CHEMISTRY
A European Journal

A Journal of
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Accepted Article

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To be cited as: *Chem. Eur. J.* 10.1002/chem.201706034

Link to VoR: http://dx.doi.org/10.1002/chem.201706034

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10.1002/chem.201706034

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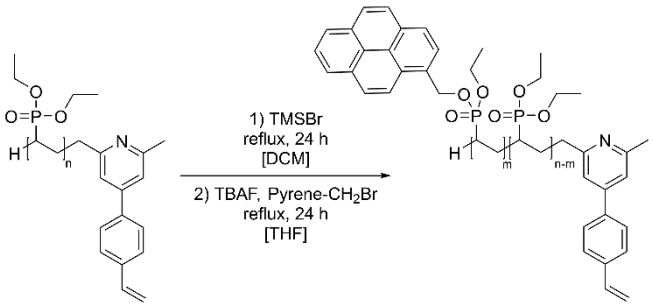
Fluorescent Polyvinylphosphonate Bioconjugates for Selective Cellular Delivery

Christina Schwarzenböck,[a] Andreas Schaffer,[a] Elfriede Nößner,[b] Peter J. Nelson,[c] Ralf Huss[d] and Bernhard Rieger*[a]

Abstract: To date, many poly(ethylene glycol) (PEG) and poly(*N*-isopropylacrylamide) (PNIPAAm) biomolecule conjugates have been described, but they often show long response times, are not bio-inert, or lose function in biological fluids. Herein, we present a modular synthetic approach to generate polyvinylphosphonate biomolecule conjugates. These conjugates exhibit a sharp phase transition temperature even under physiological conditions where few other examples with this property have been described to date. Furthermore, it was feasible to add biological functions to the polymers *via* the conjugation step. The polyvinylphosphonate cholesterol constructs are attached to the cellular membrane and the folic acid anchored polymers are shuttled into the cells. This is an exceptional finding through a straightforward synthetic approach.

Targeting of therapeutic agents to specific cells, or even to compartments in the cells, promises the optimization of therapeutic efficacy, along with minimizing the systemic side effects. Two main targeting strategies exist, of which the active targeting is mostly preferred to the less specific, passive targeting. Active targeting can be driven by antibodies, polysaccharides, biomolecules and manifold other structures.[1] Folic acid is a widely used ligand for the folic acid receptor alpha (FR-α).[2] The binding of folic acid to its receptor drives the uptake *via* endocytosis and therefore can be used to transport agents into a cell.[3] In addition, folic acid is essential for the synthesis of nucleic acids and for the metabolism of amino acids, which are required for cell division.[4] Consequently FR-α is overexpressed on many cancer cells, since they divide rapidly and have an enormous folic acid consumption.[5, 6] By contrast, the anchoring of molecules to the cellular membrane represents a less specific method for cellular engineering. The lipid cholesterol is an important component of the cell wall.[7] It is required for the structure of the cellular membrane and modulates its fluidity.[8] Additionally, a cholesterol homeostasis is maintained in the blood stream. This is mainly regulated by the lipoprotein receptors, which govern cholesterol uptake.[9]

In an earlier study we were able to connect these two disparate biomolecules, *via* thiol-ene click chemistry, to the water-soluble, biocompatible and thermoresponsive polyvinylphosphonates.[10] It was shown that the new conjugates have low toxicities and that the thermoresponsive behavior was maintained in water. However, at this stage it was not possible to report on the biological functions of the novel compounds, since there was no way of monitoring their uptake into cells. Herein, we present the fluorescent labeling of the conjugates *via* the partial transesterification of the polymer side chains. To accomplish this complex task, a strategy employed by our group for the hydrolysis of poly(diethyl vinylphosphonate) (PDEVP) was adapted.[11, 12] The functionalization proceeds *via* the trimethylsilyl ether intermediate. The consecutive deprotection is rendered by tetra-*n*-butylammonium fluoride (TBAF) and followed by the addition of pyrene to the activated positions (Scheme 1). Functionalization degrees vary from 0.38 to 1.13% with 2.00% addressed and are highly dependent on the polymer chain length. The reason for this finding can be attributed to a lower conversion of the side groups in case of long chain PDEVP during the initial ester cleavage with trimethyl silyl bromide (TMSBr). This can be explained by a higher hygroscopy of the long chain polymer resulting in the degradation of TMSBr and therefore in a lower conversion.



Scheme 1. Partial transesterification of side chain groups of poly(diethyl vinylphosphonates).

The thermoresponsive properties were then characterized. All fluorescent samples and polymers from our recent publication were measured in water, and in a medium/phosphate buffer saline solution (DMEM/PBS) containing 1% antibiotics (PS) and 10% fetal bovine serum (FBS) (Table 1).[10] This composition was used in subsequent cell culture experiments. Intriguingly, the lower critical solution temperature (LCST) was retained for all tested polymers, even under these complex conditions. And all the more

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10.1002/chem.201706034

surprising, the three pyrene functionalized long chain polymers showed no LCST transition in water, whereas they have a sharp LCST in DMEM/PBS. The reason why, in case of the 600 eq. fluorescent polymers no phase transitions in water can be measured are supposedly the π-π stacking interactions between the pyrene units and the resulting difficulties in the conformation change necessary for the LCST.[13] The heavy polymers contain more pyrene per chain and therefore pyrene interactions become more likely and their influence gets more prominent than for the lighter polymers. In not presented investigations we could observe a phase transition already in PBS and also in PBS/DMEM without PS and FBS, however the sharp reversible phase transition was only monitored in the presence of FBS. Consequently the main component affecting the thermoresponsive behaviour of the pyrene functionalized polymers is BSA, which was shown in a study of Xu *et al.*[14] to bind to pyrene. Therefore BSA can break up the interactions between the hydrophobic side groups of the functionalized PDEVPs and restore the precise thermoresponse that is known from this polymer class. To the best of our knowledge, the thermoresponsive nature of polymers in complex biological solutions has only been previously investigated by the groups of Kanazawa and Yang.[15, 16] These groups studied the effect of electrolytes and serum on the LCST of poly(*N*-isopropylacrylamide), but DMEM/PBS with antibiotics and FBS was never used before. Consequently, the LCST of our PDEVP conjugates was measured in a fluid that strongly reflects physiological conditions and is also suitable for UV/Vis measurements.

Table 1. Comparison of cloud points (T_c) of PDEVP substrates in aqueous solution and DMEM/PBS (2:1)

Entry	Substrate	T_C [°C] (H_2O)	T_C [°C] (DMEM/PBS)
	Non-fluorescent polymer substrates		
1	100 eq. DEVP	48.0	48.0
2	600 eq. DEVP	43.0	40.0
3	100 eq. DEVP – Cholesterol	40.5	40.5
4	600 eq. DEVP – Cholesterol	40.5	39.5
5	100 eq. DEVP – Folic Acid	52.0	49.0
6	600 eq. DEVP – Folic Acid	43.0	41.0
	Fluorescent polymer substrates		
7	100 eq. DEVP	46.0	38.0
8	600 eq. DEVP	n.a	45.0
9	100 eq. DEVP – Cholesterol	50.5	41.0
10	600 eq. DEVP – Cholesterol	n.a.	42.5
11	100 eq. DEVP – Folic Acid	54.0	45.0
12	600 eq. DEVP – Folic Acid	n.a	42.5

Adjacent photophysical properties were studied. Figure 1 shows the absorbance spectra of all six fluorescent samples. The absorbance of short chain polymers is stronger than of the long ones. This finding is in accordance with the obtained functionalization degrees, which are higher in case of the polymers with lower molecular weight. The absorbance spanned from 200 to a maximal 450 nm. Consequently, all excitation experiments were conducted within this UV light range.

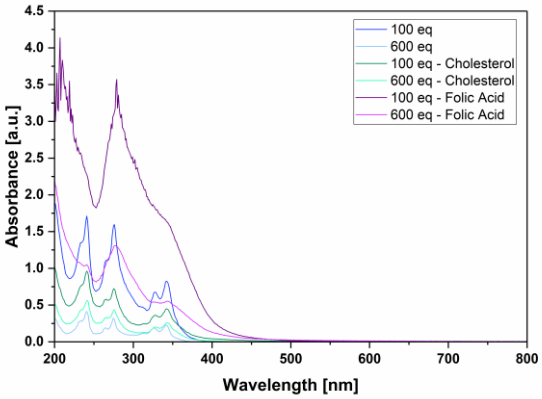

Figure 1. UV/Vis spectra of the fluorescent polymers in aqueous solution (2.5 mg/mL).

The second relevant photophysical property is the emission followed by irradiation, in this case at a 365 nm wavelength. The resulting spectra are presented in figure 2. The first high peak for most polymers results from the irradiation. Their maximum emission was measured between 450 and 500 nm, which corresponds to blue fluorescence.

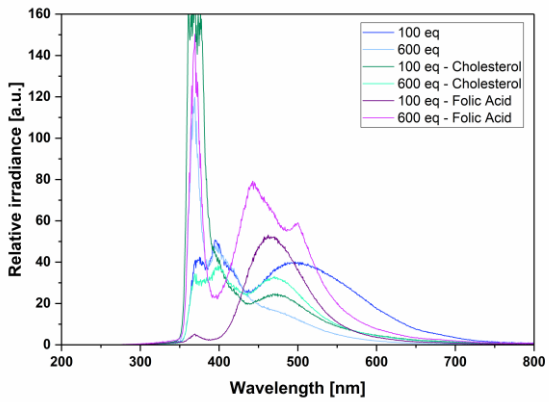

Figure 2. Photoluminescence spectra of the fluorescent polymers in aqueous solution (2.5 mg/mL).

In the next phase of the study, the location of the polymers in the treated cells was investigated. To this end, the toxicity of the fluorescent samples was first evaluated in comparison to the probes lacking pyrene. Pyrene and its metabolites are known to be cytotoxic.[17] The endothelial cell line HMEC-1, and the renal cell line HEK-293 showed reduced viability after treatment with fluorescent PDEVP for 24 or 48 hours (Figure S5-10). For cellular localization studies, a concentration of 1.25 mg/mL and an incubation time of four hours were selected as optimal conditions reflecting low cytotoxic effects and high fluorescence signals. The endothelial cells were treated with the polymers in the localization studies, since the HEK cells grow in foci and are therefore less suitable for microscopic investigations. The most important localization findings are presented in figure 3. Additional images and graphs are shown in figure S11, 12 and 13.

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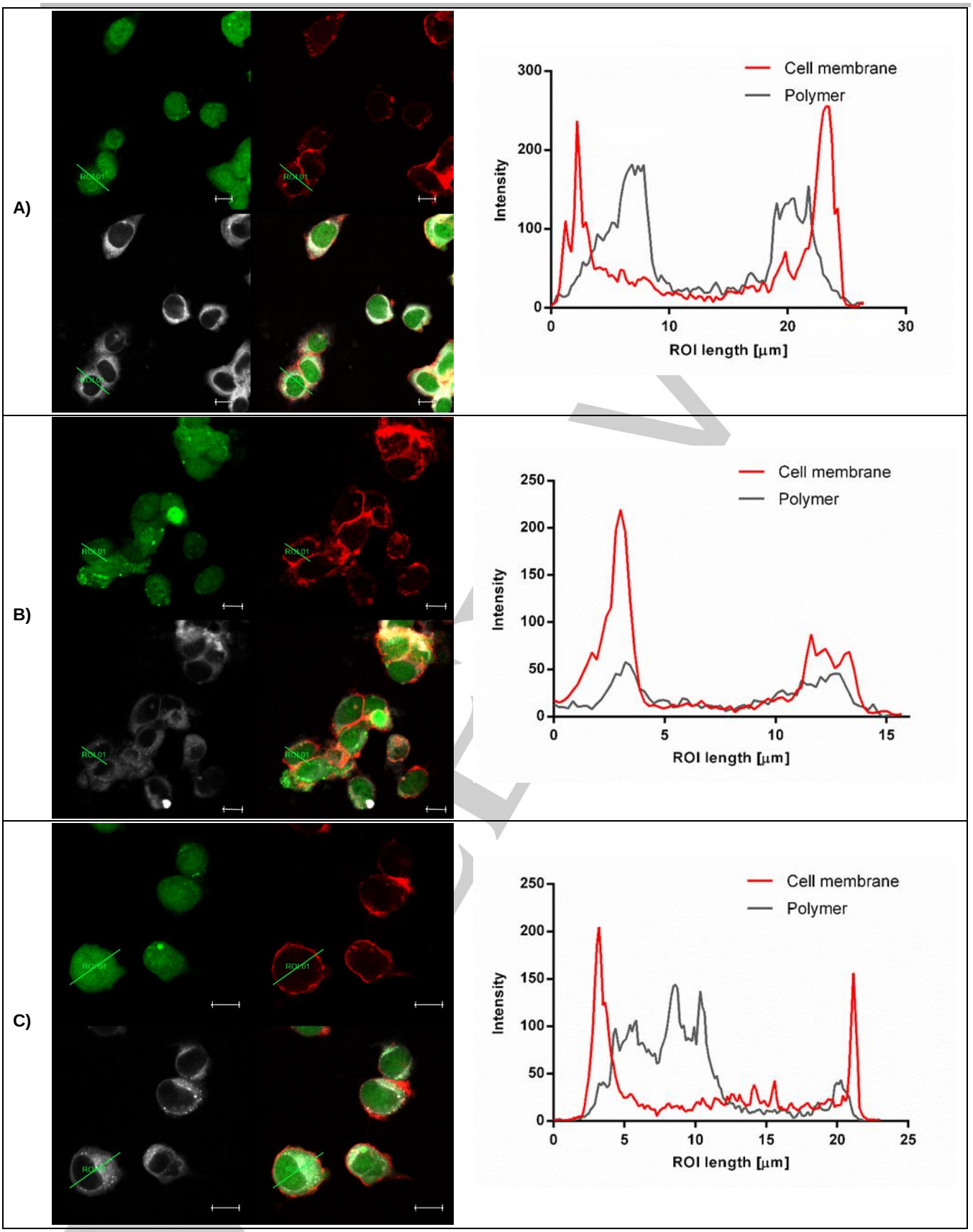

Figure 3. Confocal microscopy images and region of interest (ROI) analysis of HMEC-1 cells treated with A) polymer without anchor, B) polymer with cholesterol and C) polymer with folic acid. Scale bar represents 10 µm.

The three cellular imaging techniques using confocal microscopy are shown in figure 3. They represent green for the cellular cytoplasm, red for the cell membrane and grey for the polymer samples. Green staining was achieved with 5-chloromethylfluorescein diacetate (CMFDA)[18, 19] and red with W6/32, a major histocompatibility complex I specific monoclonal antibody[20] and an rhodamine red™-X (RRX) fluorescent-labeled secondary antibody. CMFDA enters cells through the plasma membrane where it is converted into its fluorescent derivative. W6/32 was chosen as primary antibody, since the group of Sepp[21] showed expression of MHC I antigens on the surface of HMEC-1 cells. The graphs on the right side of each image in figure 3 are the region of interest (ROI) plots of RRX and the polymers fluorescence to quantify, and locate, the fluorescence intensity of the applied polymer samples. Figure 3A shows that PDEVP without a targeting molecule can reach the inside of the cell. This is an important finding, consistent with our previous observation that micelles from 2-vinylpyridine-DEVP block copolymers can be taken up by HeLa cells.[22] A crucial observation was the localization of the cholesterol functionalized polymers at the cellular membrane demonstrated by the fluorescence co-localization of the plasma membrane stain W6/32-RRX and the polymer (Fig 3B, right panel). They are located to the cell wall and on its inner face of the membrane (See Figure 3B, S12, and 13). To the best of our knowledge, this is the first report of such an anchoring of thermoresponsive polymers to the plasma membrane. Many examples exist where cholesterol is the hydrophobic block of a polymeric micelle,[23-25] or where it has been added to liposomes for enhanced stability or uptake,[26-28] whereas our novel fluorescent PDEVP–cholesterol conjugates could be localized directly in the cellular membrane. The only studies existing about polymer-cholesterol conjugates are PEG and chitosan based ones, which are not thermoresponsive.[29-31] In contrast, the folic acid conjugates showed direct uptake into the cells (Figure 3C). Thus, it is possible to regulate the localization of PDEVP through the conjugation of one single biomolecule to the polymer chain. Consequently, a diverse potential application spectrum can be envisioned from targeted drug delivery in cancer research to the engineering of the cell membrane, including the selective addition of chemical functions to cells.

To conclude, it was possible to fluorescently label PDEVP–cholesterol and folic acid conjugates. The new macromolecules remained water-soluble and their LCST behavior was retained in biological fluids. Of capital importance was the observation that the polymer characteristics depend on their anchor unit. The attached biomolecules can regulate the localization of the polymer in the cell. Hence, this modular synthetic strategy opens the door to manifold applications and a plurality of functionalization options.

Acknowledgements

The authors thank Philipp Pahl for proof reading. We thank Anke Fischer and Sylke Rohrer for their support with the *in vitro* experiments.

Conflict of interest

The authors declare no conflict of interest.

Keywords: delivery • fluorescent polymer bioconjugate • selective cellular targeting • thermoresponsive polymer • thiol-ene click chemistry

References

[1] Y. Zhong, F. Meng, C. Deng, Z. Zhong, *Biomacromolecules* **2014**, *15*, 1955-1969.
[2] D. J. O'Shannessy, E. B. Somers, E. Albone, X. Cheng, Y. C. Park, B. E. Tomkowicz, Y. Hamuro, T. O. Kohl, T. M. Forsyth, R. Smale, Y.-S. Fu, N. C. Nicolaides, *Oncotarget* **2011**, *2*, 1227-1243.
[3] S. Gorle, M. Ariatti, M. Singh, *Int. Proc. Chem. Biol. Environ. Eng.* **2013**, *52*, 1-4.
[4] I. o. Medicine, *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline*, The National Academies Press, Washington, DC, **1998**.
[5] S. D. Weitman, R. H. Lark, L. R. Coney, D. W. Fort, V. Frasca, V. R. Zurawski, B. A. Kamen, *Cancer Res.* **1992**, *52*, 3396-3401.
[6] E. C. Calvaresi, P. J. Hergenrother, *Chem. Sci.* **2013**, *4*, 2319-2333.
[7] P. L. Yeagle, *Biochimie* **1991**, *73*, 1303-1310.
[8] P. L. Yeagle, *Biochim. Biophys. Acta* **1985**, *822*, 267-287.
[9] M. S. Brown, P. T. Kovanen, J. L. Goldstein, *Science* **1981**, *212*, 628-635.
[10] C. Schwarzenböck, A. Schaffer, P. Pahl, P. J. Nelson, R. Huss, B. Rieger, *Polym. Chem.* **2017**.
[11] S. Salzinger, U. B. Seemann, A. Plikhta, B. Rieger, *Macromol.* **2011**, *44*, 5920-5927.
[12] S. Salzinger, B. Rieger, *Macromol. Rapid Commun.* **2012**, *33*, 1327-1345.
[13] H. Ringsdorf, J. Venzmer, F. M. Winnik, *Macromol.* **1991**, *24*, 1678-1686.
[14] C. Xu, J. Gu, X. Ma, T. Dong, X. Meng, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* **2014**, *125*, 391-395.
[15] Y. Hiruta, Y. Nagumo, Y. Suzuki, T. Funatsu, Y. Ishikawa, H. Kanazawa, *Colloids Surf., B* **2015**, *132*, 299-304.
[16] C.-S. Chaw, K.-W. Chooi, X.-M. Liu, C.-w. Tan, L. Wang, Y.-Y. Yang, *Biomaterials* **2004**, *25*, 4297-4308.
[17] T. Yoshikawa, L. P. Ruhr, W. Flory, D. Giamalva, D. F. Church, W. A. Pryor, *Toxicol. Appl. Pharmacol.* **1985**, *79*, 218-226.
[18] R. G. Breuls, A. Mol, R. Petterson, C. W. Oomens, F. P. Baaijens, C. V. Bouten, *Tissue Eng.* **2003**, *9*, 269-281.
[19] V. Lulevich, Y.-P. Shih, S. H. Lo, G.-y. Liu, *J. Phys. Chem. B* **2009**, *113*, 6511-6519.
[20] P. Parham, C. J. Barnstable, W. F. Bodmer, *J. Immunol.* **1979**, *123*, 342-349.
[21] V. A. Nguyen, C. Fürhapter, N. Sepp, *Microvasc. Res.* **2001**, *62*, 204-207.
[22] P. T. Altenbuchner, P. D. L. Werz, P. Schöppner, F. Adams, A. Kronast, C. Schwarzenböck, A. Pöthig, C. Jandl, M. Haslbeck, B. Rieger, *Chem. Eur. J.* **2016**, *22*, 14576-14584.
[23] W. Y. Seow, J. M. Xue, Y.-Y. Yang, *Biomaterials* **2007**, *28*, 1730-1740.
[24] S. Sevimli, F. Inci, H. M. Zareie, V. Bulmus, *Biomacromolecules* **2012**, *13*, 3064-3075.
[25] H.-T. Jiang, K. Ding, F.-N. Meng, L.-L. Bao, Y.-D. Chai, Y.-K. Gong, *J. Mater. Chem. B* **2016**, *4*, 5464-5474.
[26] K. Kusumoto, H. Akita, A. El-Sayed, H. Harashima, *Biol. Pharm. Bull.* **2012**, *35*, 445-448.
[27] H. Yin, H. C. Kang, K. M. Huh, Y. H. Bae, *Colloids Surf., B* **2014**, *116*, 128-137.
[28] C. Martin, N. Marino, C. Curran, A. P. McHale, J. F. Callan, B. Callan, *Int. J. Pharm.* **2016**, *511*, 570-578.
[29] H.-R. Jia, H.-Y. Wang, Z.-W. Yu, Z. Chen, F.-G. Wu, *Bioconjugate Chem.* **2016**, *27*, 782-789.
[30] X. Chen, X. Zhang, H.-Y. Wang, Z. Chen, F.-G. Wu, *Langmuir* **2016**, *32*, 10126-10135.
[31] H.-Y. Wang, J. Sun, L.-Y. Xia, Y.-H. Li, Z. Chen, F.-G. Wu, *ACS Biomaterials Science & Engineering* **2017**, *3*, 2570-2578.

10.1002/chem.201706034

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Layout 2:

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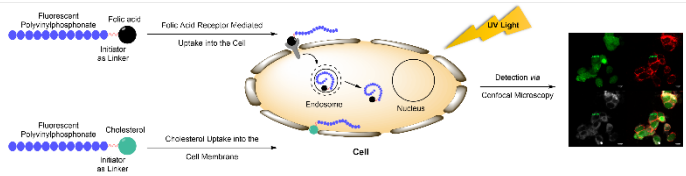

Fluorescent polyvinylphosphonates were obtained *via* post-polymerization transesterification. Thiol-ene click reactions led to fluorescent cholesterol– and folic acid–functionalized polyvinylphosphonates. Turbidity measurements in complex biological fluids could show the retention of the thermoresponsive properties. Concluding the confocal microscopy experiments proved to selective cellular uptake of the bioconjugates into the cells or its anchoring to the plasma membrane.

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