100 μM and 150 μM, respectively. Interestingly, the shorter guanidinium-peptoid **11** showed an overall higher toxicity and was the only one where viability of the cells dropped below 50% (at concentrations equal to and above 150 μM). To examine whether the observed toxicity for the highest concentrations could be associated with the coupled fluorophore, the viability tests were performed with the fluorophore-free peptoids $\{6^G,6^G,6^G,6^G,6^G\}$-NH_2 (**11 w/o**) and $\{6,6,6,6,6,6\}$-NH_2 (**10 w/o**; see SI). It can be seen (Figure 5) that such an assumption was right, as after a 48 h incubation time with the same high concentrations of peptoids, indeed the cell viability was restored or even improved. Performing the WST-1 test on the ECV304 cell line as well as an LDH assay on the A549 cells, very similar results are obtained for all the tested peptoids (see SI).

In conclusion, the synthesis of guanidinylated peptoids and biological studies of these carriers are presented compared to a peptoid with amino side chains. We have investigated these efficient carrier systems with respect to their toxicity, cellular uptake, and destination within the cells. It is demonstrated here that both systems did not show any significant toxicity. The guanidinium-peptoids caused a decrease in the viability of sensitive cell lines only at high concentrations and after long incubation times, and this effect seems to be due to the coupled fluorophore. All peptoids translocated into the cells and are, therefore, useful as carriers for drug delivery. However, certain differences in the uptake related to the cationic moiety are present. First, the amino-peptoid requires longer times to complete translocation into the cell, while the uptake rate for the guanidinium-peptoids is much faster, presumably due to a different translocation mechanism.[23–25] Second, intracellular accumulation of the peptoids is different; the amino-peptoid resides in the cytosol, while the guanidinium-peptoids accumulate preferentially in the nucleus. Thus, the uptake rate and destination of the carriers can be tuned by just modifying the side chain functionality and does not require any additional leading motif. Low cytotoxicity and high stability makes the molecules based on the peptoidic backbone attractive candidates for in vivo intracellular drug delivery. Further applications are in progress.

Peptoid-carriers selectively deliver the cargo either to the cytosol (amino-peptoid) or to the cell nucleous (guanidinium-peptoid) were synthesized and shown to be a promising tool for the use in drug delivery.

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Supporting Information Available: Detailed experimental procedures, analytical data, and results of the biological tests/assays are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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