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Liquid-Phase Synthesis of a Pegylated Adenosine–Oligoarginine Conjugate, Cell-Permeable Inhibitor of cAMP-Dependent Protein Kinase

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Abstract—An adenosine–oligoarginine conjugate (ARC) was assembled in a stepwise manner on a poly(ethylene glycol) carrier. The pegylated conjugate inhibited cAMP-dependent protein kinase with $IC_{50} = 460$ nM and the cellular uptake of its BODIPY FL derivative was demonstrated and compared to that of free ARC with fluorescence microscopy.

Recently, we described a series of inhibitors for basophilic protein kinases whose design was based on the bisubstrate analogue strategy.[1] Bisubstrate inhibitors structurally mimic both substrates of a kinase and simultaneously interact with the respective binding domains of the enzyme.[2] The inhibitors designed by us comprised moieties targeted at the ATP binding site, adenosine-5′-carboxylic acid (Adc), and the protein/peptide substrate binding site directed oligoarginine. These motifs were connected via a linker chain whose structure was optimized in QSAR studies.[1]

Later, we demonstrated that adenosine-oligoarginine conjugates (ARC) were cell-permeable[3] which pointed to the potential applicability of the bisubstrate analogue approach for rational design of potent and selective inhibitors for the regulation of the intracellular protein phosphorylation equilibrium.[2,4]

ARC-s as peptide-type compounds are prone to proteolytic degradation, therefore enhancement of their stability in order to prolong their in vivo and intracellular half-lives is of high importance. Moreover, the applicability of the compounds in vitro and in vivo experiments demands regulation of their cellular uptake and intracellular targeting.

Poly(ethylene glycol) (PEG) has been the most popular polymer for modification of peptide and protein-type drugs.[5] Pegylation (derivatization with PEG) has helped to increase the half-life of such drugs in blood plasma, reduce immunogenecity and improve resistance to proteolysis.[5] Pegylation of a peptidic drug can lead to the modification of the drug at unpredictable positions resulting in difficulties of separation and purification of these positional isomers.[6]

Liquid-phase organic synthesis (LPOS), the preparation of target compounds on soluble polymeric carriers (most often PEG), has made a remarkable progress during last decades.[7] This method is especially suitable for a stepwise synthesis of short (up to 20 units) chain-type molecules (repetitive-type synthesis). LPOS has a great advantage over solid-phase synthesis: it enables the application of sophisticated methods of structural analysis for the monitoring of synthetic reactions and quantification of the target product and intermediates. This is especially important in the case of introduction of new reaction schemes and synthetic procedures. These advantages have been convincingly demonstrated

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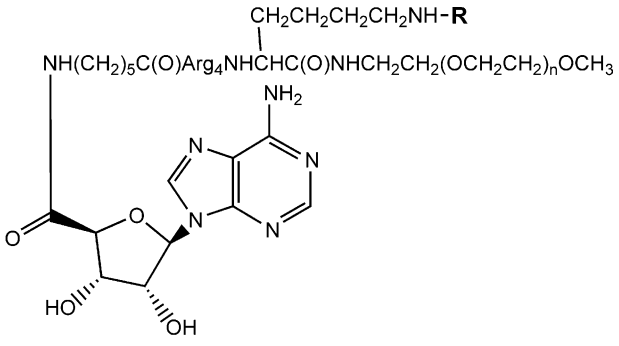
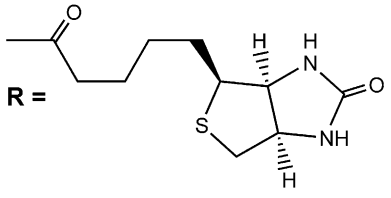
for the preparation of oligonucleotides[8] and peptides.[9] Moreover, the synthesis of a pegylated drug on polymer carriers in a stepwise manner eliminates the problem of site-specific attachment of the polymer chain to the active compound.

The aim of the present study was the production of pegylated ARC-s by the liquid-phase synthetic strategy, characterization of the ability of polymer-bound conjugates to inhibit protein kinases and establishment of cellular uptake characteristics of the compounds.

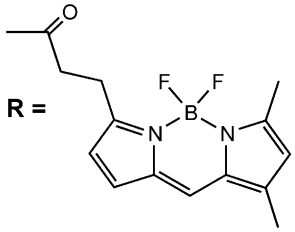
AdcAhxArg$_4$Lys-PEG$_{5000}$-OMe (Fig. 1), a pegylated adenosine–oligoarginine conjugate, was synthesized by the conventional Fmoc-peptide chemistry coupling/deprotection methods on H_2N-PEG-OMe support (average molar mass 5000).[10] Amino-PEG was chosen as the polymeric carrier because of its structural simplicity and the necessity for a stable connection between the active moiety and polymer. In addition, it provides the possibility for insertion of a suitable anchoring unit (well-known in solid-phase peptide synthesis) between the drug and the polymer in order to liberate a free target compound from the polymer, if necessary, at the final treatment.[9]



R = H
AdcAhxArg$_4$Lys-PEG-OMe



AdcAhxArg$_4$Lys(biotin)-PEG-OMe



AdcAhxArg$_4$Lys(BODIPY FL)-PEG-OMe

Figure 1. Structures of pegylated ARC-s.

The side-chain protected amino acids and Adc were coupled to the polymer in a stepwise manner (Fig. 2, cycles 1–7) to obtain the corresponding protected conjugate Adc(Ip)Ahx[Arg(Pbf)]$_4$Lys(Boc)-PEG-OMe. The removal of the protection groups with trifluoroacetic acid (TFA) treatment yielded a pegylated ARC, which was further tagged by a fluorescent marker BODIPY FL or biotin at ε-amino group of lysine for cellular uptake experiments.

The first two amino acid residues (L-Lys and L-Arg) were coupled as preformed symmetrical anhydrides. However, long reaction times (the coupling of the second arginine was not complete even after double 2-h treatment) forced us to replace it by a more efficient diisopropyl carbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) coupling. The reactions were monitored on TLC plates (visualization with ninhydrin). PEG absorbs iodine vapor and this helped us to observe free PEG and PEG-connected compounds on TLC plates. The Fmoc-protected polymer becomes visible under illumination with UV light on TLC plates with a fluorescent indicator. Fmoc-blocked polymers could also be easily quantified with UV spectrophotometry.

The polymer-bound products were separated after each coupling and deprotection step by precipitation with an excess of solvent, centrifugation and decantation. After the attachment of the first Pbf-Arg, methyl *t*-butyl ether (MTBE)-precipitated product yielded viscous, impure oil, whereas precipitation with isopropanol[11] gave a fine voluminous pellet. All precipitates were pure (TLC) after 2-fold recrystallization.

The structures of pegylated target products and intermediates could be reliably verified and the completeness of the reactions estimated (comparison of the area of new hydrogen signals with that of the signal of the terminal methyl group of PEG-OMe) by ^{1}H NMR spectroscopy (Fig. 3). A large amount of the conjugate was needed for NMR analysis but the deuterated solvent (methanol or chloroform) could be easily removed after analysis and the synthesis continued. CD_3OD was the best-suited solvent for NMR analysis of pegylated products. The results of NMR spectroscopy were corroborated by MALDI MS (Fig. 4).

It has been previously reported that chromatographic methods are not always effective for the separation and

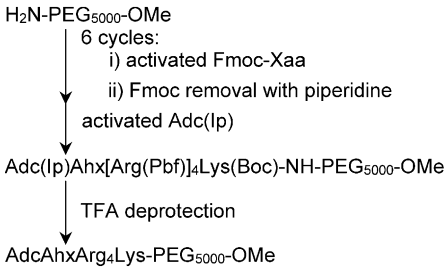


Figure 2. Synthesis of pegylated ARC. Xaa = L-Lys (Boc), L-Arg (Pbf) or Ahx (Pbf: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; Ip: 2′,3′-*O*-isopropylidene).

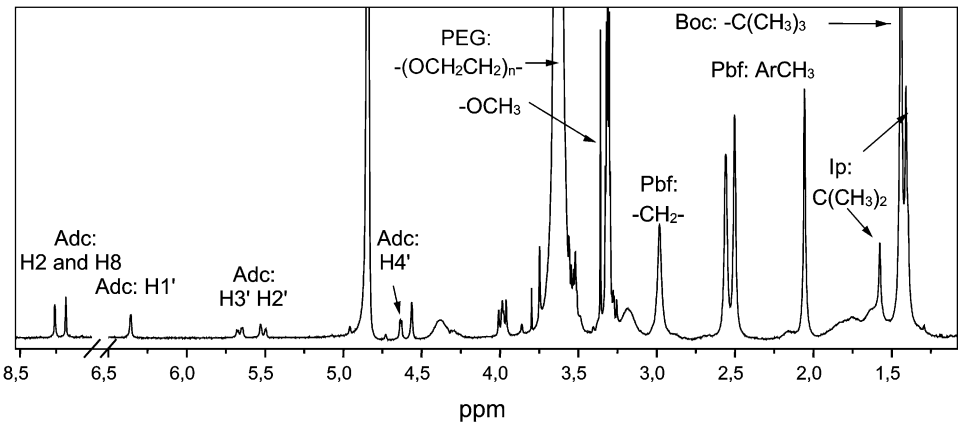


Figure 3. ^{1}H NMR spectrum (Bruker AC 200) of Adc(Ip)Ahx[Arg(Pbf)]$_4$Lys(Boc)-PEG-OMe (75 mg/mL, CD_3OD). PEG: 3.36 (s, OCH_3), 3.63 (broad, ethylene); Pbf: 2.05, 2.50 and 2.56 (3×broad, 12×$ArCH_3$), 2.98 (broad, 3×CH_2); Boc: 1.44 (s, 3×CH_3); amino acids: 1.6–2.0 (broad, side-chain CH_2), 3.0–3.3 (broad, –CH_2NH), 4.2–4.5 (broad, α-H of amino acids), Adc(Ip): 1.41 and 1.57 (2×s, C$(CH_3)_2$), 4.63 (d, J = 1.7 Hz, H-4′), 5.51 (m, J = 6.1 Hz, H-2′), 5.66 (dd, J = 6.1 and 1.7 Hz, H-3′), 6.36 (m, H-1′), 8.18 and 8.26 (2×s, H-2 and H-8); water 4.8–4.9 (broad); MeOH 3.31 (m).

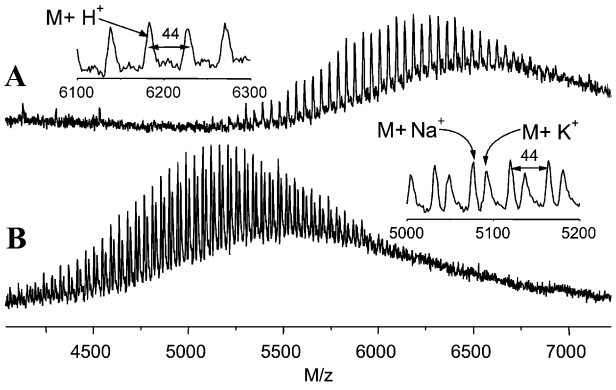


Figure 4. MS analysis (time-of-flight MALDI mass spectrometer, built at the National Institute of Chemical Physics and Biophysics, Tallinn, Estonia) of poly(ethylene glycol) derivatives. AdcAhxArg$_4$Lys-NHCH$_2$CH$_2$(OCH$_2$CH$_2$)$_n$OMe (A), M: n = 113, M_r = 6182 and NH$_2$CH$_2$CH$_2$(OCH$_2$CH$_2$)$_n$OMe (B), M: n = 113, M_r = 5053. The samples were prepared from water solutions with 2,5-dihydroxy benzoic acid (DHB) matrix.

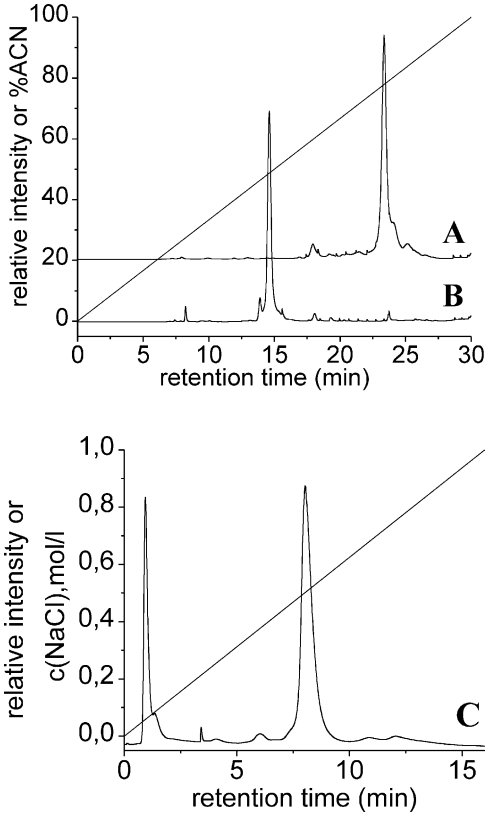


Figure 5. HPLC analysis of crude products. Adc(Ip)Ahx[Arg(Pbf)]$_4$ Lys(Boc)-PEG-OMe (A) and AdcAhxArg$_4$Lys-PEG-OMe (B), RP C18 column Inertsil ODS-3 (GL Sciences Inc.), 5 μm, linear water (0.1% TFA)–acetonitrile gradient, 1 mL/min. AdcAhxArg$_4$Lys-PEG-OMe (C), cation exchange column Mono S HR 5/5 (Pharmacia Biotech), linear gradient 0.0–1.0 M NaCl in 40 mM phosphate buffer (10% MeOH), 1 mL/min. Detection at 260 nm.

purification of pegylated compounds. We obtained good purification yields of pegylated products with both, ion exchange and reversed phase HPLC (Fig. 5).

The potency of two new pegylated ARC-s to inhibit cAMP-dependent protein kinase (PKA) was evaluated[12] by a previously used protocol for assaying kinase inhibitors.[13] The IC_{50} values (Fig. 6) for AdcAhxArg$_4$Lys-PEG-OMe (0.46 μM) and AdcAhxArg$_4$Lys(biotin)-PEG-OMe (1.2 μM) are very similar to the respective values of free ARC-s AdcAhxArg$_4$NH(CH$_2$)$_6$NH$_2$ (0.68 μM) and AdcAhxArg$_6$ (0.70 μM). It is remarkable that the attachment of a long and hydrophilic polymer to an ARC-type inhibitor has little effect on its inhibitory activity. Furthermore, the tethering of a biologically important ligand biotin to the side chain of lysine in AdcAhxArg$_4$Lys-PEG-OMe leads to a marginal (2.5 times) increase in IC_{50} value. In the same assay conditions (Fig. 6) inhibitory activities of ARC-s were compared to that of H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide, a commercially available potent inhibitor of PKA (IC_{50} = 0.135 μM[13]).

It has been established that free ARC efficiently entered cells in culture and was capable to convey a protein into the cells.[3] Pegylation of ARC renders the molecule substantially bigger and more hydrophilic and such changes in the structure of a compound are considered to reduce the cellular uptake.[14] However, pegylated ARC is taken up by HeLa cells in culture[15] as confirmed by fluorescence microscopy using the derivative labeled with

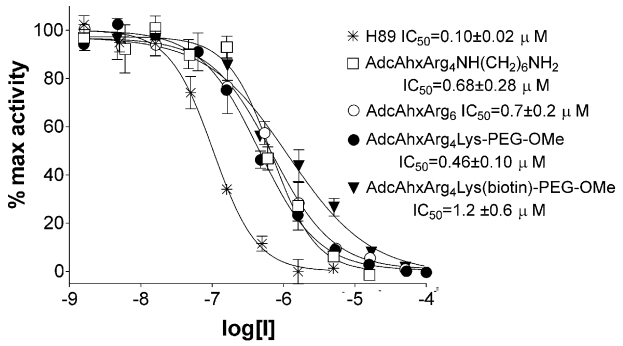


Figure 6. Inhibition of PKA by ARC-s and H-89.

BODIPY FL (Fig. 7A). As expected, upon modification of ARC with PEG_{5000}, the cell-penetrating ability is impaired and less fluorescent derivative is detected in HeLa cells after 1 h incubation as compared to uptake of the parent ARC. The cellular localization pattern of ARC(BODIPY)-PEG is also slightly different form that of ARC(BODIPY). The latter compound is rather evenly distributed over the interior of HeLa cells with some preference for vesicular structures (Fig. 7B). Pegylated ARC resides in cells mainly granularly, exhibiting perinuclear accumulation in vesicular structures. The localization of ARC(BODIPY)-PEG mostly in vesicular structures points to endocytosis as the major route of its cell entry. However, even in unfixed cells a weak uniform labeling of cytoplasm is detectable after incubation with ARC(BODIPY)-PEG.

Biotin-tagged ARC-PEG readily complexes with avidin. The supplementation of cell-culture medium with fluorescein isothiocyanate (FITC)-labeled avidin along with biotinylated ARC leads to a remarkable cellular uptake of formed fluorescent avidin-ARC complexes (Fig. 7C and D). Again, the biotin-tagged ARC promotes cellular internalization of avidin with higher efficiency than pegylated ARC. Moreover, the localization patterns of avidin complexes with ARC and pegylated ARC have substantially different character. Complexes ARC(biotin)-PEG/avidin-FITC associate readily with the plasma membrane of HeLa cells, labeling rather uniformly the cell surface, including fine structures of the membrane (Fig. 7C). ARC(biotin)/avidin-FITC complexes, on the contrary, are more confined to vesicular structures of remarkable size (Fig. 7D). The avidin-ARC containing vesicular structures seem to be mainly located beneath the plasma membrane and become concentrated in the perinuclear region. However, despite the mostly membranous localization, the avidin complexes with pegylated ARC are also taken up and concentrated in perinuclear structures of HeLa cells.

We conclude that both the adenosine–oligoarginine conjugate and its pegylated derivative penetrate into

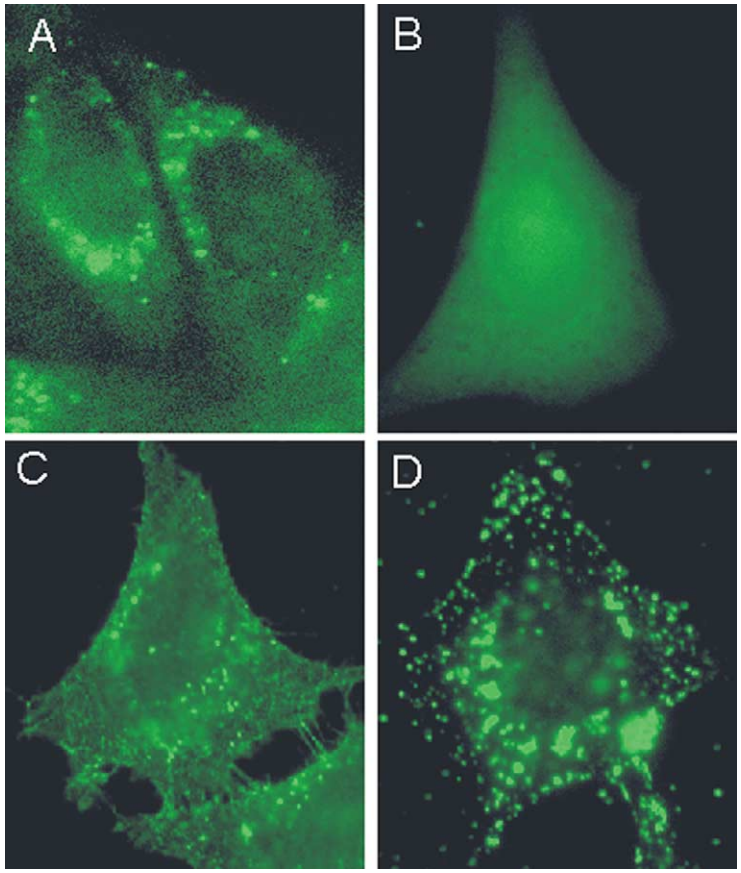


Figure 7. Uptake of adenosine-oligoarginine conjugates by HeLa cells. Localization of $AdcAhxArg_4Lys$(BODIPY FL)-PEG-OMe (A) and $AdcAhxArg_4NH(CH_2)_6NH$(BODIPY FL)[3] (B) after incubation of HeLa cells with 5 μM solution of either compound (37 °C, 1 h, cell incubator). Cellular distribution of avidin–FITC complexes with $AdcAhxArg_4Lys$(biotin)-PEG-OMe (C) or $AdcAocArg_4NH(CH_2)_6NH$(biotin)[3] (D) after coincubation of HeLa cells with 5 μM solution of the conjugate and avidin-FITC (10 μg/mL). Aoc, 8-amino-octanoic acid.

cells in culture and have the potential to be used for inhibition of protein kinases in living cells. Modification of ARC with PEG presumably increases its stability towards proteolysis, but on the other hand decreases its cellular translocation efficiency. ARC is capable of inducing cellular uptake of proteins bound to it. ARC-PEG is able to convey the complexed protein into the plasma membrane of target cells, but induces internalization of these complexes to a lower extent than unmodified ARC.

Acknowledgements

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10. **Synthetic procedures**. The synthesis was carried out in a 50-mL plastic centrifuge tube. TLC plates (Macherey-Nagel, Polygram Sil G/UV_{254}) were eluted with $CHCl_3$/MeOH 17:3 v/v. **Adc(Ip)Ahx[Arg(Pbf)]$_4$Lys(Boc)-PEG-OMe:** 0.06 mmol aminoethyl poly(ethylene glycol) monomethyl ether (Fluka, 300 mg) was coupled with a symmetrical anhydride (3 equiv, compared to terminal amino groups of the polymer), or a mixture of amino acid, DIC, HOBt (5 equiv each) in DMF (0.5–1 mL). Additionally, 10 equiv of diisopropyl ethyl amine (DIEA) was used for Adc(Ip). Reaction times were 1 h for FmocLys(Boc) (Advanced ChemTech) and 1.5 h for Fmoc-Arg(Pbf) (Novabiochem) with the first coupling system, or 20 min for FmocArg(Pbf) and 1 h for FmocAhx (Advanced ChemTech) and Adc(Ip) with DIC/HOBt activation, as monitored with ninhydrin test. Fmoc-groups were removed with piperidine/DMF (1:4 v/v, 20 min). TLC: R_f 0.7, symmetrical spot, absorbs UV and I_2. RP HPLC (Fig. 5A): elution at 76% ACN. The structure was verified with ^{1}H NMR (Fig. 3) and MALDI TOF MS (calcd: 2279 + polymer, found: wide max with average mass 7400). Yield 50%.
AdcAhxArg$_4$Lys-PEG-OMe. Boc, Ip and Pbf groups were removed from the previous product with TFA/H_2O/TIS/DCE (85:5:5:5 by volume, 1 mL, 2 h, TIS: triisopropyl silane, DCE: 1,2-dichloroethane). The reaction was monitored with TLC (R_f 0.0, absorbs I_2 and UV, purple color with ninhydrin). RP HPLC (Fig. 5B): symm. peak at 49% ACN. Ion-exchange HPLC (Fig. 5C): symm. peak at 0.5 M NaCl. MS (Fig. 4A): calcd 1129 + polymer, found protonated molecular ions with average mass 6200. ^{1}H NMR (CD_3OD): polymer: 3.36 (s, OCH_3), 3.63 (broad, ethylene); amino acids: 1.6–2.0 (broad, side-chain CH_2), 3.0–3.4 (broad, –CH_2NH), 4.2–4.4 (broad, α-H); Adc: 4.44 (dd, J = 2.4 and 4.5 Hz, H-3′), 4.53 (d, J = 2.4 Hz, H-4′), 6.17 (d, J = 6.6 Hz, H-1′) 8.53 and 8.69 (2×s, H-2 and H-8); water 4–5.0 (broad). Cleavage yield ~70%.
AdcAhxArg$_4$Lys(BODIPY FL)-PEG-OMe. AdcAhxArg$_4$Lys-PEG-OMe (3.3 mg, 0.5 μmol) was treated with BODIPY FL succinimidyl ester (0.2 mg, 1 equiv, Molecular Probes) and DIEA (5 μL) in DMF (55 μL) for 2 h. DMF was removed under vacuum and the obtained material purified by RP HPLC (peak at 46% ACN). TLC: R_f 0.0, pink spot. UV–vis λ_{max}: 260 and 502 nm. MS: calcd 1404 + polymer, found M + H$^+$ peaks with average mass 6600.
AdcAhxArg$_4$Lys(biotin)-PEG-OMe. AdcAhxArg$_4$Lys-PEG-OMe (5.5 mg, 0.89 μmol) was reacted with biotin 4-nitrophenyl ester (1.0 mg, 3 equiv, Aldrich) and DIEA (5 μL) in DMF (50 μL) for 12 h. No reaction with ninhydrin on TLC plate revealed disappearance of reactive amino groups. The product was separated analogously to BODIPY-labeled compound. RP HPLC: symm. peak at 47% ACN. MS: calcd 1356 + polymer, found M + H$^+$ peaks with average mass 6400. UV λ_{max}: 260 nm.
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15. Internalization of ARC, its derivatives and their complexes with avidin-FITC by HeLa cells, was assessed and documented essentially as previously described.[3] HeLa cells were fixed with 3% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3 in order to maintain intact cell plasma membranes.