1. Introduction

Poly(2-oxazoline)s (POx) are a family of polymers that can be obtained via living cationic ring opening polymerization of 2-oxazoline monomers, which allows for excellent synthetic control over the molar mass and preparation of multi-block copolymers [1,2]. POx are structurally versatile, for example, both chain termini can be independently equipped with a chemical (e.g., –OH, –NH, etc.) or structural (e.g. lipid) functionality [3]. A number of 2-oxazoline monomers are commercially available (e.g. 2-methyl-, 2-ethyl-, 2-isopropyl, 2-phenyl-2-oxazoline) and a wide range of 2-oxazoline monomers carrying other aliphatic side chains and functional side chains are readily accessible. Recently, in addition to the long known side chain functionalities such as –OH and –COOH [4], we and other researchers introduced a number of functional side chains, which can be employed in chemoselective ligations, such as oxime formation [5] and click chemistry [6] as well as the possibility to obtain star-like POx using pluritriflile initiators [7]. Moreover, molecular brushes based on POx have been introduced and complete the structural tool kit available with POx [8,9].

POx are attractive for biomedical applications [10]. For therapeutic and analytical purposes, Saegusa and co-workers have suggested the use of POx for catalase conjugation (POxylation) already in 1990 [11] and more recently a similar approach has been used to conjugate trypsin and cytosine arabinose [12]. We recently reported on the modification of cellular uptake of horseradish peroxidase by attachment of amphiphilic POx [13]. Also, we discovered recently that in particular 2-butyl-2-oxazoline (BuOx) based POx amphiphiles are interesting candidates for formulation and delivery of highly water-insoluble drugs [14].

POx with C2 and C3 side chains are thermosensitive in aqueous solutions and can be tuned to yield cloud points over a broad range of temperatures [10,15–17].

Hydrophilic poly(2-methyl-2-oxazoline) (PMeOx) and poly(2-ethyl-2-oxazoline) (PEtOx) can impose a stealth effect similar to PEG when grafted on liposomes and surfaces [18,19]. Also, amphiphilic POx block copolymers exhibit only very limited interactions with human serum proteins [20]. Accordingly, low molar mass PMeOx and PEtOx are excreted very rapidly via the kidneys and show no significant unspecific uptake in vivo after intravenous administration [21]. At the same time, POx can be tailored to exhibit antimicrobial effects [22,23]. As a result, POx are discussed as a potential alternative for the current biomaterial “gold standard” poly(ethylene glycol) (PEG) to overcome its limitations [10,24].

Structure-property relationship in cytotoxicity and cell uptake of poly(2-oxazoline) amphiphiles

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Overall, these polymer carriers are chemically well-defined, non-toxic, exhibit low immunogenicity and their sufficiently small size allows them to be easily excreted from the body.

Cellular uptake of synthetic polymers and nanomaterials is a central interest in drug delivery. The vast structural diversity among POx makes this platform an ideal candidate to decipher structure-uptake relationships in cells. Therefore, we synthesized a number of POx amphiphiles comprising 2-butyl-2-oxazoline (BuOx), 2-nonyl-2-oxazoline (NOx), 2-n-propyl-2-oxazoline (nPrOx), 2-isopropyl-2-oxazoline (iPrOx) as the hydrophobic blocks and investigated their cytotoxicity in human cancer cell lines (MCF7, MCF7-ADR) and immortalized canine kidney epithelial cells (Madin-Darby Canine Kidney, MDCK). After conjugation with fluorescent labels (tetramethylrhodamine isothiocyanate (TRITC) or ATTO425, respectively) we investigated the cellular uptake of these polymer amphiphiles by flow cytometry and laser scanning confocal microscopy to evaluate their feasibility for intracellular drug delivery. Our results suggest that POx based amphiphiles are generally non-toxic even at high concentrations of up to 20 g/L and show, depending on their structure, rapid, energy dependent cellular uptake.

2. Materials and Methods

2.1. Materials and Instrumentations

All substances were purchased 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-ethyl-2-oxazoline (EtOx), acetonitrile (ACN) and other solvents were dried by refluxing over CaH₂ under dry nitrogen atmosphere and subsequent distillation prior to use. NMR spectra were recorded on a Bruker ARX 300 (1H: 300.13 MHz) or a Bruker AC 250 (1H: 250.13 MHz) at room temperature. The spectra were calibrated using the solvent signals (CDCl₃: 7.26 ppm; D₂O: 4.67 ppm). Gel permeation chromatography (GPC) was performed on a Waters system (pump mod. 510, RI-detector mod. 410, precolumn PLgel and two PL Resipore columns (3 µm, 300 x 7.5 mm)) with N,N-dimethyl acetamide (DMAc) [57 mmol/L LiBr, 80 °C, 1 mL/min] as eluent and calibrated against polymethylmethacrylate standards. Microwave supported polymerizations were performed using a CEM Discover microwave with a maximum power setting to 150 W. The microwave was set to reaction temperature of 130 °C which was continuously monitored by an external infra-red detector.

MCF7-ADR cells derived from human breast carcinoma cell line, MCF7 (ATCC HT-B22) by selection with Doxorubicin, were kindly presented by Y.L. Lee (William Beaumont Hospital, Royal Oak, MI) unless otherwise stated. Cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM), containing 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin as described elsewhere. All cell culture materials were from Gibco Life Technologies, Inc. (Grand Island, NY) unless otherwise stated. Cells were used 2 days after seeding unless otherwise stated.

2.2. Synthesis of polymer amphiphiles

2.2.1. Preparation of bifunctional initiator

The bisoxazoline 1,2-bisoxazolinylethane was prepared according to literature procedure [25].

2.2.2. Preparation of polymer amphiphiles

The polymerizations and work-up procedures were carried out according to the procedures described previously for H1 [21], T1-T3 and D3 [14].

Exemplary, the preparation of T1 is described as follows.

Under dry and inert conditions 32.2 mg (0.2 mmol, 1 eq) of methyl trifluoromethylsulfonate (methyl triflate, MeOTf) and 440 mg (5.17 mmol, 26 eq) of 2-methyl-2-oxazoline (MeOx) were dissolved in 3 mL dry acetonitrile at room temperature. The mixture was subjected to microwave irradiation (150 W maximum, 130 °C) for 15 min. After cooling to room temperature, the monomer for the second block, 2-butyl-2-oxazoline (256 mg, 2.01 mmol, 10 eq) was added and the mixture was irradiated the same way as for the first block. The procedure was repeated for the third block with 442 mg MeOx (5.19 mmol, 26 eq). Finally, T1 was terminated by addition of 0.1 mL piperidine (1.01 mmol, 5 eq) at room temperature. After stirring over night, an access of K2CO₃ was added and the mixture was allowed to stir for several hours. After centrifugation, the solvent was removed from the supernatant under reduced pressure, the residue was taken up by addition of 3 mL of chloroform. After precipitation from cold diethyl ether (approx. 10 times the amount of polymer solution) the product was obtained by centrifugation. The precipitation was repeated twice and the polymer was obtained as colorless powder (792 mg, 67%, Mn = 5.8 kg/mol) after lyophilization from water. GPC (DMAc): M_w = 8.5 kg/mol (Đ = 1.21).

Accordingly, the other polymers were prepared with the appropriate initiators, monomers and terminating reagents.

2.2.2.3. Fluorescent labeling of polymer

Fluorescent labels (tetramethylrhodamine isothiocyanate and ATTO425-NHS ester) were coupled to amine terminated polymers in dry dimethylformamide using a 1.2 fold excess of dye and disopropylethylamine as base. Free dye was removed by repeated gel filtration (Sephadex LH20, mobile phase methanol). Typically after 2–3 filtrations, no free dye band was observed. Degree of labeling was obtained spectrophotometrically and was found to be typically 30–60%.

2.3. Cytotoxicity assay

Cells were plated in 96 well plates (Corning Inc., Corning NY) and incubated with polymer solutions in assay buffer (2 h incubations) containing 122 mM NaCl, 25 mM NaHCO₃, 10 mM glucose, 10 mM HEPES, 3 mM KCl, 1.2 mM MgSO₄, 1.4 mM CaCl₂ and 0.4 mM K₂HPO₄. The mean fluorescence intensity was analyzed using a Becton Dickinson LSRII flow cytometer operating under FACSDiva software (San Jose, CA). Data were acquired in linear mode and visualized in logarithmic mode. Data from 10,000 events were gated using forward and side scatter parameters to exclude debris and dead cells as well as control cells incubated with media alone as control for autofluorescence.
Diblock Copolymers

\[
\begin{align*}
R_1 &= \text{Me} \quad \rightarrow \quad \text{NH} \\
R_2 &= \text{Bu} \\
R_3 &= \text{Me} \quad \rightarrow \quad \text{NH} \\
R_4 &= \text{Bu} \\
R_5 &= \text{Me} \quad \rightarrow \quad \text{NH} \\
R_6 &= \text{Bu} \\
R_7 &= \text{Me} \\
R_8 &= \text{Bu}
\end{align*}
\]

triblock copolymers were prepared and studied (T7-T12). Water soluble homopolymers were synthesized from 2-methyl-2-oxazoline and 2-ethyl-2-oxazoline.

For example, triblock copolymers of ABA type (e.g. T5) are compared with diblock copolymers AB and BA (i.e. D1 and D2) of the same monomer composition, in which A comprises MeOx or EtOx whereas B represents a more hydrophobic block (e.g., comprising nPrOx, iPrOx, BuOx etc.). In addition, we prepared polymers with different termini. For example, polymer T7-T12 are derived from one batch of polymerization, but obtained by addition of different amines to aliquots of the mixture after the polymerization. In particular, T10 is a polymer terminated with N,N-dimethyl-N-dodecylamine, generating an amphiphilic block copolymer with a hydrophobic terminus bearing a quaternized amine. Tiller and co-workers have shown in previous accounts that similar structures are effective antimicrobials [22,23]. We were interested in their ability to integrate into mammalian cell membranes leading to cytotoxicity. Other termini at the very same polymers included N-Boc-piperazine (a protected amine terminal group) (T8), piperidine (T7) and ethyl isonpeicotate (a protected carboxylic acid terminal group) (T11). T9 represents the same polymer bearing a piperazine terminus, obtained by deprotection of T8. Accordingly, T12 is saponified T11 (Fig. 1).

In general, the polymers are of good to medium definition (D<1.3) with some exceptions where we obtained relatively broad distributions (D = 1.3-1.5) (Table 2).

To study the endocytosis of the polymers piperazine-terminated polymers were modified with Atto425 (polymers indexed with t) or TRITC (index *). These dyes were chosen due to their small molar mass and zero net charge. Thus, we expected minimal influence of the fluorophore on the HLB of the polymer-dye conjugate. However, some influence on the interaction of the polymers with cell membranes cannot be ruled out.

Five different diblock copolymers were investigated with respect to their concentration-dependent uptake. Four comprised MeOx as hydrophilic monomer (D1*, D2*, D4* and D5*) while D3* comprised EtOx. The hydrophobic block varied from BuOx (e.g. D1* - D3*) to iPrOx and nPrOx (D4*, D5*). The triblock copolymers included in the flow cytometry study were T5*, T6*, T9* and T9*. In addition, endocytosis of H3*, a homopolymer of EtOx was studied by flow cytometry.

3.2. Cytotoxicity studies

The cytotoxicity of the polymers was investigated in different cell lines. We investigated the cell viability using the MTT assay after treatment with polymer solution at different concentrations for 2 h and 24 h, in either human breast cancer cells (MCF7 and MCF7-ADR)

Table 1

<table>
<thead>
<tr>
<th>Polymer</th>
<th>1st Block</th>
<th>2nd Block</th>
<th>3rd Block</th>
<th>Terminus</th>
<th>Polymer</th>
<th>1st Block</th>
<th>2nd Block</th>
<th>Terminus</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>MeOx</td>
<td>BuOx</td>
<td>MeOx</td>
<td>Pid</td>
<td>T15</td>
<td>NOx</td>
<td>MeOx</td>
<td>Pid</td>
</tr>
<tr>
<td>T2</td>
<td>MeOx</td>
<td>BuOx</td>
<td>MeOx</td>
<td>Pid</td>
<td>T16</td>
<td>NOx</td>
<td>MeOx</td>
<td>Pid</td>
</tr>
<tr>
<td>T3</td>
<td>MeOx</td>
<td>BuOx</td>
<td>MeOx</td>
<td>Pid</td>
<td>T17</td>
<td>BuOx</td>
<td>MeOx</td>
<td>Pid</td>
</tr>
<tr>
<td>T4</td>
<td>MeOx</td>
<td>BuOx</td>
<td>MeOx</td>
<td>BocPip</td>
<td>T18</td>
<td>BuOx</td>
<td>MeOx</td>
<td>Pid</td>
</tr>
<tr>
<td>T5</td>
<td>MeOx</td>
<td>BuOx</td>
<td>MeOx</td>
<td>Pip</td>
<td>T19</td>
<td>MOP</td>
<td>MeOx</td>
<td>Pid</td>
</tr>
<tr>
<td>T6</td>
<td>MeOx</td>
<td>nPrOx</td>
<td>MeOx</td>
<td>Pip</td>
<td>T10</td>
<td>BuOx</td>
<td>MeOx</td>
<td>Pid</td>
</tr>
<tr>
<td>T7</td>
<td>BuOx</td>
<td>MeOx</td>
<td>n.a.</td>
<td>Pid</td>
<td>T11</td>
<td>BuOx</td>
<td>MeOx</td>
<td>Pid</td>
</tr>
<tr>
<td>T8</td>
<td>BuOx</td>
<td>MeOx</td>
<td>n.a.</td>
<td>BocPip</td>
<td>T12</td>
<td>BuOx</td>
<td>MeOx</td>
<td>Pid</td>
</tr>
<tr>
<td>T9</td>
<td>BuOx</td>
<td>MeOx</td>
<td>n.a.</td>
<td>Pip</td>
<td>T9</td>
<td>BuOx</td>
<td>MeOx</td>
<td>Pid</td>
</tr>
<tr>
<td>T10</td>
<td>BuOx</td>
<td>MeOx</td>
<td>n.a.</td>
<td>DMDOd</td>
<td>D5</td>
<td>MeOx</td>
<td>nPrOx</td>
<td>Pip</td>
</tr>
<tr>
<td>T11</td>
<td>BuOx</td>
<td>MeOx</td>
<td>n.a.</td>
<td>ENp</td>
<td>H1</td>
<td>BuOx</td>
<td>MeOx</td>
<td>BocPip</td>
</tr>
<tr>
<td>T12</td>
<td>BuOx</td>
<td>MeOx</td>
<td>n.a.</td>
<td>ENp</td>
<td>H2</td>
<td>BuOx</td>
<td>MeOx</td>
<td>BocPip</td>
</tr>
<tr>
<td>T13</td>
<td>PynOx</td>
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<td>n.a.</td>
<td>Pid</td>
<td>T13</td>
<td>H3</td>
<td>MeOx</td>
<td>n.a.</td>
</tr>
<tr>
<td>T14</td>
<td>PynOx</td>
<td>MeOx</td>
<td>BocPip</td>
<td>H4</td>
<td>T14</td>
<td>H3</td>
<td>MeOx</td>
<td>Pid</td>
</tr>
</tbody>
</table>

Monomers: MeOx:2-methyl-2-oxazoline, EtOx:2-ethyl-2-oxazoline, nPrOx:2-n-propyl-2-oxazoline, iPrOx:2-isopropyl-2-oxazoline, NPrOx:2-nonyl-2-oxazoline, PynOx:2-(4-pentynyl)-2-oxazoline, MOP: methyl-(3-oxazol-2-yl)propionate.

MCF7-ADR cells show no cytotoxicity for the majority of polymers.

0.01 mM, respectively) the apparent cell viability increased slightly with concentration within the cells[26,27]. We therefore used fluorescently labeled polymers and investigated their cellular uptake using flow cytometry in the multi-drug resistant cell line MCF7-ADR.

Also for MCF7 cells, no adverse effects were observed with up to 1 wt.% or 2 wt.% (for example T1–T3, 20 g/L; 3.6 mM, 3.1 mM and 2.6 mM, respectively) (Fig. 3). Finally, we investigated cytotoxicity of polymers in adriamycin-resistant cell line MCF7-ADR (Fig. 4). As can be clearly seen, only one polymer sample, the quaternized triblock copolymer T10 showed a marked inhibition of cell proliferation after 2 h incubation with an IC50 of approx. 0.05 wt.% (0.07 mM). After 24 hours of incubation, the IC50 value of T10 remained practically unchanged. It should be noted that T10 is a triblock copolymer obtained from a bifunctional initiator and therefore also bears two termini.

3.3. Cellular uptake of fluorescently labeled polymers

In general it is of importance to understand the fate of non-degradable materials after injection. Besides pharmacokinetic studies in animals, studies of the endocytosis of such materials are warranted. Although hydrophobic drugs can enter cells by diffusion once the carrier reaches the target, it is also of interest whether the carrier itself can enter the target cells. If this is the case, it may help drugs to bypass drug resistance mechanisms, increasing the effective drug concentration within the cells [26,27]. We therefore used fluorescently labeled polymers and investigated their cellular uptake using flow cytometry in the multi-drug resistant cell line MCF7-ADR.

### Table 2

Analytical data of polymers investigated in this study.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Mw,0,a</th>
<th>McPC,b</th>
<th>D0,0</th>
<th>Mw,0/c</th>
<th>DP0,a</th>
<th>DP0,c</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>5.8</td>
<td>8.5</td>
<td>1.21</td>
<td>6.6</td>
<td>54</td>
<td>12</td>
<td>67</td>
</tr>
<tr>
<td>T2</td>
<td>6.4</td>
<td>10.4</td>
<td>1.18</td>
<td>7.3</td>
<td>51</td>
<td>19</td>
<td>80</td>
</tr>
<tr>
<td>T3</td>
<td>7.1</td>
<td>9.9</td>
<td>1.23</td>
<td>7.0</td>
<td>48</td>
<td>20</td>
<td>55</td>
</tr>
<tr>
<td>T4</td>
<td>5.9</td>
<td>5.8</td>
<td>1.19</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
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<td>14.7</td>
<td>1.22</td>
<td>10.0</td>
<td>88</td>
<td>18</td>
<td>69</td>
</tr>
<tr>
<td>T6</td>
<td>8.2</td>
<td>6.2</td>
<td>1.47</td>
<td>6.7</td>
<td>47</td>
<td>23</td>
<td>53</td>
</tr>
<tr>
<td>T7</td>
<td>6.7</td>
<td>n.d.</td>
<td>n.d.</td>
<td>6.7</td>
<td>52</td>
<td>16</td>
<td>&gt;95%</td>
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<tr>
<td>T8</td>
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<td>6.9</td>
<td>52</td>
<td>16</td>
<td>&gt;95%</td>
</tr>
<tr>
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<td>6.7</td>
<td>10.2</td>
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<td>6.7</td>
<td>52</td>
<td>16</td>
<td>n.d.</td>
</tr>
<tr>
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<td>n.d.</td>
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<td>52</td>
<td>16</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>T11</td>
<td>6.8</td>
<td>7.5</td>
<td>1.24</td>
<td>6.8</td>
<td>52</td>
<td>16</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>T12</td>
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<td>51</td>
<td>19</td>
<td>80</td>
</tr>
<tr>
<td>D1</td>
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<td>6.9</td>
<td>54</td>
<td>17</td>
<td>&gt;95%</td>
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<tr>
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<td>10.4</td>
<td>1.29</td>
<td>8.7</td>
<td>94</td>
<td>5</td>
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<td>60</td>
<td>n.a.</td>
<td>88</td>
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<tr>
<td>H1</td>
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<td>n.a.</td>
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<tr>
<td>H2</td>
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</tr>
<tr>
<td>H4</td>
<td>4.2</td>
<td>6.4</td>
<td>1.22</td>
<td>5.2</td>
<td>56</td>
<td>n.a.</td>
<td>66</td>
</tr>
</tbody>
</table>

a as determined from [Mw]/[I]0.

b as determined by gel permeation chromatography (solvent DMAc, PMMA calibration).

c as estimated by 1H-NMR by end-group and side chain analysis.

3.3.1. Flow cytometry study of temperature dependent uptake of copolymers

Comparison of the uptake at 37 °C and 4 °C after 60 min exposure revealed that the uptake was strongly inhibited at 4 °C for all polymers investigated (Fig. 5). For the majority of polymers the uptake appears to be completely diminished as about 0% gated cells are observed. In contrast, two polymers, D11 and D31 still show considerable uptake at 4 °C with 25% and 65% gated cells, respectively.

3.3.2. Flow cytometry study of concentration dependent uptake of copolymers

The uptake study was performed after 60 min incubation. All polymers showed a pronounced concentration dependent cellular uptake, albeit with markedly differing uptake behavior (Fig. 6A, Table 3). The effective concentration resulting in 50% gated cells (EC50) ranges from high nanomolar (D11, D31) to micromolar (D21, D41, D51) values, with D31 having the lowest value of 0.1 μM. In contrast, block copolymers with the 2-propyl-2-oxazoline blocks...
EC50 values of clear that the uptake continues after that with an essentially linear rather high concentrations with an EC50 value of approx. 0.07 mM.

amphiphilic in nature, it was able to enter the cells, albeit only at more or much less material than the main population.

homogenous population, i.e. we were unable to detect subpopulations of cells, which differ in their uptake behavior, e.g. taking up much more or much less material than the main population.

Also in the case of triblock copolymers (T9/1, T6/1 and T9/1/9*) a marked difference with respect to the concentration dependence of the endocytosis was observed (Fig. 6B). The EC50 values range from 1 μM to approx. 0.06 mM. T5*, the polymer with the lowest EC50 values also comprised the longest hydrophobic block. T9*/T9*, with the hydrophobic block length of only approx. 2/3 of the hydrophobic block in T5*, had a slightly higher EC50 of approx. 6 μM, irrespective of the nature of the fluorescent dye. As with the diblock copolymers, the triblock copolymer with moderately hydrophobic nPrOx (T6*) block exhibited the highest EC50 value of approx. 0.06 mM.

Finally, H3* was studied by flow cytometry. Being itself slightly amphiphilic in nature, it was able to enter the cells, albeit only at rather high concentrations with an EC50 value of approx. 0.07 mM.

3.3.3. Flow cytometry study of time dependent uptake of copolymers

To determine the kinetics of cellular uptake of POx in greater detail we also performed a time course experiment. Cells were incubated with the polymers for predetermined times of 1 min to up to 60 min. D3* showed an extremely fast uptake as already after 1 min more than 50% gated cells were obtained and after only 10 min all cells were gated (Fig. 7A). As observed by the mean fluorescence it becomes clear that the uptake continues after that with an essentially linear

**Fig. 3.** Cell viability of block copoly(2-oxazoline) in MCF7 cells after 2 h incubation as determined by MTT assay. Experiments were performed in quadruplicate and data is expressed as means±SEM.

(D4/D5*), which are partly water soluble enter the cells only at 50 – 60 times higher concentrations (EC50 5–6 μM). Also the point of attachment of the fluorescent dye may have some influence, as the EC50 values of D1* and D2* differ somewhat (0.4 vs 2 μM).

It should be noted that the cells reacted as one relatively homogenous population, i.e. we were unable to detect subpopulations of cells, which differ in their uptake behavior, e.g. taking up much more or much less material than the main population.

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**Fig. 4.** Cell viability of block copoly(2-oxazoline) in MCF7-ADR cells after 2 h (A-I) or 24 h (J-L) incubation as determined by MTT assay. Experiments were performed in quadruplicate and data is expressed as means±SEM.
increase of the mean fluorescence over time. In comparison, the time course of the uptake of T5 appears somewhat slower (Fig. 7B). Even after 60 min, 100% gated cells were not obtained while 50% gated cells were reached after around 12 min. In the example of T9 it took about 30 min to obtain 100% gated cells in this cell line with 50% gated cells (MDCK) reached within less than 5 min (Fig. 7C).

3.3.4. Confocal study of cellular uptake of amphiphilic block copolymers

In order to confirm that the fluorescence signals obtained in flow cytometry experiment do not stem from merely cell membrane-associated polymers, laser scanning confocal microscopy of live cells was performed. In no case we observed a considerable amount of membrane-associated polymer. In the case of T9 we performed a 5 min pulse, 55 min chase experiment (with unlabeled polymer T9) as well as a 60 min incubation experiment in MCF7-ADR cells and a 60 min incubation experiment in MDCK cells (Fig. 8).

It is evident that little portion of the fluorescent material is bound with cell membranes. In contrast, the fluorescence appears to be distributed over the entire cell especially in the perinuclear region but did not show in the nucleus. No apparent differences in the fluorescence patterns between the pulse-chase experiment, the 60 min incubation and the non-resistant cell line can be observed. A z-stack of a cell cluster of MCF7-ADR cells incubated with T9 confirms the distribution over the entire cytoplasm with the exception of the nucleus as well as the perinuclear enrichment (Fig. 9).

### Table 3

Comparison of EC50 values for the different di- and triblock copolymers (D1-D5, T5, T6, T9) and EtOx homopolymer H3 as obtained from flow cytometry and subsequent Boltzmann fitting of data.

<table>
<thead>
<tr>
<th>Polymer ID</th>
<th>Polymer composition</th>
<th>EC50 (µM)</th>
<th>EC50 (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1*</td>
<td>MeOx52-b-BuOx21</td>
<td>0.4</td>
<td>0.003</td>
</tr>
<tr>
<td>D2*</td>
<td>BuOx52-b-MeOx54</td>
<td>2</td>
<td>0.01</td>
</tr>
<tr>
<td>D3*</td>
<td>EtOx52-b-BuOx21</td>
<td>0.1</td>
<td>0.0008</td>
</tr>
<tr>
<td>D4*</td>
<td>MeOx93-b-iPOx38</td>
<td>6</td>
<td>0.07</td>
</tr>
<tr>
<td>D5*</td>
<td>MeOx93-b-nPOx38</td>
<td>5</td>
<td>0.06</td>
</tr>
<tr>
<td>T5*</td>
<td>MeOx26-b-BuOx15-b-MeOx26</td>
<td>8</td>
<td>0.06</td>
</tr>
<tr>
<td>T9*</td>
<td>MeOx26-b-nPOx15-b-MeOx25</td>
<td>8</td>
<td>0.06</td>
</tr>
<tr>
<td>H3*</td>
<td>EtOx43</td>
<td>72</td>
<td>0.4</td>
</tr>
</tbody>
</table>
Experiments were performed in triplicate and data is expressed as means±SEM.

Although several publications have dealt with biomedical use of POxylated horseradish peroxidase, which was dependent on the structure of POx [13]. Preliminary results suggested that amphiphilic POx alone also entered cells rather efficiently. To understand this uptake in detail we decided to investigate the temperature, concentration and time dependence of a series of fluorescently labeled polymers (Table 3).

The fluorescently labeled polymers show a drastically different temperature dependence of cellular uptake as compared to the free dye (TRITC) as verified for T9*. While the polymer appears to be taken up by endocytosis (strongly diminished at 4°C), the small dye molecule freely diffuses into MCF7-ADR cells as expected (no temperature dependence observed, data not shown). This difference shows that no or only negligible amount of free dye is present in the incubation solution of T9*. Moreover, this results demonstrates how drug loaded POx micelles could help to overcome multi-drug resistance in cancer therapy. While the uptake of free small molecule and Pgp substrate TRITC is markedly reduced in the MDR phenotype, the POx micelles are still entering the cells effectively.

Comparing the uptake of diblock and triblock copolymers with different block sizes and different hydrophobic blocks we were able to identify clear structure–property relationships, supporting previous endocytosis studies with homo-, random and block copolymers of 2-hydroxypropylmethacrylamide (HPMA) in MCF7-ADR cells [28]. Within the structural limits we investigated, the more hydrophobic the polymer is, the more readily it enters the cells. It should be noted, however, that all polymers were excellently water-soluble with solubility exceeding 100 g/L in all cases. The polymers with the lowest EC50 values were D3† (0.1 μM) and D1† (0.4 μM), both comprising 20 units of BuOx as the hydrophobic block and about 50 units of EtOx or MeOx as the hydrophilic block. Using the concentration dependence of the endocytosis of D1† and D3† it can be estimated that the uptake of the polymers at 4°C as compared to 37°C is comparable to a 50-fold decrease of the polymer concentration. Interestingly, the structural isomer of D1† with the opposite sequence of hydrophilic and hydrophobic block (D2†) has a slightly higher EC50 of 2 μM. A possible explanation of this may be the resulting different attachment

4.2. Endocytosis

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point of the fluorescent dye. In the case of D1, the dye is attached to the hydrophobic block while in D2 it is attached to the hydrophilic block. It is known that terminal groups can have significant effects on the physicochemical properties of POx [16]. In this context, one can argue that the hydrophobic dye elongates the hydrophobic block in D1. In contrast, the hydrophobic character of the dye may not influence the uptake as much when it is attached to the hydrophilic MeOx block in the case of D2.

Reducing the hydrophobicity of the hydrophobic block further also diminishes the uptake of the polymers as observed with D4 and D5, which bear iPrOx and nPrOx, respectively. Both monomers yield thermoresponsive polymers with cloud points around 45 °C and 25 °C (at 20 g/L), respectively [13]. At the investigated temperature and low concentrations, both polymers are presumably fully water-soluble. Accordingly, their uptake was observed only at higher concentrations with EC50 values being 6 and 5 μM, respectively.

A similar trend was observed for triblock copolymers T5, T6 and T9/T9'. The monomer composition of T5 is similar to the one of D1. However, T5 is an ABA type triblock with a central hydrophobic core and flanking hydrophilic blocks. The effect of this structural difference appears to be minor with virtually identical EC50 values of 0.4 and 1 μM observed for D1 and T5, respectively. As the size of the hydrophobic block was decreased by approx. 25% (22 vs 15 units), the uptake was shifted towards higher concentrations (T9/T9'; EC50 = 8 μM). T6 exhibited the highest EC50 values of all copolymers investigated (0.06 mM). Interestingly, here a pronounced difference between a diblock copolymer and triblock copolymer (D5 vs T6)

**Fig. 8.** Representative laser scanning confocal microscopy images of MCF7-ADR (columns A and B) and MCF7 (column C) cells incubated with T9'. Row 1 shows overlay of fluorescence observed at 561 nm (row 2) and DIC (row 3). Scale bars represent 20 μm.

**Fig. 9.** Representative laser scanning confocal microscopy z-stack image of MCF7-ADR cells incubated with T9'.
was observed with the EC50 value of D5 (5 μM) being about one order of magnitude less than that of the T6. D5 has a significantly longer hydrophilic block as compared to T6. Therefore, one could expect a higher EC50 value for D5, contrary to what is observed. This observation suggests that the length of the hydrophilic blocks play a minor role for the cellular uptake, similarly as the length of hydrophobic blocks has limited influence on the critical micelle concentration or critical aggregation concentration. Finally, a homopolymer of EtOx was studied and we found very low endocytosis even at rather high concentrations (EC50 = 0.07 mM). In summary, the order of EC50 values of the polymers investigated in this study was D3 - D1 - T5 - D4 - D5 - T9 = T9 - T6 - H3 and are summarized in Table 3.

It should be noted that the cmc values for all polymers are higher than the respective EC50 values. Moreover, under the current experimental conditions, we were unable to observe any apparent change in the uptake behavior when crossing into the micellar region. Thus, it is safe to assume that the individual unimers and micelles are taken into the cells. However, to investigate whether unimers and micelles use different uptake mechanisms, more detailed studies with endocytosis inhibitors and markers of individual uptake mechanisms would be necessary [29,30].

In the present study, we found the uptake of POx based amphiphiles to be rather fast. In the case of D3, the polymer with the lowest EC50 value, the uptake was also particularly fast with 50% of cells positively gated after only 1 min of incubation.

Direct comparison to other studies is difficult as only relatively few groups studied the endocytosis of POx based materials. For example, DeSimone and co-workers recently reported endocytosis of PEtOx based nanoparticles. In this study we could confirm the limited uptake of hydrophilic polymers, while we are unable to confirm the lower uptake for polymers of a more pronounced hydrophobic character. Future studies should therefore include polymers with a considerably stronger hydrophobic character than D3.

A comparison with the previously published concentration dependent uptake of HPMA based random and block copolymers and HPMA homopolymer reveals that the EC50 values of POx-based polymers are in the similar range. A random copolymer of HPMA and laurylacrylate (molar monomer ratio 80/20) of 15 kg/mol had an EC50 of 0.2 μM while a block copolymer of comparable size and monomer composition gave an EC50 = 7 μM. Similar as in the present report HPMA homopolymers had increased EC50 values of about 30 μM [28].

DeSimone and co-workers reported recently on the uptake of PEG-containing particles synthesized by the PRINT technique. It was observed that the concentration dependent uptake of the nanoparticles was cell-type dependent, suggesting further uptake studies of the introduced POx block copolymers in a wider range of cell lines. Although no EC50 values are given in this account, it is apparent that the internalization of the 1 μm large particles occurred in the same order of magnitude of concentration [33]. The same group also studied the size effects on the internalization. In this particular case, no concentration (dependence) is given, but fastest uptake is observed for particles with a diameter of 150 nm and length of 450 nm (~50% gated cells in ~15 min) [34]. Haag and co-workers report on the cellular uptake of dendritic polyglycerols in A549 lung epithelial cells. At low concentrations of 10 μM (4 h incubation), a pronounced difference between charged and non-charged derivatives was observed, the latter giving only little uptake while the former where internalized efficiently [35].

Laser scanning confocal microscopy of live cells clearly shows that all polymers enter the cells and no fluorescence is fixed to cell membranes (Figs. 8 and 9). Although the fluorescence is distributed throughout the entire cells, the polymers appear to be enriched in vesicular compartment(s) of the cells. However, in detail investigation of the subcellular distribution and intracellular trafficking of POx amphiphiles is beyond the scope of this article and will be published in a separate account. We hypothesize a specific uptake mechanism for these rapidly incorporated POx amphiphiles similar but possibly distinct to the uptake mechanism we postulated for Pluronic® P85, a triblock copolymer of similar molar mass (4.6 kg/mol) of a central poly(propylene oxide) and two flanking PEG blocks [29].

5. Conclusion

In summary, we studied the cytotoxicity of a range of POx based homo and block copolymers. We could confirm that in general POx appear to be well tolerated by mammalian cells, a result suggested earlier in a number of studies, albeit using only a few examples. More importantly, we performed a quantitative screening of the endocytosis of amphiphilic POx which already have demonstrated great potential in micellar drug delivery [14]. The synthetic possibilities given by the living cationic ring-opening polymerization of POx give an ideal tool to manufacture defined polymers and investigate structure property relationships of their interaction with biological entities. The results of our study underline the importance of thorough investigation of the concentration dependent uptake of nanomaterials, in particular to allow for a comparison of nanomaterials of different origin, structure and properties.

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References


