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Cationic Oligopeptides with Amino Groups as Synthetic Nucleolar Localization Signals for the Rational Design of Nucleolus-Staining Probes

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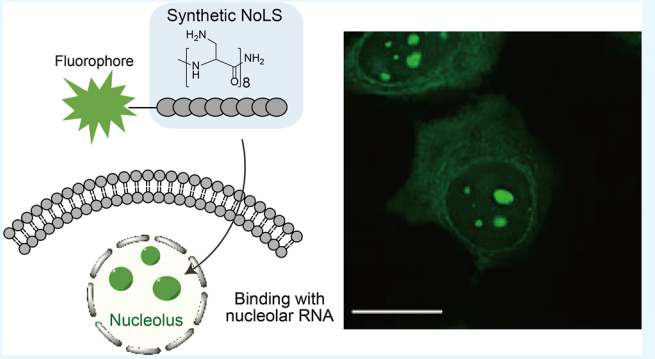
ABSTRACT: Cationic oligopeptides with amino groups were found to function as synthetic nucleolar localization signals for directing various fluorophores to the nucleolus with high selectivity in the cells with a view toward the development of nucleolus-staining probes.



INTRODUCTION

The nucleolus is the largest substructure in the nucleus, where ribosome biogenesis occurs.[1] Besides, the nucleolus is involved in a variety of cellular functions including cell cycle regulation and cellular stress response.[2,3] Much attention has been paid to its great potential as a diagnostic biomarker for various diseases as well as a drug target in the treatment of cancers.[4] Fluorescent probes targeting specific organelles are useful tools for understanding their intracellular behaviors.[5] At present, there is one commercially available probe for nucleolus imaging, which is the SYTO RNA select. This probe can stain the nucleolus upon simple incubation with the cells as it shows the binding-induced fluorescence enhancement to nucleolar RNA. However, the usefulness of SYTO RNA select for the nucleolus analysis has been severely limited because of several drawbacks.[6] Numerous efforts have been made to develop a new class of small molecule-based fluorescent probes, in which most of these works focused on the improvement of fluorescent properties such as the photostability and the excitation/emission wavelength.[7] On the other hand, SYTO RNA select also suffered from moderate selectivity toward the nucleolus over the nucleoplasm and other organelles,[6,7e,g] which should be problematic for the accurate analysis of the nucleolus. Nonetheless, less attention has been paid to the nucleolus selectivity of the fluorescent probes, presumably due to the difficulty in the rational probe design to achieve this.

Organelle-targeting molecular motifs emerged as powerful tools for directing various kinds of molecules including small molecules, nucleotides, and proteins to the specific organelle.[8] As for targeting the nucleolus, several peptide sequences enriched with positively charged amino acids proved to be crucial for the localization of the proteins at the nucleolus.[9] Such a nucleolar localization signal (NoLS) facilitated the accumulation of the protein of interest in the nucleolus by fusing the peptides to the protein.[10] While native NoLS peptides were never examined for small molecules, of particular interest for us is the report by Cardoso's group, where various synthetic NoLSs were designed based on native sequences in order to assess the molecular requirements for the nucleolus localization.[10a] They demonstrated that the conjugation of short cationic peptides with arginine residues (R5-R12) resulted in the nucleolar localization of the fluorophore (FITC: fluorescein isothiocyanate) through the binding to nucleolar RNAs. Although the localization in the nucleolus was relatively moderate, their results motivated us to develop highly selective nucleolus-staining probes based on synthetic NoLS peptides with RNA-binding properties.

In this work, we explored a series of 8-mer cationic oligopeptides with amino groups (Figure 1) for the rational molecular design of the imaging probes with high nucleolus selectivity. Given the fact that lysine residues are enriched in

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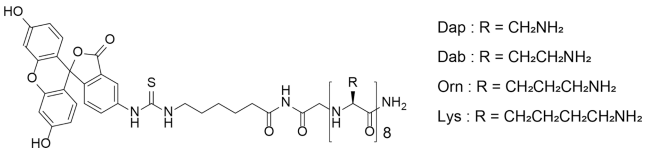

Figure 1. Chemical structures of FITC-labeled oligopeptides with amino groups.

the native NoLS sequences,[10a] these peptides are expected to exert the nucleolus-targeting property due to their RNA-selective binding properties.[11] Here, we synthesized FITC-labeled oligopeptides composed of Dap (L-2,3-diaminopropionic acid), Dab (L-2,4-diaminobutyric acid), Orn (L-ornithine), or Lys (L-lysine) and systematically examined their properties for fluorescence imaging of the cells. Significantly, FITC-Dap8 was shown to be useful for superior selective staining of the nucleolus in the cells compared to SYTO RNA select. FITC-Dap8 even performed much better than an FITC-labeled 8-mer cationic peptide consisting of arginine residues (FITC-Arg8) under experimental conditions in this study. We also demonstrate the versatility of the Dap8 unit for directing various fluorophores toward the nucleolus, resulting in the expansion of the emission color repertoire of the nucleolus-staining probes.

RESULTS AND DISCUSSION

Probe Synthesis. All probes were synthesized by Fmoc-based solid phase synthesis. After completing the assembly of the peptide, FITC was labeled with the N-terminal of the peptides through 6-aminohexanoic acid and glycine spacers. Crude probes were purified by reverse-phase HPLC, followed by the characterization by MALDI TOF-MS (Figure S1 and Table S1).

Fluorescence Imaging of Cells. Figure 2A shows the fluorescence image of fixed-permeabilized MCF7 cells after 60 min of incubation with 1.0 μM FITC-Dap8. Strong emission of FITC-Dap8 was observed at the nucleolus, which indicates FITC-Dab8 selectively stains the nucleolus over other organelles. As FITC itself has no cell-staining ability (data not shown), it is highly likely that the Dap8 unit serves as a synthetic NoLS peptide for directing FITC to the nucleolus, as observed for the previous arginine-rich peptides.[10a] From the quantitative analysis of the intracellular emission intensity, the emission at the nucleolus was found to be more than 4.7-fold larger than that of the nucleoplasm (Figure 2B). These results show a highly selective nucleolus-staining property of FITC-Dap8. It should be noted that the number of Dap units has a large impact on the nucleolus-staining property (Figure S2). The results for FITC-Dap8 were then compared with those of SYTO RNA select, the commercially available probe, under identical conditions (Figures 2B and S3). The nucleolus selectivity of SYTO RNA select is much inferior to that of

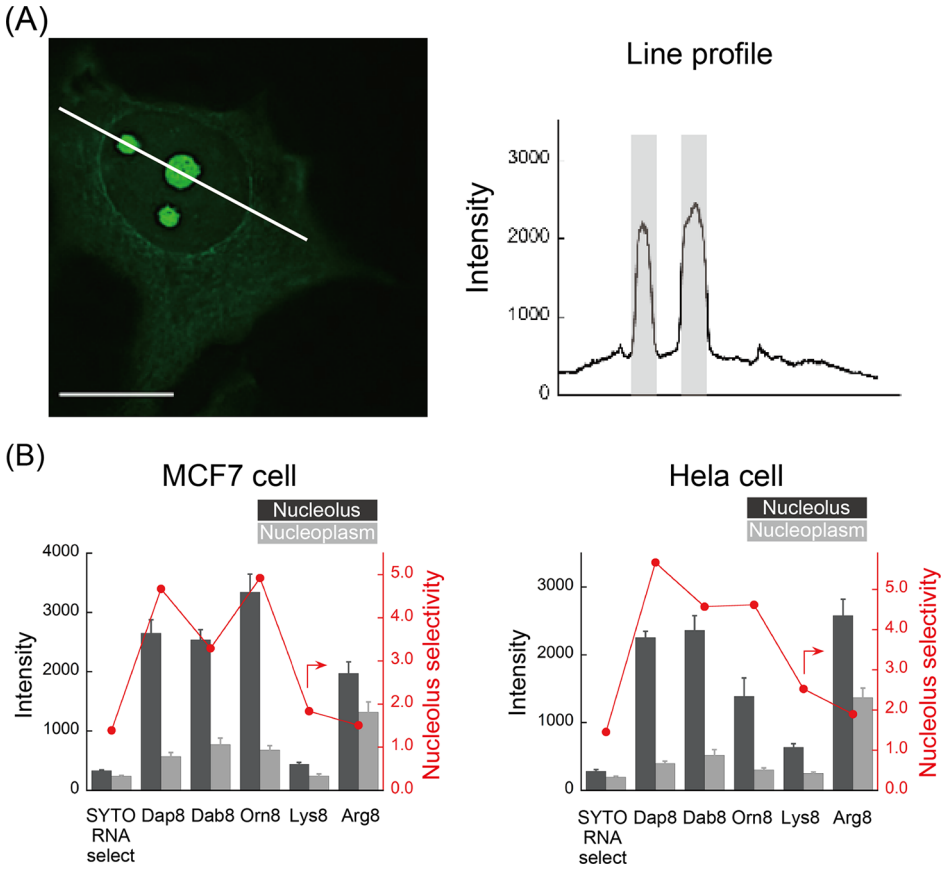

Figure 2. (A) Fluorescence image of MCF7 cells stained by 1.0 μM FITC-Dap8. The fluorescence intensity profile along the white line is also shown, where the fluorescence in the nucleolus is highlighted. Scale bar: 15 μm. (B) Evaluation of the nucleolus-staining ability of the fluorescent probes. Left ordinate: fluorescence emission intensity of the nucleolus and the nucleoplasm for cells stained by the fluorescence probes. Right ordinate: nucleolus selectivity over the nucleoplasm for the fluorescent probes.

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FITC-Dap8. Furthermore, its emission at the nucleolus is 8.0-fold smaller compared to that of FITC-Dap8. Apparently, FITC-Dap8 facilitates more selective and sensitive imaging of the nucleolus over SYTO RNA select.

We next investigated the impact of the kind of amino acid residues in the probes on the staining performance for MCF7 as well as Hela cells (Figures S4 and S5). Similar to FITC-Dap8, three other probes (FITC-Dab8, FITC-Orn8, and FITC-Lys8) displayed better performance for nucleolus staining in both cells relative to SYTO RNA select (Figure 2B). All oligopeptides examined here are thus useful as synthetic NoLS peptides for the transportation of FITC toward the nucleolus. Meanwhile, we found that the nucleolus-staining performance significantly differed depending on the amino acid residues of the probes. FITC-Dap8, FITC-Dab8, and FITC-Orn8 had superior selectivity and sensitivity for nucleolus imaging over FITC-Lys8. In addition, the emission intensity of FITC-Orn8 at the nucleolus was significantly different between these cells, where the intensity for the Hela cells decreased by 2.4-fold compared to MCF7 cells (Figure 2B). We further compared it with the FITC-labeled cationic peptide consisting of arginine residues (FITC-Arg8) that was previously developed by Cardoso's group.[10a] Although FITC-Arg8 could also stain the nucleolus (Figures S4 and S5), its selectivity toward the nucleolus over the nucleoplasm was even worse than that of FITC-Lys8 (Figure 2B). This indicates that cationic oligopeptides with amino groups are more suitable for the development of nucleolus-selective staining probes. From these results, both FITC-Dap8 and FITC-Dab8 were quantified as the prominent probes in terms of the nucleolus-staining property and versatility for various cells.

We next carried out deoxyribonuclease (DNase) and ribonuclease (RNase) digestion experiments in order to investigate the origin of the nucleolar emission from FITC-Dap8 as a typical peptide probe (Figure S6). After DNase treatment, strong emission was observed in the nucleolus similar to the control condition (buffer). In sharp contrast, the emission at the nucleolus dramatically decreased in the case of RNase treatment. These results show that the observed emission originated from FITC-Dap8 bound to the RNAs that were abundant in the nucleolus such as ribosomal RNAs.[7] Hence, it is highly likely that FITC-Dap8 is able to stain the nucleolus based on the binding to nucleolar RNA.

It should be noted that FITC-Dap8 has good counter-staining compatibility with Hoechst 33342, a blue-emissive DNA-staining probe (Figure S7). The emission of FITC-Dap8 did not overlap with that of Hoechst 33342 (Pearson's correlation coefficient: 0.24). Thus, the combination of these probes allows the simultaneous imaging of DNA and RNA distribution. We demonstrated the low cytotoxicity of FITC-Dap8 under the present condition (1.0 μM, 60 min incubation) by the Alamar blue assay (Figure S8). Also, we observed no morphological changes of the cells upon staining with FITC-Dap8. FITC-Dap8 thus displays a negligible influence on the cell viability.

Binding Ability to Nucleic Acids. The obtained results along with the literature on arginine-rich peptides[10a] suggest the importance of RNA-binding ability of the probes for effective staining of the nucleolus. We thus sought to assess the binding functions of our peptide probes for biological nucleic acids by fluorescence anisotropy titration experiments. Figure 3 shows the change in the anisotropy r of FITC-Dap8 upon addition of *Escherichia coli* total RNA or calf thymus DNA. The

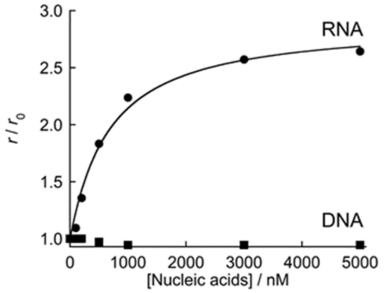

Figure 3. Titration curves for the binding of FITC-Dap8 (1.0 μM) to the biological nucleic acids. r and r_0 denote the fluorescence anisotropy of FITC-Dap8 in the presence and absence of nucleic acids. The obtained curve was analyzed with the fitting equation.

r value increased when the concentration of RNA increased. This can be explained by the decreased rotation of the FITC unit due to the increase in the effective molecular volume of FITC-Dap8 upon binding to RNAs. From the analysis of the obtained titration curve, the apparent dissociation constant K_d was estimated as 490 ± 110 nM (N = 3). Meanwhile, we did not observe any anisotropy change for DNAs. Apparently, FITC-Dap8 does have the binding selectivity to RNAs over DNAs. We also observed RNA-selective binding for other peptide probes, but the K_d value for RNA significantly differs among the probes (Figure S9, K_d/μM; FITC-Dab8, >70; FITC-Orn8, 2.0 ± 0.6). The K_d value of FITC-Lys8 could not be determined due to the weak binding, which seems correlated with its relatively poor nucleolus-staining property (cf. Figure 2B). Although other chemical properties such as the molecular size and the hydrophobicity would be partly involved in the staining performance, selective and effective binding of the peptide probes toward RNAs would be necessary for targeting the nucleolar RNA with a view toward the nucleolus-staining applications.

Expansion of the Emission Color Repertoire. Furthermore, we investigated whether the Dap8 unit could direct other fluorophores to the nucleolus or not. We prepared two kinds of Dap8-based probes carrying TAMRA (TAMRA-Dap8) or Cy5 (Cy5-Dap8) at the N-terminal, where the linker length was set to be the same as FITC-Dap8 (Figure 4). Both probes could effectively stain the nucleolus with high selectivity for MCF7 cells. Note that 0.5 μM Cy5-Dap8 is suitable for better nucleolus staining because the fluorescence intensity dramatically decreases at higher probe concentrations (>1.0 μM) due to the possible dimerization of the Cy5 unit.[12] Accordingly, the present probe design based on conjugation with Dap8 unit is useful and versatile for the development of nucleolus-staining probes. This would be particularly useful for the multicolor cellular imaging applications in combination with other organelle-staining probes as our design features tunable fluorescence properties by adopting suitable organic fluorophores.

CONCLUSIONS

In summary, we proposed a new molecular design of nucleolus-staining probes by using RNA-binding cationic oligopeptides with amino groups as synthetic NoLS peptides. FITC-Dap8 served as a more sensitive and selective probe compared to commercially available SYTO RNA select. RNA-binding properties of the probes were found to be important for effective staining of the nucleolus. Moreover, we

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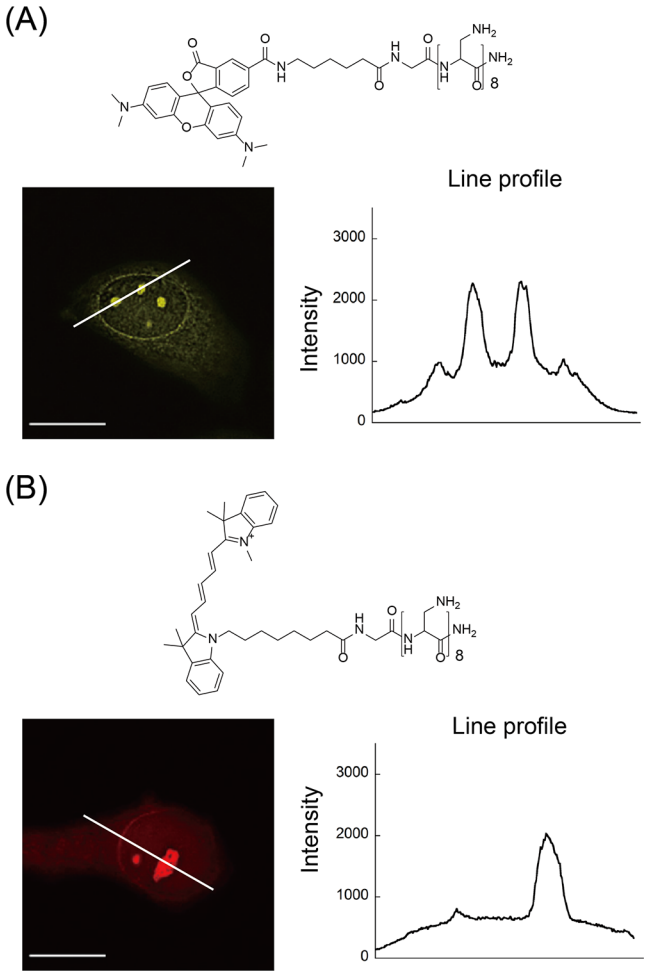

Figure 4. Fluorescence images of MCF7 cells stained by (A) TAMRA-Dap8 (1.0 μM) or (B) Cy5-Dap8 (0.5 μM). Fluorescence intensity profiles along the white line are also shown. Scale bar: 15 μm.

demonstrated that the Dap8 unit could be applied for directing various organic fluorophores to the nucleolus. On the other hand, our probes are unlikely to be used for living cells due to the difficulty in penetration into the membranes in living cells, which is an issue to be addressed in our next studies toward the live-cell imaging applications.[6,7b−g,13] We believe that this work provides valuable insights into the NoLS-based molecular design toward the nucleolus-staining probes as well as the delivery tools targeting the nucleolus.[8]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c00116.

Experimental details, probe synthesis, cellular imaging experiments, fluorescence anisotropy titration experiments, and cytotoxicity test (PDF)

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Author Contributions

M.S. and Y.S. conceived the study. M.S. designed and performed all experiments in cooperation with N.T.. M.S., Y.S., and S.N. wrote the manuscript. Y.S. and S.N. supervised the research.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Lindström, M. S.; Jurada, D.; Bursac, D.; Orsolic, I.; Bartek, J.; Volarevic, S. Nucleolus as an emerging hub in maintenance of genome stability and cancer pathogenesis. *Oncogene* **2018**, *37*, 2351−2366.

(2) Wang, K.-N.; Chao, X.-J.; Liu, B.; Zhou, D.-J.; He, L.; Zheng, X.-H.; Cao, Q.; Tan, C.-P.; Zhang, C.; Mao, Z.-W. Red fluorescent probes for real-time imaging of the cell cycle by dynamic monitoring of the nucleolus and chromosome. *Chem. Commun.* **2018**, *54*, 2635−2638.

(3) Andersen, J. S.; Lam, Y. W.; Leung, A. K.; Ong, S.-E.; Lyon, C. E.; Lamond, A. I.; Mann, M. Nucleolar proteome dynamics. *Nature* **2005**, *433*, 77−83.

(4) Weeks, S. E.; Metge, B. J.; Samant, R. S. The nucleolus: a central response hub for the stressors that drive cancer progression. *Cell. Mol. Life Sci.* **2019**, *76*, 4511−4524.

(5) (a) Gao, P.; Pan, W.; Li, N.; Tang, B. Fluorescent probes for organelle-targeted bioactive species imaging. *Chem. Sci.* **2019**, *10*, 6035−6071. (b) Lin, J.; Yang, K.; New, E. J. Strategies for organelle targeting of fluorescent probes. *Org. Biomol. Chem.* **2021**, *19*, 9339−9357.

(6) Li, Q.; Kim, Y.; Namm, J.; Kulkarni, A.; Rosania, G. R.; Ahn, Y.-H.; Chang, Y.-T. RNA-selective, live cell imaging probes for studying nuclear structure and function. *Chem. Biol.* **2006**, *13*, 615−623.

(7) (a) Law, A. S.-Y.; Lee, L. C.-C.; Lo, K. K.-W.; Yam, V. W.-W. Aggregation and supramolecular self-assembly of low-energy red luminescent alkynylplatinum (II) complexes for RNA detection, nucleolus imaging, and RNA synthesis inhibitor screening. *J. Am. Chem. Soc.* **2021**, *143*, 5396−5405. (b) Cao, C.; Wei, P.; Li, R.; Zhong, Y.; Li, Y.; Xue, F.; Shi, Y.; Yi, T. Ribosomal RNA-selective light-up fluorescent probe for rapidly imaging the nucleolus in live cells. *ACS Sens.* **2019**, *4*, 1409−1416. (c) Yoshino, Y.; Sato, Y.; Nishizawa, S. Deep-red light-up signaling of benzo[*c, d*]indole−quinoline monomethine cyanine for imaging of nucleolar RNA in living cells and for sequence-selective RNA analysis. *Anal. Chem.* **2019**, *91*, 14254−14260. (d) Higuchi, K.; Sato, Y.; Togashi, N.; Suzuki, M.; Yoshino, Y.; Nishizawa, S. Bright and light-up sensing of benzo[*c, d*]indole-oxazolopyridine cyanine dye for RNA and its application to highly sensitive imaging of nucleolar RNA in living cells. *ACS Omega* **2022**, *7*, 23744−23748. (e) Lu, Y.-J.; Deng, Q.; Hu,

D.-P.; Wang, Z.-Y.; Huang, B.-H.; Du, Z.-Y.; Fang, Y.-X.; Wong, W.-L.; Zhang, K.; Chow, C.-F. A molecular fluorescent dye for specific staining and imaging of RNA in live cells: a novel ligand integration from classical thiazole orange and styryl compounds. *Chem. Commun.* **2015**, *51*, 15241−15244. (f) Zhou, B.; Liu, W.; Zhang, H.; Wu, J.; Liu, S.; Xu, H.; Wang, P. Imaging of nucleolar RNA in living cells using a highly photostable deep-red fluorescent probe. *Biosens. Bioelectron.* **2015**, *68*, 189−196. (g) Yao, Q.; Li, H.; Xian, F.; Xu, J.; Xia, J.; Fan, J.; Du, J.; Wang, X.; Peng, X. Differentiating RNA from DNA by a molecular fluorescent probe based on the ″door-bolt″ mechanism biomaterials. *Biomaterials* **2018**, *177*, 78−87.

(8) Louzoun-Zada, S.; Jaber, Q. Z.; Fridman, M. Guiding drugs to target-harboring organelles: stretching drug-delivery to a higher level of resolution. *Angew. Chem., Int. Ed.* **2019**, *58*, 15584−15594.

(9) (a) Dang, C. V.; Lee, W. M. Nuclear and nucleolar targeting sequences of c-erb-A, c-myb, N-myc, p53, HSP70, and HIV tat proteins. *J. Biol. Chem.* **1989**, *264*, 18019−18023. (b) Scott, M. S.; Boisvert, F.-M.; McDowall, M. D.; Lamond, A. I.; Barton, G. J. Characterization and prediction of protein nucleolar localization sequences. *Nucleic Acids Res.* **2010**, *38*, 7388−7399.

(10) (a) Martin, R. M.; Ter-Avetisyan, G.; Herce, H. D.; Ludwig, A. K.; Lättig-Tünnemann, G.; Cardoso, M. C. Principles of protein targeting to the nucleolus. *Nucleus* **2015**, *6*, 314−325. (b) Musinova, Y. R.; Kananykhina, E. Y.; Potashnikova, D. M.; Lisitsyna, O. M.; Sheval, E. V. A charge-dependent mechanism is responsible for the dynamic accumulation of proteins inside nucleoli. *Biochim. Biophys. Acta* **2015**, *1853*, 101−110.

(11) Maeda, Y.; Iwata, R.; Wada, T. Synthesis and properties of cationic oligopeptides with different side chain lengths that bind to RNA duplexes. *Bioorg. Med. Chem.* **2013**, *21*, 1717−1723.

(12) Gruber, H. J.; Hahn, C. D.; Kada, G.; Riener, C. K.; Harms, G. S.; Ahrer, W.; Dax, T. G.; Knaus, H. G. Anomalous fluorescence enhancement of Cy3 and Cy3.5 versus anomalous fluorescence loss of Cy5 and Cy7 upon covalent linking to IgG and noncovalent binding to avidin. *Bioconjug. Chem.* **2000**, *11*, 696−704.

(13) Schröder, T.; Niemerier, N.; Afonin, S.; Ulrich, A. S.; Krug, H. F.; Bräse, S. Peptoidic amino- and guanidinium-carrier systems: targeted drug delivery into the cell cytosol or the nucleus. *J. Med. Chem.* **2008**, *51*, 376−379.

https://doi.org/10.1021/acsomega.3c00116
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