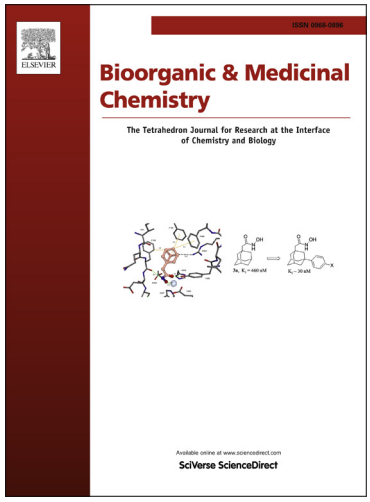


Fine tuning of the pH-dependent drug release rate from polyHPMA-ellipticinium conjugates

Ondřej Sedláček, Martin Studenovsky, David Větvička, Karel Ulbrich, Martin Hrubý

PII: S0968-0896(13)00659-7
DOI: http://dx.doi.org/10.1016/j.bmc.2013.07.038
Reference: BMC 11002

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 16 May 2013
Revised Date: 17 July 2013
Accepted Date: 19 July 2013

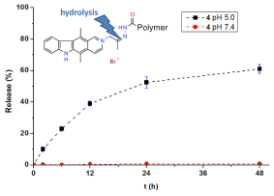
Please cite this article as: Sedláček, O., Studenovsky, M., Větvička, D., Ulbrich, K., Hrubý, M., Fine tuning of the pH-dependent drug release rate from polyHPMA-ellipticinium conjugates, *Bioorganic & Medicinal Chemistry* (2013), doi: http://dx.doi.org/10.1016/j.bmc.2013.07.038

Graphical Abstract

Fine Tuning of the pH-Dependent Drug Release Rate from PolyHPMA-Ellipticinium Conjugates

Leave this area blank for abstract info.

Ondrej Sedlacek, Martin Studenovsky, David Vetvicka, Karel Ulbrich, Martin Hruby
Institute of Macromolecular Chemistry AS CR, v.v.i., Heyrovsky Sq. 2, 162 06 Prague 6, Czech Republic



Bioorganic & Medicinal Chemistry
journal homepage: www.elsevier.com

Fine tuning of the pH-dependent drug release rate from polyHPMA-ellipticinium conjugates

Ondřej Sedláček[a], Martin Studenovsky[a], David Větvička[b], Karel Ulbrich[a], Martin Hrubý [a*]

[a] *Institute of Macromolecular Chemistry AS CR, v.v.i., Heyrovsky Sq. 2, 162 06 Prague 6, Czech Republic*
[b] *Institute of Biophysics and Informatics, First Faculty of Medicine, Charles University in Prague, Salmovska 1, 120 00 Prague 2, Czech Republic*

ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
ellipticine
controlled release
hydrazone
polyHPMA
drug delivery

ABSTRACT

Polymer conjugates of anticancer drugs have shown high potential for assisting in cancer treatments. The pH-labile spacers allow site-specific triggered release of the drugs. We synthesized and characterized model drug conjugates with hydrazide bond-containing poly[N-(2-hydroxypropyl)methacrylamide] differing in the chemical surrounding of the hydrazone bond-containing spacer to find structure-drug release rate relationships. The conjugate selected for further studies shows negligible drug release in a pH 7.4 buffer but released 50% of the ellipticinium drug within 24 h in a pH 5.0 phosphate saline buffer. The ellipticinium drug retained the antiproliferative activity of the ellipticine.

1. Introduction

Ellipticine **(**5,11-dimethyl-6H-pyrido[4,3-b]carbazole**)** is an isoquinoline-type alkaloid that naturally occurs in the *Ochrosia elliptica* plant.[1] Ellipticine shows a strong antineoplastic effect based mainly on the DNA intercalation and topoisomerase II inhibition.[2-6] However, the low water solubility of ellipticine prevented it from entering clinical trials.[7] Therefore, its water-soluble derivatives with a quaternary ammonium structural motif were synthesized.[8] These include elliptinium (Celiptium®, i.e., 2-methyl-9-hydroxyellipticine),[9, 10] methoxycelliptium (9-methoxy-2-methyl-ellipticine)[11] datelliptium [2(diethylamino)ethyl-9-hydroxyellipticine],[12] retelliptine (1-diethyl-aminopropylamino-9-methoxyellipticine),[13] elliprabin (ε-arabinosyl-9-hydroxyellipticine)[14, 15] or the bis-ellipticinium derivative ditercalinium.[15] From these, elliptinium and datelliptium have been used for the treatment of advanced breast cancer.[9] However, severe side effects, including nephrotoxicity,[16] renal toxicity, hemolysis,[17] xerostomia, nausea and vomiting,[18] have been observed.

The targeted delivery and controlled release using water-soluble polymer carriers became a widely studied approach to improve the solubility and circumvent the side effects of anticancer drugs.[19-22] The drug is attached to a polymer carrier. This biologically inactive prodrug conjugate is delivered to the cancer tissue, where it accumulates through e.g., Enhanced Permeation and Retention (EPR)[23, 24] of macromolecules or ligand-based targeting.[25, 26] The conjugate releases its cargo in a biologically active form in the tissues or cells of tumors. A suitable linker between the drug and the carrier is essential for such a delivery system.[27] This linker must be stable during the transport through the blood and must rapidly hydrolyze to release the drug in the tumor, triggered by various factors such as a pH change after crossing from the blood into the tumor or tumor cell environments.[1]

A hydrazone bond was first used as a part of the spacer connecting doxorubicin to a polymer carrier,[28-30] and it quickly became popular for the synthesis of polymer conjugates with pH-controlled drug release.[31] It is relatively stable in pH of blood (7.4), thus allowing the derivative to reach the tumor tissue without a substantial chemical change. However, in the mildly acidic environment of the tumor interstitial space (pH variable around 6.5 depending on actual oxygen supply and metabolic activity in each part of the tumor) or even in the pH of late endosomes (as low as pH 5.0), the drug cargo rapidly releases from the polymer.[32, 33] The release profile of the drug can be strongly influenced by variations in the chemical structure of the spacer in the vicinity of the hydrazone bond, and it can be fine-tuned for the delivery of each drug.[34, 35] Due to this and because ellipticine lacks the oxo group needed for conjugation with hydrazine or the hydrazide group-bearing polymer carrier, functionalization of the drug with suitable oxo group-containing linker is necessary. However, when modifying it, one must take care not to hamper the biological activity of the original drug

*Corresponding author, Tel: +420 296 809 230, Fax: +420 296 809 410, E-mail: mhruby@centrum.cz

because it will then be released in its substituted form. To connect ellipticine to the polymer, the drug may be converted to its derivative by quaternization of the ellipticines' isoquinoline-type nitrogen using a suitable linker, as previously described by our group.[36] A higher affinity between these derivatives and DNA (due to the permanent positive charge in the molecule) also benefits this function.[37]

In this paper, we describe the effect of the structural changes of the linker that is used to connect the ellipticinium drug with the polymer carrier on the pH-dependent release profile of the drug. Understanding these structural changes will allow us to fine-tune the ellipticinium drug delivery systems. To the best of our knowledge, no such study on ellipticine derivatives has been published so far, and the only study on ellipticinium hydrazone conjugates was our paper on the multilevel targeting of an Auger electron emitter ^{125}I.[36]

2. Materials and Methods

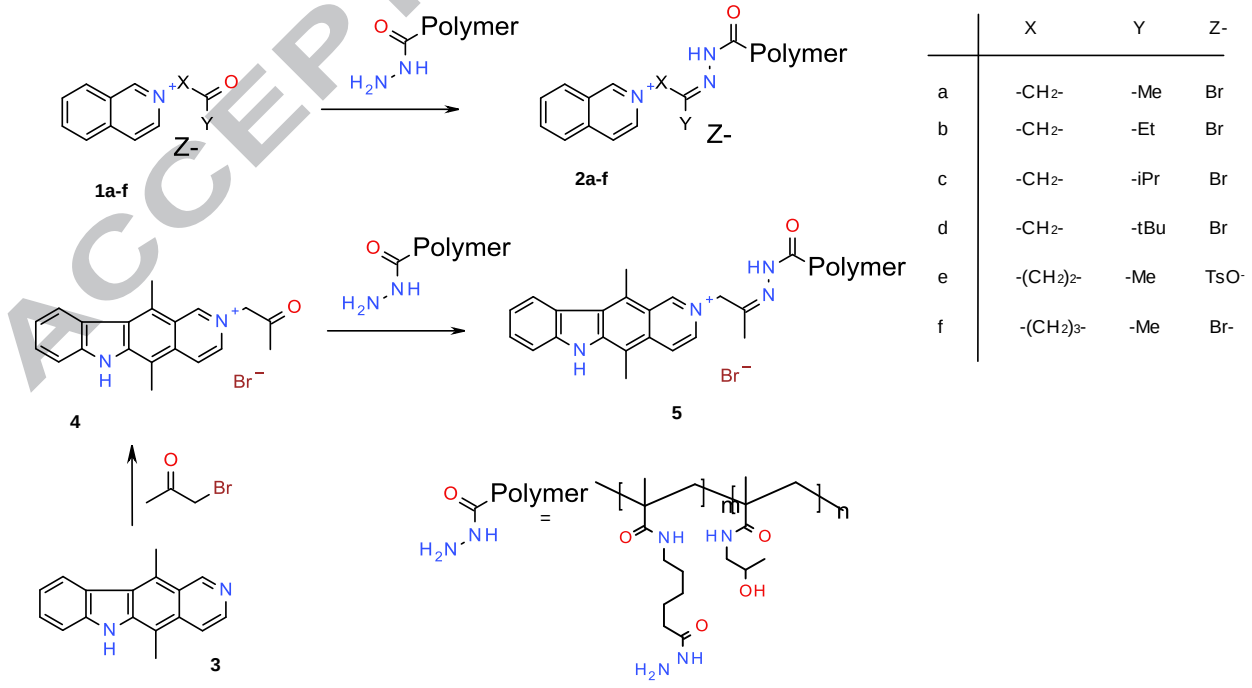
Detailed desctiption of the materials, characterization of products and experimental procedures could be found in **Supplementary Material**.

3. Results and Discussion

Isoquinoline was used as a cheap and relatively non-toxic model for the release profile screening of ellipticine. This compound was quaternized readily with plethora bromo- or tosyloxy ketones to produce oxo-alkyl isoquinolinium salts **1a-f**. These were conjugated with hydrazide groups-containing HPMA copolymer (M_w = 24.5 kDa, M_w/M_n = 1.87) by acetic acid-catalyzed condensation,[33] forming derivatives **2 (Figure 1)**. The molecular weights and polydispersity indexes of all conjugates confirmed no cross-linking of chains, and the content of isoquinolinium salts was 0.8 – 5.6% wt. See supporting materials for detailed experimental procedures and characterization data.

To assess the influence of steric hindrance on the cleavage of acidic hydrazone, the isoquinolinium conjugates containing methyl (**2a**), ethyl (**2b**), isopropyl (**2c**) and *tert*-butyl (**2d**) groups adjacent to the ketone were synthesized, and their hydrolytic release profiles were determined. In the phosphate buffer that was used to model the pH of blood (pH 7.4), nearly no low molecular weight isoquinolinium models were released in any instance. This could be explained by the strong electron-withdrawing effect of the permanent positive charge in the beta position to the ketone. The release of the drug was significantly faster when exposed to a pH of 5.0, which simulated the pH in late endosomes. Sterical hindrance had a dramatic effect on the release rate, the polymer conjugate of the methyl derivative **2a** had the fastest release rate, and the polymer conjugate of *tert*-butyl derivative **2d** had the slowest release rate. This slow release rate could be ascribed to the steric hindrance of the transition state, which is most likely to have hybridization close to the sp^2 state.[38]

To determine the influence of adjacent permanent positive charge on the rate of hydrolysis, we compared the release of the aforementioned derivative **1a**, which contained a positive charge in the β-position respective to the oxo-group, with the derivatives with positive charges in the γ- (**1e**) and δ- (**1f**), respectively δ- (**1f**) positions, from their conjugates. It can be clearly observed in **Figures 2** and **3** that the presence of a positive charge proximal to the original ketone substantially reduces its release rate. This decrease of release rate made the derivative with the closest charge (β-oxo derivative **2a**) the most stable derivative, whereas the conjugate with most remote charge (δ-oxo derivative **2f**) was the most labile conjugate, even at a pH of 7.4 (77 % of the drug released within 24 h). The release rate of the latter two conjugates (i.e., **2e-f**) is comparable with the release rates obtained with common aliphatic linkers (e.g., levulinic acid) studied for the conjugation of drugs to polymers via the hydrazone bond.[34]



	X	Y	Z-
a	-CH2-	-Me	Br
b	-CH2-	-Et	Br
c	-CH2-	-iPr	Br
d	-CH2-	-tBu	Br
e	-(CH2)2-	-Me	TsO-
f	-(CH2)3-	-Me	Br-

Figure 1. Syntheses and structures of the polymer conjugates.

This behavior could be explained by the electrostatic disinclination of hydrazones with proximate positive charges towards their protonation as the first step of the hydrolysis mechanism. A similar explanation was also used in the case of the hydrolysis of charged acetals.[39]

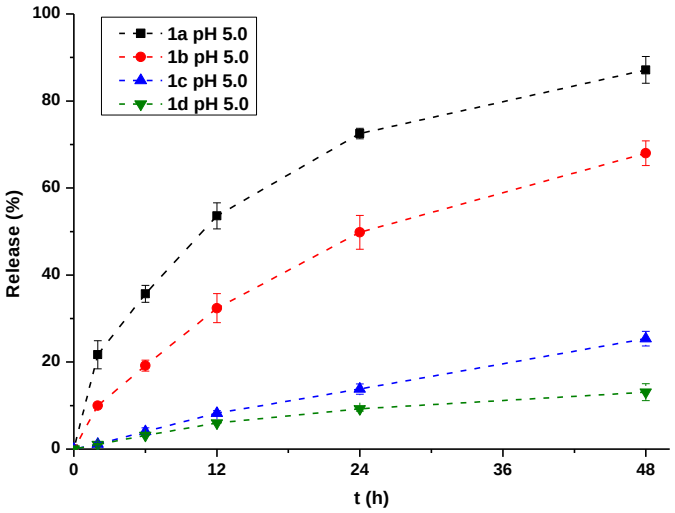


Figure 2. The release profile of derivatives **1a-d** from their conjugates **2a-d** in phosphate buffered media at 37°C. The release in the pH 7.4 phosphate buffer was under 2% after 24 h.

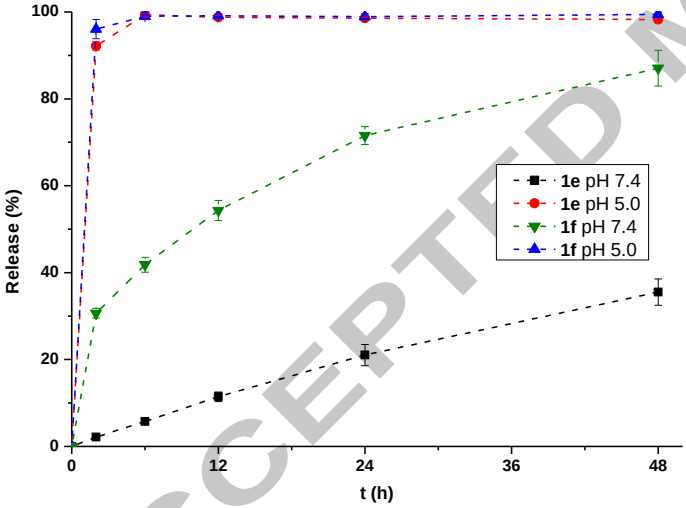


Figure 3. The release profile of derivatives **1e-f** from their conjugates **2e-f** in phosphate buffer media at 37°C.

Of all the linkers described above, the simplest 2-oxopropyl linker showed the best release profile for cancer applications (negligible at pH 7.4 and sufficiently fast in a slightly acidic milieu) and was thus chosen to connect ellipticine to the hydrazide-containing pHPMA polymer in the same manner as described for the model isoquinolinium derivatives above. The drug release profile of the modified ellipticinium polymer conjugate (**5**) was determined (**Figure 4**). At a pH of 7.4, conjugate **5** has shown remarkable stability, and only a negligible amount of ellipticinium **4** was released. At a pH of 5.0, over 50% of the drug was released from the polymer within 24 h. This slightly slower release rate than that of the conjugate **2a** can be explained by the enhanced electron delocalization of hydrazones' π-electrons in the aromatic system of the ellipticinium in comparison with isoquinolinium. However, the release of the drug is still more than two-fold faster than in the case of the conjugate with the oxobutyl-linker described in our previous work.[36]

To assure the DNA-intercalation ability, the solution of ellipticinium derivative **4** was titrated with the solution of calf-thymus DNA, and the fluorescence of the mixture was measured.[40] As a result, the fluorescence emission of the solution gradually rises with the addition of DNA, which confirms intercalation. The blue shift in fluorescence emission maximum is also consistent with more hydrophobic microenvironment of the intercalated molecule compared to free molecule in aqueous solution. The DNA affinity constant of the ellipticinium **4** and of ellipticine was determined using a Scatchard plot.[41] As described previously, the ellipticine derivatives exhibit two different binding modes depending on the drug/DNA ratio.[42] At a low drug/DNA ratio, ellipticinium derivative **4** binds with the intercalation constant $K=2.17\times10^{7}$ M^{-1}(bp) (see **Table 1** and supplementary information), with an average of one molecule of ellipticine to 4.57 DNA base pairs (bp), which is in the same range as the ellipticine standard ($K=3.81\times10^{7}$ M^{-1}(bp) and a ratio of 5.42 bp per molecule of the drug). Lower (0.57-times) stability of the complex of derivative **4** with DNA compared to similar complex with protonated ellipticine may be explained by sterical reasons on the quaternary amine, which is sterically more demanding than just protonated ellipticinium.

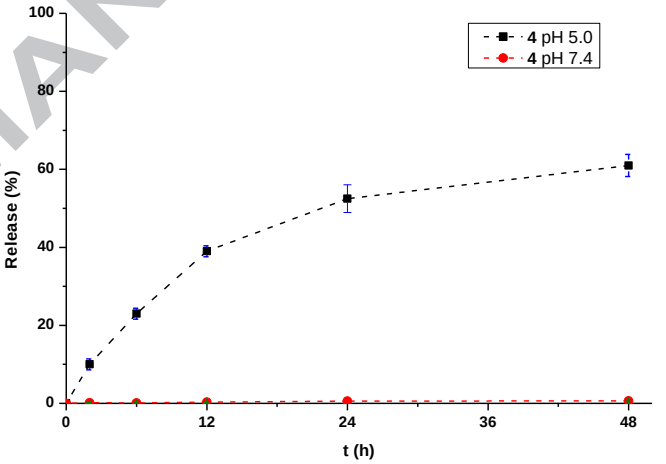


Figure 4. The release profile of derivative **4** from conjugate **5** in phosphate buffer media at 37°C.

At high drug/DNA ratios, the second binding mode occurs, and the higher intercalation density of 2.18 bp per molecule of the ellipticinium **4**, as well as the lower intercalation constant $K=6.12\times10^{5}$ M^{-1}(bp), are observed (for the ellipticine standard, the values $K=5.9\times10^{5}$ M^{-1}(bp) and a ratio of 2.65 bp/drug were determined). All values are consistent with those described in the literature for similar compounds and thus confirm the retention of the intercalation ability of **4**.[42]

Furthermore, we tested the antiproliferative activity of 2-*N*-(2-oxopropyl)ellipticinium bromide (**5**) on selected cell lines (4T1, Raji and EL4, respectively).[34] Cytotoxicities, expressed as the IC_{50} values, were in the range of 2.7 - 7.1 μmol/L for **4**, whereas those of ellipticine were in the range of 1.0 - 8.3μmol/L (**Table 1**). The EL4 cell line was the most sensitive, and the 4T1cell line was the least sensitive to both compounds. The differences between the IC_{50} values for different cell lines were statistically significant (analysis of variance, ANOVA on the level $\alpha = 0.05$) for both ellipticine and **4**. However, the IC_{50} values were not statistically significantly different when comparing ellipticine and **4** for any cell line tested (analysis of variance, ANOVA on the level $\alpha = 0.05$). One can thus conclude that ellipticine and its low molecular weight derivative **4** possess equal antiproliferative

Compound	K [10^7 M^{-1}(bp)][a]	IC_{50}[b], μmol/L		
		4T1	Raji	EL4
4	2.17 ± 0.29	7.1 ± 0.8	2.8 ± 0.7	2.7 ± 0.5
ellipticine	3.81 ± 0.62	8.3 ± 0.8	7.7 ± 1.1	1.0 ± 0.2

Table 1. DNA binding and antiproliferative activity of ellipticinium derivative **4** and ellipticine. [a] DNA-intercalation constant obtained from the compound fluorescence changes upon the addition of CT-DNA (K ± standard deviation). [b] Concentrations caused a 50% inhibition in the MTT test (IC_{50} ± standard deviation, n = 5) in μmol/L.

activities for all of the tested cell lines and thus that quaternization does not lead to the loss of activity.

The cytotoxicity of the conjugates was not tested because we showed by the HPLC that the free drug in its original form was released from its conjugates without side reactions. The data on the *in vitro* cytotoxicity of the conjugates may thus be misleading due to the significantly different concentrations of the drug released into the media during incubation with the cells compared with the *in vivo* situation.[30] This is because in an *in vivo* situation, the system is opened, i.e., the released drug is being continuously removed by internalization into cells or diffusion out of the tumor tissue. In addition, the pH of tumor tissue is generally slightly acidic but varies according to the exact location in the tumor by 1 – 1.5 pH units, which has a dramatic effect on the drug release rate and therefore the published IC_{50} values of hydrazone conjugates are not relevant. The values of hydrazone conjugates are typically one order of magnitude higher than the IC_{50} values of free drugs and generally do not correspond with *in vivo* antitumor effectiveness.[30]

Conclusions

We have shown that ellipticinium derivatives with antiproliferative activity can be bound to a polymer carrier by a hydrolytically labile bond with a widely tunable release rate. The key structural features that determine the release rate are proximity of the positive charge and the sterical hindrance. The optimized derivative showed no less antiproliferative activity when compared with ellipticine. The optimized derivative also exhibited a negligible release rate at pH modeling blood plasma (pH 7.4) and a sufficient release rate in an environment that modeled pH in late endosomes (pH 5.0; 50 % drug released within 24 h of incubation). The rules found for the described system have the potential to aid further designs of biodegradable spacers for biomedicinal applications also in other drug delivery systems.

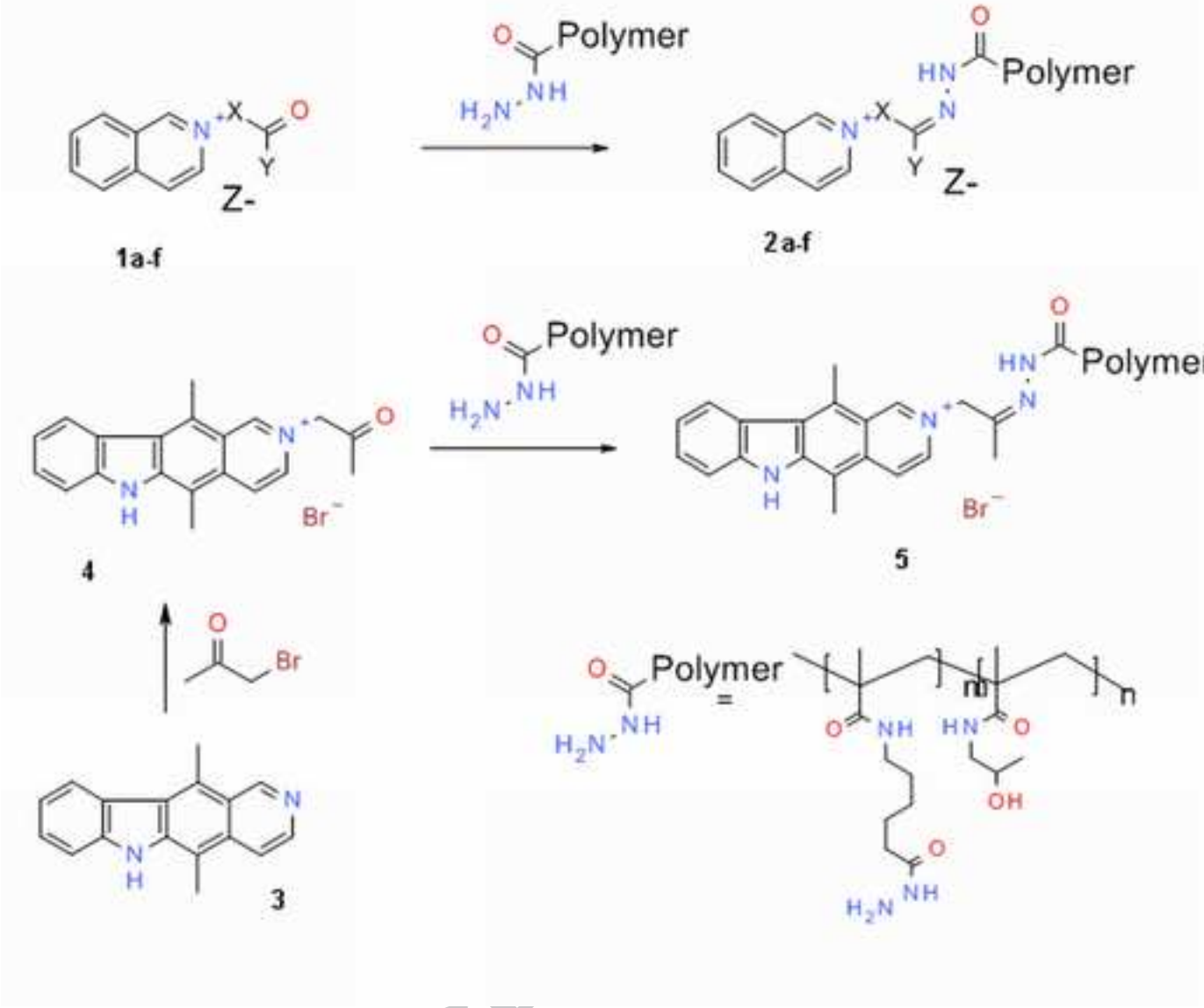
Aknowledgements

The financial support from the Grant Agency of the Czech Republic (grant # P304/12/0950), from the Ministry of Industry and Trade of the Czech Republic (grant # MPO TIP FR-TI4/625) and from the Academy of Sciences of the Czech Republic (grant # M200501201) is gratefully acknowledged.

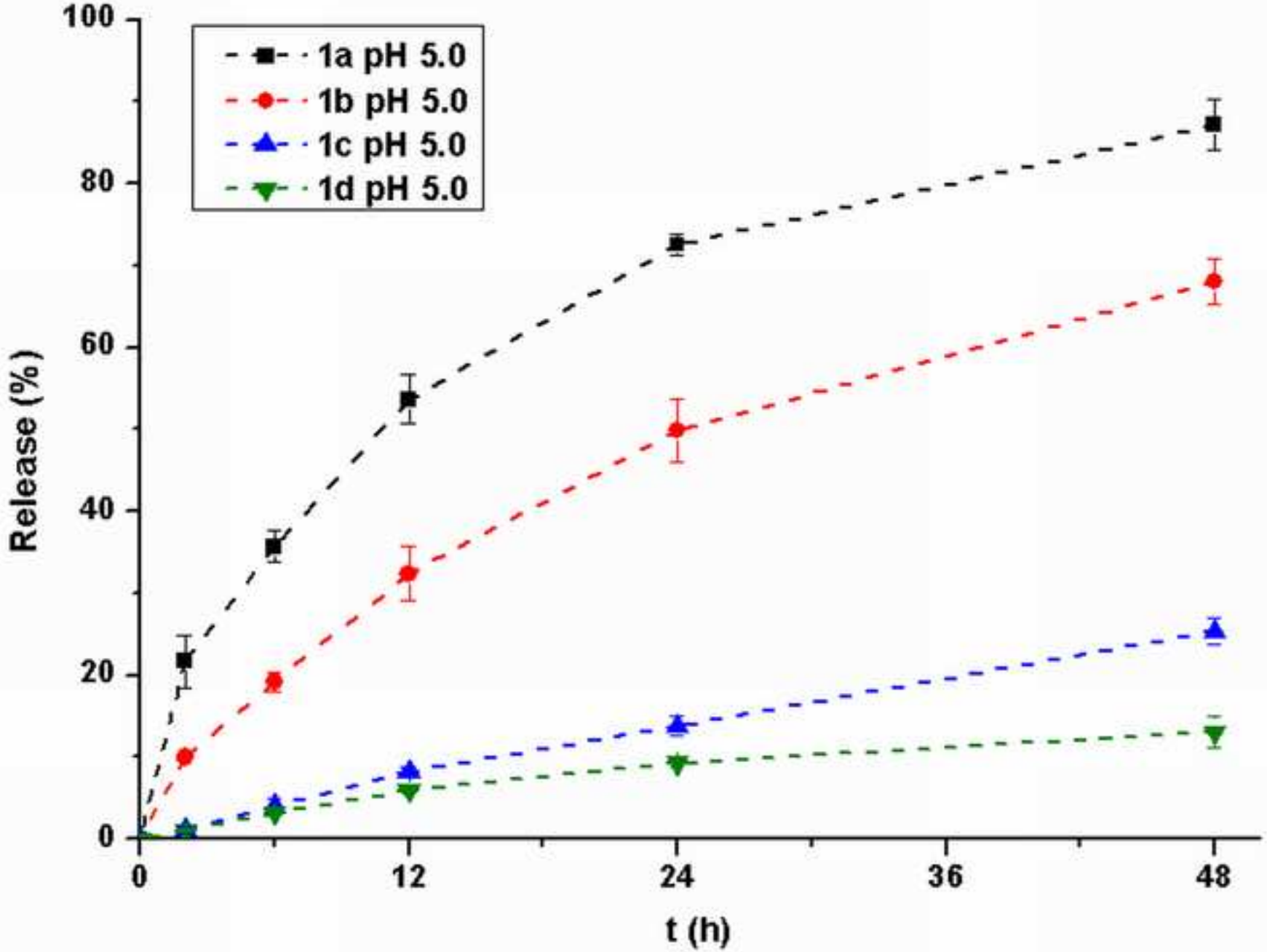
References

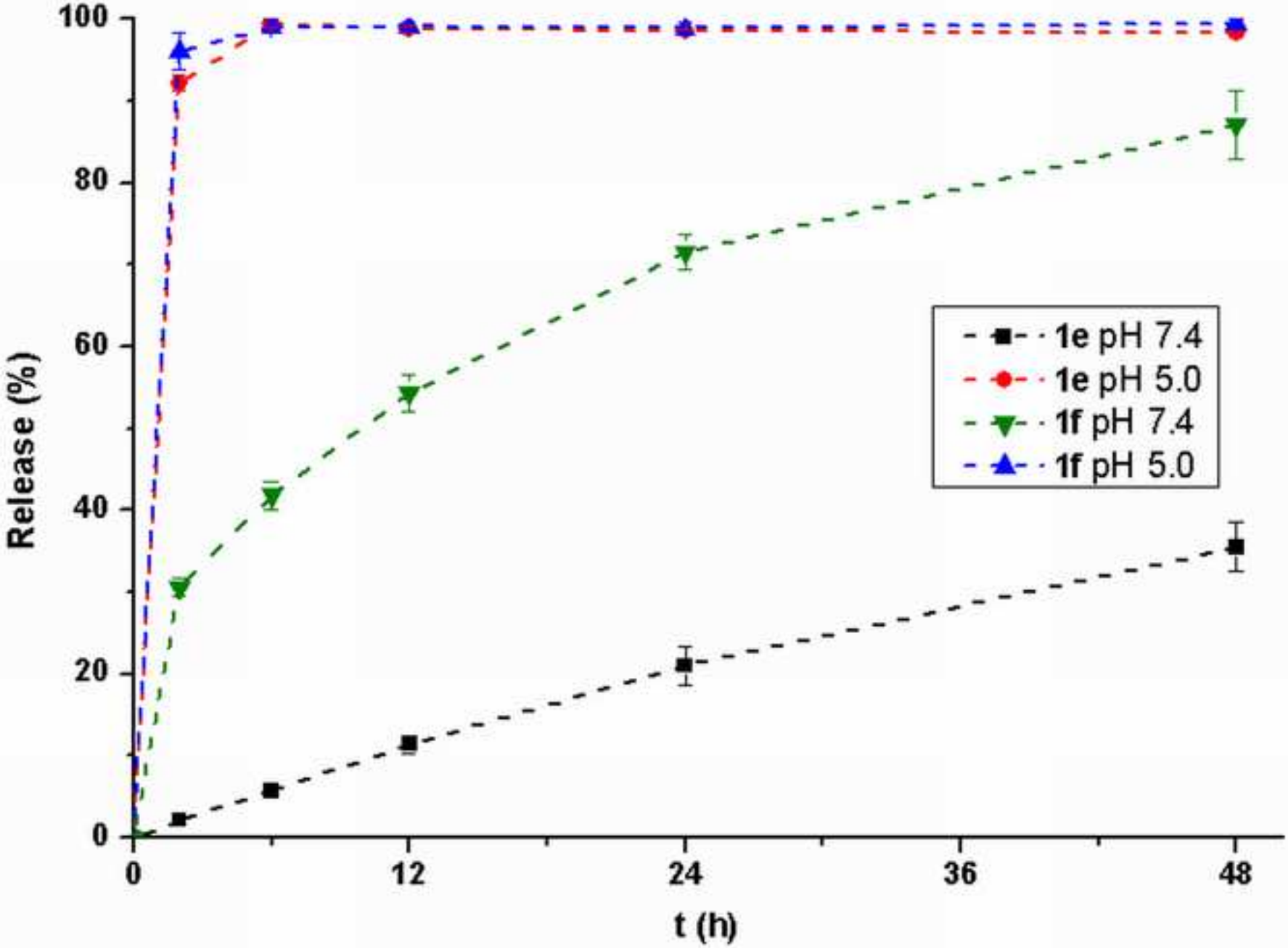
1. Schoenmakers, R. G.; van de Wetering, P.; Elbert, D. L.; Hubbell, J. A. *Journal of Controlled Release* **2004,** *95*, 291.
2. Reha, D.; Kabelac, M.; Ryjacek, F.; Sponer, J.; Sponer, J. E.; Elstner, M.; Suhai, S.; Hobza, P. *Journal of the American Chemical Society* **2002,** *124*, 3366.
3. Stiborova, M.; Sejbal, J.; Borek-Dohalska, L.; Aimova, D.; Poljakova, J.; Forsterova, K.; Rupertova, M.; Wiesner, J.; Hudecek, J.; Wiessler, M.; Frei, E. *Cancer Research* **2004,** *64*, 8374.
4. Froelichammon, S. J.; Patchan, M. W.; Osheroff, N.; Thompson, R. B. *Journal of Biological Chemistry* **1995,** *270*, 14998.
5. Malonne, H.; Atassi, G. *Anti-Cancer Drugs* **1997,** *8*, 811.
6. Monnot, M.; Mauffret, O.; Simon, V.; Lescot, E.; Psaume, B.; Saucier, J. M.; Charra, M.; Belehradek, J.; Fermandjian, S. *Journal of Biological Chemistry* **1991,** *266*, 1820.
7. Liu, J. B.; Xiao, Y. H.; Allen, C. *J. Pharm. Sci.* **2004,** *93*, 132.
8. Paoletti, C.; Cros, S.; Xuong, N. D.; Lecointe, P.; Moisand, A. *Chemico-Biological Interactions* **1979,** *25*, 45.
9. Rouesse, J. G.; Lechevalier, T.; Caille, P.; Mondesir, J. M.; Sanchogarnier, H.; Maylevin, F.; Spielmann, M.; Dejager, R.; Amiel, J. L. *Cancer Treatment Reports* **1985,** *69*, 707.
10. Samadibaboli, M.; Favre, G.; Canal, P.; Soula, G. *British Journal of Cancer* **1993,** *68*, 319.
11. Acton, E. M.; Narayanan, V. L.; Risbood, P. A.; Shoemaker, R. H.; Vistica, D. T.; Boyd, M. R. *Journal of Medicinal Chemistry* **1994,** *37*, 2185.
12. Khayat, D.; Borel, C.; Azab, M.; Paraisot, D.; Malaurie, E.; Bouloux, C.; Weil, M. *Cancer Chemotherapy and Pharmacology* **1992,** *30*, 226.
13. Kattan, J.; Durand, M.; Droz, J. P.; Mahjoubi, M.; Marino, J. P.; Azab, M. *American Journal of Clinical Oncology-Cancer Clinical Trials* **1994,** *17*, 242.
14. Ohashi, M.; Oki, T. *Expert Opinion on Therapeutic Patents* **1996,** *6*, 1285.
15. Markovits, J.; Pommier, Y.; Mattern, M. R.; Esnault, C.; Roques, B. P.; Lepecq, J. B.; Kohn, K. W. *Cancer Research* **1986,** *46*, 5821.
16. Thomas, N.; Moulin, B.; Raguenezviotte, G.; Fillastre, J. P. *Renal Failure* **1991,** *13*, 243.
17. Lee, I. P. *Journal of Pharmacology and Experimental Therapeutics* **1976,** *196*, 525.
18. Rouesse, J.; Spielmann, M.; Turpin, F.; Lechevalier, T.; Azab, M.; Mondesir, J. M. *European Journal of Cancer* **1993,** *29A*, 856.
19. Duncan, R. *Anti-Cancer Drugs* **1992,** *3*, 175.
20. Duncan, R. *Nature Reviews Cancer* **2006,** *6*, 688.
21. Langer, R. *Nature* **1998,** *392*, 5.
22. Sedlacek, O.; Monnery, B. D.; Filippov, S. K.; Hoogenboom, R.; Hruby, M. *Macromolecular Rapid Communications* **2012,** *33*, 1648.
23. Maeda, H. In *Advances in Enzyme Regulation, Vol 41;* Weber, G., Ed., 2001; Vol. 41, pp. 189.
24. Maeda, H.; Wu, J.; Sawa, T.; Matsumura, Y.; Hori, K. *Journal of Controlled Release* **2000,** *65*, 271.
25. Kircheis, R.; Kichler, A.; Wallner, G.; Kursa, M.; Ogris, M.; Felzmann, T.; Buchberger, M.; Wagner, E. *Gene Therapy* **1997,** *4*, 409.
26. Torchilin, V. P. *Cellular and Molecular Life Sciences* **2004,** *61*, 2549.
27. Uhrich, K. E.; Cannizzaro, S. M.; Langer, R. S.; Shakesheff, K. M. *Chemical Reviews* **1999,** *99*, 3181.
28. Bae, Y.; Nishiyama, N.; Fukushima, S.; Koyama, H.; Yasuhiro, M.; Kataoka, K. *Bioconjugate Chemistry* **2005,** *16*, 122.
29. Hruby, M.; Konak, C.; Ulbrich, K. *Journal of Controlled Release* **2005,** *103*, 137.
30. Ulbrich, K.; Etrych, T.; Chytil, P.; Jelinkova, M.; Rihova, B. *Journal of Controlled Release* **2003,** *87*, 33.
31. Binauld, S.; Stenzel, M. H. *Chemical Communications* **2013,** *49*, 2082.
32. Binauld, S.; Scarano, W.; Stenzel, M. H. *Macromolecules* **2012,** *45*, 6989.
33. Etrych, T.; Jelinkova, M.; Rihova, B.; Ulbrich, K. *Journal of Controlled Release* **2001,** *73*, 89.
34. Sedláček, O.; Hrubý, M.; Studenovský, M.; Větvička, D.; Svoboda, J.; Kaňková, D.; Kovář, J.; Ulbrich, K. *Bioorganic & Medicinal Chemistry* **2012**.

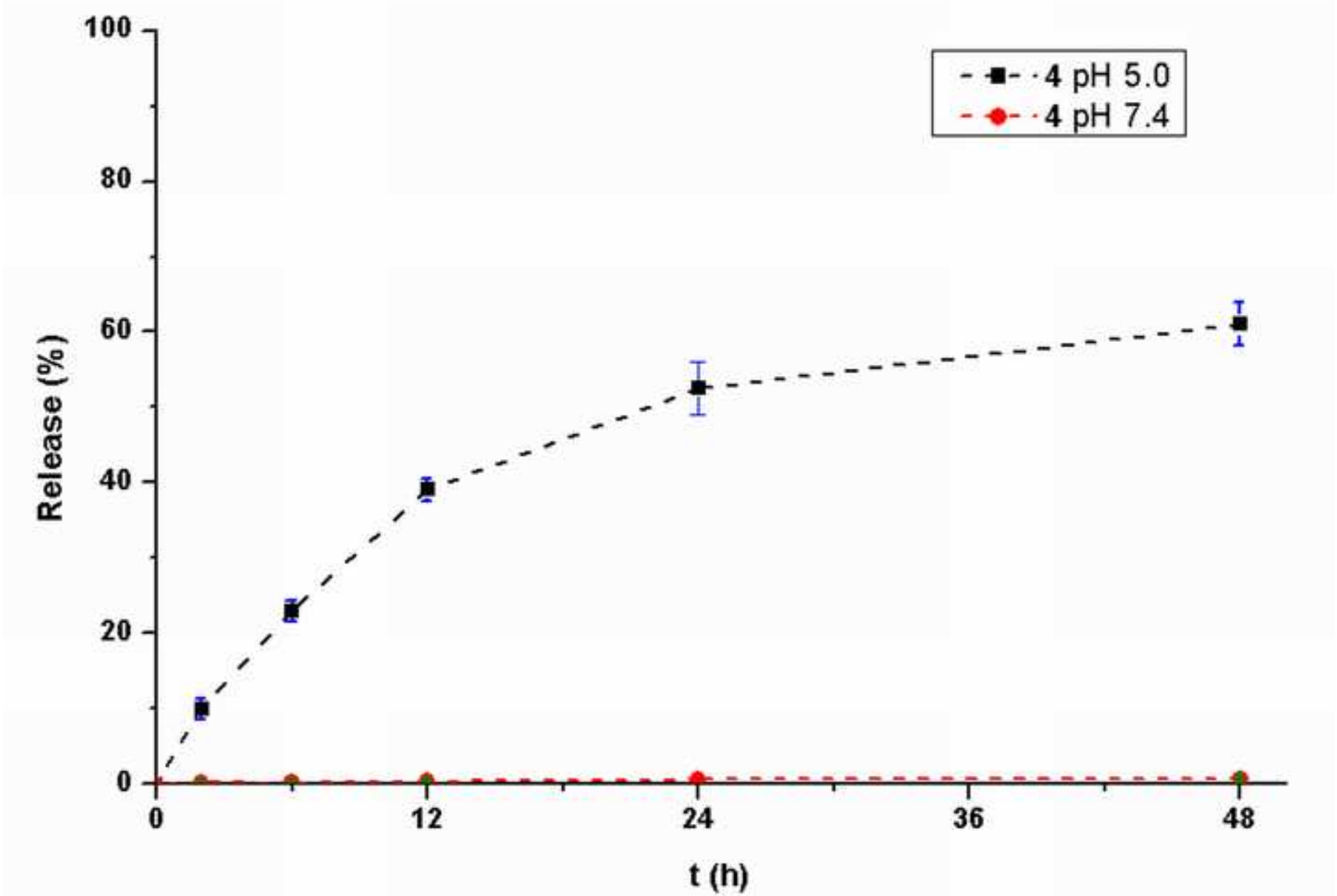
35. Alani, A. W. G.; Bae, Y.; Rao, D. A.; Kwon, G. S. *Biomaterials* **2010,** *31*, 1765.
36. Sedlacek, O.; Hruby, M.; Studenovsky, M.; Kucka, J.; Vetvicka, D.; Kovar, L.; Rihova, B.; Ulbrich, K. *Bioconjugate Chemistry* **2011,** *22*, 1194.
37. Grinstaff, M. W. *Angewandte Chemie-International Edition* **1999,** *38*, 3629.
38. Custer, T. G.; Kato, S.; Bierbaum, V. M.; Howard, C. J.; Morrison, G. C. *Journal of the American Chemical Society* **2004,** *126*, 2744.
39. Ciufolini, M. A.; Byrne, N. E. *Journal of the American Chemical Society* **1991,** *113*, 8016.
40. Renault, E.; Fontaine-Aupart, M. P.; Tfibel, F.; Gardes-Albert, M.; Bisagni, E. *Journal of Photochemistry and Photobiology B: Biology* **1997,** *40*, 218.
41. Bailly, C.; Ohuigin, C.; Houssin, R.; Colson, P.; Houssier, C.; Rivalle, C.; Bisagni, E.; Henichart, J. P.; Waring, M. J. *Mol. Pharmacol.* **1992,** *41*, 845.
42. Mauffret, O.; Rene, B.; Convert, O.; Monnot, M.; Lescot, E.; Fermandjian, S. *Biopolymers* **1991,** *31*, 1325.



	X	Y	Z-
a	$-CH_2-$	-Me	Br
b	$-CH_2-$	-Et	Br
c	$-CH_2-$	-iPr	Br
d	$-CH_2-$	-tBu	Br
e	$-(CH_2)_2-$	-Me	TsO-
f	$-(CH_2)_3-$	-Me	Br-


1a pH 5.0
1b pH 5.0
1c pH 5.0
1d pH 5.0
Release (%)
t (h)
0
20
40
60
80
100
0
12
24
36
48


100
80
60
40
20
0
Release (%)
0
12
24
36
48
t (h)
1e pH 7.4
1e pH 5.0
1f pH 7.4
1f pH 5.0



*Graphical Abstract (for review)