POLY(2-OXAZOLINE)S AS HIGH-CAPACITY MULTI-DRUG DELIVERY SYSTEMS

Anita Schulz[1], Yingchao Han[2], Zhijian He[2], Tatiana K. Bronich[2], Alexander V. Kabanov[2,3], Robert Luxenhofer[1], Rainer Jordan[1]

[1] Professur für Makromolekulare Chemie, Department Chemie, Technische Universität Dresden, Zellescher Weg 19, 01069 Dresden, Germany
[2] Center for Drug Delivery and Nanomedicine, 985830 Nebraska Medical Center, Omaha, Nebraska 68198-5830, USA
[3] Laboratory of Chemical Design of Bionanomaterials, Faculty of Chemistry, M.V. Lomonosov Moscow State University, Moscow 119992, Russia

Introduction

The family of POx is being increasingly explored for biomedical applications.[1,2] Similar to PEG, hydrophilic POx such as poly(2-methyl-2-oxazoline) and poly(2-ethyl-2-oxazoline) exhibit stealth properties[3], good biodistribution[4], and fast renal excretion when administered alon[5]. Recently, we presented amphiphilic block copolymers based on poly(2-oxazoline)s (POx) as drug delivery systems with high loading capacities for poorly water–soluble drugs such as paclitaxel (PTX)[6]. We contributed this extraordinary high loading to the particular molecular structure of the core forming hydrophobic block, poly(2-butyl-2-oxazoline), which provides polar moieties within the hydrophobic block. However, only one or a few mutations within the tumor are necessary to adopt resistance towards a single drug. A combination of drugs, each addressing different pathways, should impede the development of resistance against therapy. Therefore, we investigated the simultaneous solubilization of various binary and ternary drug combinations of PTX, docetaxel (DTX), 17-allylamino-17-demethoxygeldanamycin (17-AAG), etoposide (ETO) and bortezomib (BTZ) by amphiphilic POx micelles. In addition, combination of multiple drugs within one vehicle potentially makes patient treatment less hazardous and simple.

Experimental

Materials. PTX, DTX, 17-AAG and BTZ were purchased from LC Laboratories (Woburn, MA) ETO and all other substances were obtained from Aldrich (München, Germany) and Acros (Geel, Belgium) and were used as received unless otherwise stated. Methyl trifluoromethylsulfonate (MeOTf), 2-methyl-2- oxazoline (MeOx), 2-butyl-2-oxazoline (BuOx), chlorobenzene (ClBz) and acetonitrile (ACN) were refluxed over CaH_2 and distilled under nitrogen.

Instrumentation. NMR spectra were recorded on a Bruker DRX 500 P (^{1}H: 500.13 MHz) at room temperature (RT). The spectra were calibrated to the signals of residual protonated solvent signals (ACN: 1.94 ppm). Gel permeation chromatography (GPC) was performed on a Polymer Laboratories GPC-120 (1x PSS GRAM analytical 1000 and 1x PSS GRAM analytical 100) with N,N-dimethyl acetamide (5 mmol/L LiBr, 70 °C, 1 mL/min) as eluent and polymethylmethacrylate as standards. Microwave supported polymerization were performed using a CEM Discover microwave with a maximum power setting to 150 W and reaction temperature of 100 °C. HPLC analysis was performed via an Agilent Technologies 1200 Series HPLC system using a Nucleosil C18-5µ column (250 mm x 4 mm). Dynamic light scattering (DLS) was determined with a Nano-ZS (Malvern Instruments Inc., UK).

Synthesis of Methyl-P[MeOx$_{40}$-b-BuOx$_{21}$-b-MeOx$_{34}$]-piperazine. Under dry and inert conditions 0.29 g (1.76 mmol, 1eq) MeOTf and 5.24g (61.6 mmol, 35 eq) MeOx were dissolved in 30 mL dry ACN/ClBz (50/50, v/v) at RT. The reaction mixture was irradiated in the microwave for 60 min. After cooling to RT 4.37 g (34.4 mmol, 20 eq) BuOx were added and the mixture was irradiated for another 60 min. The procedure was repeated with 5.13 g (60.3 mmol, 34 eq) MeOx. Termination was carried out with 1.00 g (5.4 mmol, 3 eq) 1-BOC-piperazine at RT and stirred over night. An excess of potassium carbonate was added and the mixture was stirred for several hours. After filtration the mixture was concentrated, added into a mixture of chloroform and methanol (75/25, v/v) and precipitated in cold diethylether (10-20 fold of volume of polymer solution). After centrifugation and removal of diethylether the precipitation was repeated two more times. The residual was lyophilized and 13.2 g of a colorless powder were obtained. ^{1}H-NMR (ACN, 300 K): δ[ppm] = 3.38 (br, 379H, N-CH_2CH_2); 2.98/2.85 (m, 3H, N-CH_3^{Ini}); 2.33-2.18(m, 59H, CO-CH_2^{butyl}, CH_2^{Pip}); 2.04-1.08 (m, 222H, CO-CH_3); 1.52 (br, 42H, CH_2-CH_2-CH_2-); 1.42 (br, 7H, CH_3^{BOC}); 1.32 (br, 42H, CH_2-CH_3); 0.90 (br, 62H, CH_3^{butyl}). GPC (DMAc): M_n= 10.2 kg/mol ($Đ_M = M_w/M_n$ =1.21)
5.1 g of the obtained polymer were dissolved in 32 mL of a mixture of trifluoroacetic acid (TFA), triisobutylsilane and water (95/2.5/2.5, v/v/v) and stirred for 3h at RT. The volatiles were removed under fine vacuum. The residual was dissolved in 25 mL deionized water, transferred into a dialysis bag (MWCO 3500 g/mol) and dialyzed against 3 L deionized water (water exchanged after 2h, 24h and 48h). The solution was recovered from the bag, lyophilized and 3.7 g of a colorless powder were obtained. GPC (DMAc): M_n= 11.6 kg/mol ($Đ_M$ =1.14).

Synthesis Methyl-P[MeOx$_{33}$-b-BuOx$_{26}$-b-MeOx$_{45}$]-piperazine. Synthesis was done accordingly using 0.30 g (1.82 mmol, 1 eq) MeOTf, 5.44 g (63.9 mmol, 35 eq) MeOx, 4,57 g (35.9 mol, 20 eq) BuOx, 5,34 g (62.7 mmol, 34 eq) MeOx and 1.03 g (5.6 mmol, 3 eq) 1-BOC-piperazine. 13.65 g of a colorless powder were obtained. ^{1}H-NMR (ACN, 300 K): δ[ppm] = 3.43 (br, 387H, N-CH_2CH_2); 2.98/2.85 (m, 3H, N-CH_3^{Ini}); 2.33-2.18(m, 48H, CO-CH_2^{butyl}, CH_2^{Pip}); 2.04-1.08 (m, 226H, CO-CH_3); 1.51 (br, 44H, CH_2-CH_2-CH_2-); 1.41 (br, 9H, CH_3^{BOC}); 1.31 (br, 45H, CH_2-CH_3); 0.89 (br, 65H, CH_3^{butyl}).
9.92 g of the polymer were deprotected with 60 mL TFA. 7.19 g colorless powder were obtained. GPC (DMAc): M_n= 11.4 kg/mol ($Đ_M$ =1.14).

Drug solubilization. Pre-determined amounts of ethanolic solutions of POx and drugs were mixed. After removal of ethanol, the formed thin films were further dried *in vacuo* and subsequently redispersed with appropriate amounts of deionized water. The solutions were heated to 50-60 °C for 5-20 min. For all samples the polymer concentration was 10 g/L and each drug concentration 4 g/L. Quantification of drug loading is defined via loading capacity LC (m_{drug}/m_{total}) and loading efficiency LE ($m_{recovered\ drug}/m_{added\ drug}$).

HPLC analysis of drug solubilization. PTX, 17-AAG containing samples were analyzed under isocratic conditions of ACN/MeOH/H_2O (39/38/23, v/v/v). Similarly, DTX and 17-AAG samples were quantified with ACN/ H_2O (55/45, v/v). The column temperature was 30 °C and the flow rate 1 ml/min. HPLC analysis of ETO containing samples were performed with a step-wise gradient. First the analyte was eluted with ACN/MeOH/H_2O (5/5/90, v/v/v) for 10 min followed by ACN/ H_2O (60/40, v/v) for another 10 min. Column temperature was 40 °C. For BTZ analysis the mobile phase started for one minute with ACN/ H_2O (90/10, v/v) followed with ACN/MeOH/H_2O (35/35/30, v/v/v) for 10 min. The flow rate was 2.0 mL/min and column temperature was 55 °C. Detection was performed at 227 nm for PTX, DTX,ETO, 333 nm for 17-AAG and 270 nm for BTZ.

***In vitro* cytotoxicity.** Cytotoxicity was determined using standard MTT assay. Cells were treated for 24 h with drug formulation.[6]

Results and Discussion

Polymer Synthesis and characterization. POx are synthesized by means of living cationic polymerization, therefore the polymer can be precisely tailored. To verify appropriate reproducibility of the synthesis two batches were prepared. The analytical data and compositions of both

triblock copolymers are in good agreement, also in comparison with an earlier batch from previous work (**Table 1**).

Table 1: Analytical data and composition of triblock copolymers used in this study.

Polymer composition[a]	M_n[a] [kg/mol]	M_n[b] [kg/mol]	Đ[b]
P[$MeOx_{40}$-b-$BuOx_{21}$-b-$MeOx_{34}$]	9.1	11.6	1.14
P[$MeOx_{33}$-b-$BuOx_{26}$-b-$MeOx_{45}$]	10.0	11.4	1.14
P[$MeOx_{37}$-b-$BuOx_{23}$-b-$MeOx_{37}$][c]	9.3	10.8	1.18

[a] As determined by endgroup analysis from ^{1}H NMR spectroscopy.
[b] As determined by gel permeation chromatography.
[c] Data from Ref. 6.

Both newly prepared polymers display similar compositions and dispersities, confirming the control of the polymerization. As no differences in their solubilization capabilities were observed, they will not be further distinguished in the following discussion.

Drug solubilization. Confirming our previous results, solubilization of PTX and DTX alone at drug concentration of 4 g/L resulted in high LE of 97 %. While ETO, 17-AAG and BTZ were somewhat less efficiently solubilized, remarkably high concentrations of 3.62±0.18, 3.45±0.21 and 3.12±0.12 respectively, were obtained, increasing the solubilities of the drugs by at least two orders of magnitude. Interestingly, adding a second and third drug to the formulation decreased the LE of each drug only slightly or not at all (**Figure 1**). Thus the total LC of the formulations increased from single drugs at an average of 26.4 ± 1.7 wt.%, over 41.5 ± 1.5 wt.% for binary up to 48.6 ± 0.2 wt.% for ternary formulations.

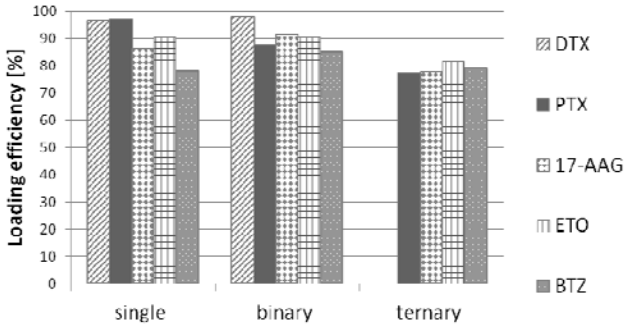

Figure 1: Averaged loading efficiencies of each drug in single, binary and ternary formulations (drug concentration: 4 g/L, polymer concentration: 10 g/L).

Size distribution and stability of drug loaded POx micelles. While the polymer alone displays different populations of aggregates when measured with DLS, the addition of PTX, DTX and 17-yielded monomodal micelles with hydrodynamic radii of 9-18 nm and moderate size distribution (PDI<0.3). In contrast, solubilization of single ETO and BTZ resulted in multimodal DLS profiles with broad size distributions. Combinations of PTX or 17-AAG with ETO or BTZ generated more homogeneous micelles. PTX containing binary formulations were in general smaller in size (r_H ~ 20 nm) than combinations with 17-AAG (r_H ~ 30-40 nm). Mixing ETO or BTZ with both PTX and 17-AAG generated narrow distributed (PDI < 0.19), ternary formulations with hydrodynamic radii of 26 nm (ETO/PTX/17-AAG) and 50 nm (BTZ/PTX/17-AAG) which were stable for at least two weeks. In general the more uniform and smaller the drug loaded micelles were, higher stability of the aggregates over time was observed. Thus single drug solubilization of PTX and 17-AAG as well as their combination were stable for at least two weeks, while other binary formulations with PTX or 17-AAG ensured at least one week of unaltered aggregates. In contrast single drug solubilization of ETO and BTZ precipitated after 2 days.

***In vitro* cytotoxicity study.** The cytotoxicity of various POxs has been recently investigated by us.[7] POx are in general non-toxic up to concentrations of 20 g/L in various cell lines. Thus the observed toxicities seen in **Figure 2** correspond to the released drugs. The 50 % inhibition concentrations (IC_{50}) for PTX, 17-AAG and ETO loaded micelles in MCF-7 breast cancer cells ranged from 0.013 ± 0.008 µg/mL, 0.357 ± 0.087 µg/ml and 2.730 ± 1.209 µg/ml respectively. For the binary formulation PTX/17-AAG and ETO/17-AAG IC_{50} values of 0.023 ± 0.007 µg/mL and 0.745 ± 0.111 µg/mL respectively were determined, which do not vary significantly from their single drug equivalent. It is well known, that in order to observe synergism, incubation time, cell line and drug ratios are crucial and need to be further investigated.

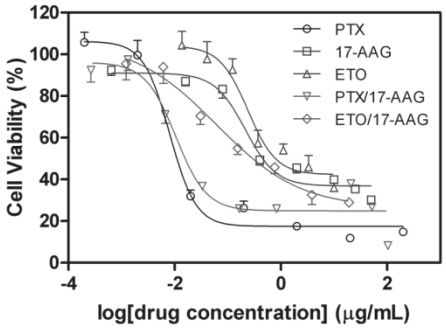

Figure 2: Dose dependent cell viability of MCF7 cells exposed to various micellar drug formulations. Data is presented as mean ± SEM (n=6) for each drug concentration.

Conclusion

In summary, POx appear to be a well-suited multi-drug delivery platform. We have shown that incorporation of up to three different hydrophobic agents in different combinations results in highly stable and well-defined micelles with high loading capacities close to 50 wt.%. Thus only 1 g of polymer is needed to solubilize 1 g of the drug cocktail.

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References

(1) Hoogenboom, R. *Angew Chem Int Ed* **2009**, *48*, 7978.
(2) Schlaad, H.; Diehl, C.; Gress, A.; Meyer, M.; Demirel, A.L.; Nur, Y.; Bertin, A. *Macromol Rapid Comm* **2010**, *31*, 511.
(3) Konradi, R.; Pidhatika, B.; Mühlebach, A.; Textor, M. *Langmuir* **2008**, *24*, 613.
(4) Zalipsky, S.; Hansen, C.B.; Oaks, J.M.; Allen, T.M. *J. Pharm. Sci.* **1996**, *85*, 133.
(5) Gaertner, F.C.; Luxenhofer, R.; Blechert, B.; Jordan, R.; Essler, M. *J. Control. Release* **2007**, *119*, 219.
(6) Luxenhofer, R.; Schulz, A.; Roques, C.; Li, S.; Bronich, T.K.; Batrakova, E.V.; Jordan, R.; Kabanov, A.V. *Biomaterials* **2010**, *31*, 4972.
(7) Luxenhofer, R.; Sahay, G.; Schulz, A.; Alakhova, D.; Bronich, T. K.; Jordan, R.; Kabanov, A.V. *J. Control. Release* **2011**, *153*, 73.