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 from Aldrich (München, Germany) and Acros (Geel, Belgium) and were used as received unless otherwise stated.

Synthesis of Methyl-P[MeOx35-b-BuOx25-b-MeOx45]–piperazine.

Under dry and inert conditions 0.29 g (1.76 mmol, 1 eq) MeOTf and 5.24 g (63.9 mmol, 35 eq) MeOx, 4.57 g (35.9 mol, 20 eq) BuOx, 5.44 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. 1H-NMR (ACN, 300 K): δ [ppm] = 3.43 (br, 38H, N–CH2–); 2.98/2.85 (m, 3H, N–CH3); 2.33–2.18 (m, 48H, CO–CH2–); 1.42 (br, 7H, CH2–); 1.32 (br, 42H, CH2–CH2–); 0.90 (br, 62H, CH2–); 0.89 (br, 65H, CH3–).

9.92 g of the polymer were deprotected with 60 mL TFA. 7.19 g colorless powder were obtained. GPC (DMAc): Mw = 11.6 kg/mol (Dw = 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 (\(m_{\text{drug}}/m_{\text{total}}\)) and loading efficiency LE (\(m_{\text{recovered drug}}/m_{\text{added drug}}\)).

HPLC analysis of drug solubilization. PTX, 17-AAG containing batches were prepared. The analytical data and compositions of both 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

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 diethylther (10-20 fold of volume of polymer solution). After centrifugation and removal of diethylthether the precipitation was repeated two more times. The residual was lyophilized and 13.2 g of a colorless powder were obtained. 1H-NMR (ACN, 300 K): δ [ppm] = 3.38 (br, 379H, N–CH2(CH2)2–); 2.98/2.85 (m, 3H, N–CH3(CH2)2–); 2.33–2.18 (m, 59H, CO–CH2(CH2)2–); 1.52 (br, 42H, CH2–CH2–CH2–); 1.42 (br, 7H, CH2–); 1.32 (br, 42H, CH2–CH2–); 0.90 (br, 62H, CH2–); 0.89 (br, 65H, CH3–).

In vitro cytotoxicity. Cytotoxicity was determined using standard MTT assay. Cells were treated for 24 h with drug formulation.3

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.

<table>
<thead>
<tr>
<th>Polymer composition</th>
<th>M_n [kg/mol]</th>
<th>M_w [kg/mol]</th>
<th>D_b</th>
</tr>
</thead>
<tbody>
<tr>
<td>P[MeOx40-b-BuOx21-b-MeOx37]</td>
<td>9.1</td>
<td>11.6</td>
<td>1.14</td>
</tr>
<tr>
<td>P[MeOx33-b-BuOx26-b-MeOx45]</td>
<td>10.0</td>
<td>11.4</td>
<td>1.14</td>
</tr>
<tr>
<td>P[MeOx40-b-BuOx21-b-MeOx37]</td>
<td>9.3</td>
<td>10.8</td>
<td>1.18</td>
</tr>
</tbody>
</table>

a As determined by endgroup analysis from 1H 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.

**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 homogenous micelles. PTX containing binary formulations were in general smaller in size (t~20 nm) than combinations with 17-AAG (t~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.1 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 (IC50) 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 IC50 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.

**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**