Biodegradable star HPMA polymer–drug conjugates: Biodegradability, distribution and anti-tumor efficacy

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Herein, new biodegradable star polymer–doxorubicin conjugates designed for passive tumor targeting were investigated, and their synthesis, physico-chemical characterization, drug release, biodegradation, biodistribution, and in vivo anti-tumor efficacy are described. In the conjugates, the core formed by poly(amideamine) (PAMAM) dendrimers was grafted with semitelechelic N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers bearing doxorubicin (Dox) attached by hydrazone bonds, which enabled intracellular pH-controlled drug release. The described synthesis facilitated the preparation of biodegradable polymer conjugates in a broad range of molecular weights (200–1000 g/mol) while still maintaining low polydispersity (≈1.7). The polymer grafts were attached to the dendrimers through either stable amide bonds or enzymatically or reductively degradable spacers, which enabled intracellular degradation of the high-molecular-weight polymer carrier to excretable products. Biodegradability tests in suspensions of EL4 T-cell lymphoma cells showed that the rate of degradation was much faster for reductively degradable conjugates (close to completion within 24 h of incubation) than for conjugates linked via an enzymatically degradable oligopeptide GFLG sequence (slow degradation taking several days). This finding was likely due to the differences in steric hindrance in terms of the accessibility of the small molecule glutathione and the bulky enzyme cathepsin B to the polymer substrate. Regarding drug release, the conjugates were fairly stable in buffer at pH 7.4 (model of blood stream) but released doxorubicin under mild acidic conditions that model the tumor cell microenvironment. The star polymer–Dox conjugates exhibited significantly prolonged blood circulation and enhanced tumor accumulation in tumor-bearing mice, indicating the important role of the EPR effect in its anti-cancer activity. The star polymer conjugates showed prominently higher in vivo anti-tumor activities than the free drug or linear polymer conjugate when tested in mice bearing EL4 T-cell lymphoma, with a significant number of long-term surviving (LTS). Based on the results, we conclude that a Mw of HPMA copolymers of 200,000 to 600,000 g/mol is optimal for polymer carriers designed for the efficient passive targeting to solid tumors. In addition, an expressive therapy-dependent stimulation of the immune system was observed.

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1. Introduction

Water-soluble polymer drug carriers based on N-(2-hydroxypropyl) methacrylamide (HPMA) copolymers have been widely studied over the last 30 years. Most of these drug carriers were developed for the treatment of tumors, with a special focus on the site-specific delivery of anticancer and anti-inflammatory agents into tumor tissues or cells. Proper selection of the molecular weight and structure of the polymer carrier, the spacer between the drug and carrier and the targeting approach are all essential for achieving an effective tumor- or tumor cell-specific drug targeting followed by controlled drug release, which is important for the subsequent anti-tumor activity of the drug [1,2].

The most frequently studied HPMA-based conjugates contain oligopeptide spacers (e.g., GFLG sequences) between the drug and carrier designed for enzymatic drug release by lysosomal enzymes [3,4]. In the last decade, the synthesis and properties of HPMA-based conjugates, in which the drug is bound to a carrier by pH-sensitive hydrazone bonds, have been intensively studied [5–8]. While the hydrazone bond is relatively stable at neutral pH, which corresponds to the blood environment, the drug is released under mildly acid conditions, such as in endosomes or lysosomes of tumor cells, with half-lives in the range of hours. These conjugates are highly cytotoxic for cancer cells in vitro and exhibit an enhanced accumulation in solid tumor tissue (e.g., mouse EL4 T-cell lymphoma) and superior anti-tumor activity.

The anti-tumor activity of HPMA-based conjugates can be improved by either introducing targeting moieties (e.g., monoclonal antibodies [9–11] or oligopeptides [12,13]), or by enhancing their “passive” targeting due to the EPR (enhanced permeability and retention) effect,
on which the present work will focus. Maeda first described that high-molecular-weight (HMW) polymer carriers are effectively accumulated in solid tumors [14]. The high molecular weight of polymer carriers prevents fast elimination of the drug from the organism by renal filtration; thus, it enables prolonged blood circulation and increased tumor uptake. Some reviews have been published that describe the EPR effect in large detail [15–17]. It can be summarized that the extent of polymer carriers that passively accumulate in solid tumors primarily depends on their molecular weight or, more precisely, on the size of their coil in aqueous solutions. However, this trend reverses at very high molecular weights. These features were also observed in the case of HPMA-based copolymers [18–20].

Ideally, polymer drug carriers should be eliminated from the body after the drugs are delivered and released. Nevertheless, only nondegradable polymers (i.e., HPMA-based copolymers) of molecular weight under ~50,000 g/mol are small enough to undergo renal filtration. Thus, several structures of biodegradable HMW HPMA-based polymer drug carriers have been proposed and studied, such as the branched [21,22] or graft [18] polymer carriers, containing either enzymatically or reductively degradable spacers that connect short polymer chains into the HMW structure. Some of these carriers showed prolonged blood circulation, enhanced tumor accumulation and, most importantly, excellent anti-tumor activity. The graft copolymers also proved to be biodegradable in vitro in cell cultures [23]. However, these carriers have shown some drawbacks (e.g., high polydispersity and low reproducibility of their synthesis), which might be overcome using the grafting of dendrimers with semitelechelic copolymers. The first HMW star polymer carriers exhibited relatively low polydispersity, but contained no biodegradable linkages between the PAMAM dendrimer core and polymer grafts [24]. Recently, we reported a study describing the synthesis and physico-chemical properties of new star polymer drug conjugates differing in the nature of the biodegradable spacers, both between the polymer and drug and between the polymer and dendrimer core [25].

Herein, the synthesis, physico-chemical and detailed biological evaluations of novel biodegradable star HPMA copolymer–Dox conjugates tailored for efficient passive tumor targeting are described. The major aim was to design and prepare HMW star polymer–drug conjugates that are suitable as an efficient drug delivery system with reproducible synthesis, precise control over molecular weights, sufficient molecular weight for effective tumor accumulation and a defined molecular structure with a narrow distribution of molecular weights. Moreover, focus has been placed on the synthesis and characterization of novel star conjugates and on the impact of the conjugate’s structure on in vivo anti-tumor activity, immunoprotection, biodegradability, tumor accumulation and blood clearance.

2. Experimental section

Experimental section is described in Supplementary information.

3. Results and discussion

It has been more than 15 years since the EPR effect was verified using HPMA copolymers [19,27]. Recently, we have demonstrated significant in vivo anti-tumor activities of micellar [28] and graft [18] HMW HPMA copolymer–Dox conjugates in the treatment of solid tumors in mice, which were attributed to the EPR effect. The apparent drawbacks of these systems include the rather high polydispersity of the conjugates and the poor reproducibility of their synthesis, which does not enable the preparation of larger batches of the same quality. In this paper, we have focused on the design, synthesis and evaluation of the biological characteristics of new HMW HPMA copolymer–Dox conjugates with well-defined architecture, proper control of molecular weight and controlled biodegradation. The new HMW polymer carrier was prepared by grafting the semitelechelic HPMA copolymers onto a PAMAM dendrimer core to form star-like structures. To ensure controlled elimination of the polymer carrier system, the semitelechelic polymers were grafted onto the PAMAM dendrimer via biodegradable linkages containing reductively or enzymatically degradable spacers. Moreover, the results of drug release, star carrier degradation, biodistribution and in vivo anti-tumor activity are presented and discussed. Typical experiments were performed with groups of four animals.

3.1. Synthesis of polymer precursors and modified dendrimers

The semitelechelic polymer precursor $P_{NH}$-TT was prepared by radical solution copolymerization of HPMA with Ma-ah-NHNH-Boc initiated with ABIC-TT, a bifunctional azo-initiator containing reactive TT groups. The polymerization conditions were chosen to keep the molecular weight of copolymers under the renal threshold [26]. The functionality (number of chain-terminating TT groups per polymer) of the polymer was higher than 1 ($M_p/M_n,T = 1.24$), in spite of the preference for disproportionation in the termination reaction. The semitelechelic copolymer bearing an end-chain PDS group (polymer $P_{NH}$-PDS) was prepared by an aminolytic reaction of the TT groups of the polymer $P_{NH}$-TT with PDEA. The molecular weight and polydispersity of the copolymers remained almost unchanged after aminolysis (Table S11). Slightly lower yields of the modification reaction led to a decrease in the functionality of polymer $P_{NH}$-PDS to 1.07. The subsequent semitelechelic copolymer bearing an end-chain thiol group (polymer $P_{NH}$-SH) was prepared by the reduction of the PDS groups of polymer $P_{NH}$-PDS with an excess of DTT. The molecular weight of the polymer was only slightly increased, and its functionality was slightly decreased. These changes in characteristics were not significant, and polymer $P_{NH}$-SH was used for further reactions. The semitelechelic copolymer bearing an enzymatically degradable oligopeptide end-chain (polymer $P_{NH}$-COOH) was prepared by the aminolysis of polymer $P_{NH}$-TT with an oligopeptide H-GFLG-OH. Analogous to aminolysis with PDEA, the slightly lower yield of the reaction resulted in a small decrease in the functionality of polymer $P_{NH}$-COOH without significant changes in molecular weight and polydispersity.

Later, the semitelechelic polymers $P_{NH}$-TT and $P_{NH}$-COOH terminating in TT or GFLG-OH groups were used for aminolytic grafting to the $D_{NH2}$ dendrimer. The thiol-terminated semitelechelic polymer $P_{NH}$-SH was used for grafting to the $D_{NH2}$ dendrimer. The structures of the semitelechelic polymers and the scheme of synthesis are given in Fig. S11.

A $D_{NH2}$ dendrimer containing reactive PDS groups was prepared in methanol by the aminolysis of the active ester of SPDP with amino groups of the $D_{NH2}$ dendrimer [25]. The modified dendrimer was characterized by the number of PDS groups (degree of substitution). In five independent measurements, we observed an average of 15.7 PDS groups per molecule of dendrimer, which signified nearly complete functionalization.

3.2. Synthesis of star polymer precursors and conjugates

The star polymer conjugates were prepared by grafting semitelechelic polymers $P_{NH}$-TT, $P_{NH}$-SH and $P_{NH}$-COOH onto the dendrimers that contain amino ($D_{NH2}$) or thiol-reactive PDS ($D_{NH2}$) groups. Three different types of star polymer conjugates differing in the detailed structure were synthesized. The first type contained a stable amide bond between the polymer grafts ($P_{NH}$-TT) and the central $D_{NH2}$ dendrimer and was non-degradable under body conditions ($D_2$-$P_{NH}$ and $D_4$-$P_{NH}$). On the contrary, the other two types were biodegradable polymers containing either reductively degradable disulfide bonds ($D_2$-$SS$-$P_{NH}$) or an enzymatically degradable oligopeptide GFLG sequence ($D_2$-$GFLG$-$P_{NH}$). The products of the grafting reaction were HMW polymers with a star structure and a relatively narrow distribution of molecular weights ($M_n = 180,000$–
1,079,000 g/mol; $M_w/M_n ≈ 1.7$). These characteristics indicate that they are good drug carrier candidates for the passive targeting to solid tumors. The influence of the dendrimer structure on the molecular characteristics of the star polymer conjugates was studied with the 2nd and 4th generation D$_{NH2}$ conjugates containing 16 and 64 surface amino groups (D$_2$-P$_{NH2}$ and D$_4$-P$_{NH2}$). It is evident that an increased number of surface groups enhanced the loading capacity for the semitelechelic copolymers, which was shown by the significant increase in the molecular weight (from 196,000 to 711,000 g/mol) and hydrodynamic radius ($R_h$) (from 12.6 to 27.9 nm) of the star polymers. The analysis of molecular weights signposted that an average of seven to eight semitelechelic polymers were attached in star polymer D$_2$-P$_{NH2}$, and almost twenty-seven were attached in star polymer D$_4$-P$_{NH2}$. The ratio of grafted to original amino groups of D$_{NH2}$ slightly decreased with the increasing generation of dendrimers and is in agreement with published results [25]. This observation can be ascribed to the increased steric hindrance for HPMA copolymers in accessing more dense amino groups of the higher generation dendrimers. With the aim to prepare star polymer conjugates with a broad range of molecular weights for in vivo biodistribution and anti-tumor activity studies, we have fractionated the star polymer D$_4$-P$_{NH2}$. After fractionation using gel filtration with Sephacryl S-300, we obtained two star polymer precursors, D$_4$-P$_{NH2}$-F1 ($M_w = 587,000$, $M_n/M_w = 1.45$) and D$_4$-P$_{NH2}$-F2 ($M_w = 1,023,000$, $M_n/M_w = 1.63$), with significantly different molecular weights.

For further studies and the synthesis of biodegradable star polymer carriers, we have chosen the 2nd generation of dendrimers because they enable the preparation of star polymers with sufficient molecular weights to achieve effective passive tumor targeting while limiting the residual amino groups with potential toxicity.

Molecular characteristics of star polymers and conjugates D$_2$-SS-P$_{NH2}$, D$_2$-SS-P-Dox, D$_2$-GFLG-P$_{NH2}$, and D$_2$-GFLG-P-Dox, which were prepared from the 2nd generation dendrimers, corresponded with that of star polymer D$_2$-P$_{NH2}$. Neither the type of the dendrimer (different core or surface groups) nor the method of synthesis (non-degradable or degradable structure with S-S bond or GFLG sequence) significantly influenced the molecular weight, polydispersity or $R_h$ of the dendritic polymers prepared from the 2nd generation PAMAM dendrimers.

Removing the protective 80c groups from the hydrazides in precursors D$_2$-P$_{NH2}$, D$_2$-P$_{NH2}$-F1, D$_2$-P$_{NH2}$-F2, D$_2$-SS-P$_{NH2}$, and D$_2$-GFLG-P$_{NH2}$ with TFA did not significantly change their molecular weight and polydispersity. All star polymer–Dox conjugates (Table 1) containing Dox attached by a pH-sensitive hydrazone bond were prepared as previously described [21], using star polymer precursors. Conversion of the reaction was nearly complete and attachment of the drug had no significant influence on the molecular characteristics of the polymer conjugates.

The $R_h$ of the star polymer conjugates was much higher than the $R_h$ of the linear polymer–drug conjugate P-Dox in aqueous buffer solution. One point grafting of the dendrimers with the semitelechelic polymers led to an increase in $R_h$ from approximately 4.3 nm to a radius that was 3–7-fold higher. This increase in molecular weight, together with the biodegradability of star polymer structures, fulfilled the prerequisite criteria for the enhanced accumulation of polymers in solid tumors due to the EPR effect and the subsequent elimination of polymer degradation products from body. Schemes of the grafting reactions and structures of the HMW polymer precursors and conjugates are shown in Fig. 1.

### 3.3. In vitro drug release

Results of the pH-dependent chemical hydrolysis (Fig. S12) showed that the hydrazone bond used for drug attachment in the linear polymer P-Dox and star polymer conjugates D$_2$-P-Dox, D$_2$-P-Dox-1, D$_2$-P-Dox-2, and D$_2$-SS-P-Dox was fairly stable in buffer solutions that model the blood environment (pH 7.4 and 37 °C). Only a slight release of Dox (up to 8%) was observed within 24 h of incubation. On the contrary, more than 90% of Dox was released from the conjugates within 24 h incubation in a buffer that simulates conditions in the endosomes of target cells (pH 5 at 37 °C). It is clear that the effect of the size and structure of the polymer–drug conjugates on the rate of Dox release is not significant in this case. All polymer conjugates showed approximately the same release profile, independent of their molecular weight and the detailed structure of the polymer carrier.

To sum up the drug release studies, these novel star polymer–drug conjugates complied with basic requirements for acceptable anticancer prodrugs. They exhibit fair stability under conditions that model blood circulation and release an active drug in an environment that mimics conditions in tumor cells or tissues.

### 3.4. In vitro incubation of labeled polymer conjugates with EL4 lymphoma cells

Previously, we described the degradation of star polymer carriers in a buffer containing glutathione in the concentration found in the cytoplasm [25], resulting in polymer fragments with molecular weights of the polymer precursors. The susceptibility of the star polymers containing disulfide linkages to the reduction with glutathione is a very important factor; nevertheless, the intracellular degradability of the star polymer carriers is a more comprehensive problem. To confirm the degradability of star polymer carriers in a real biological system, the polymers were incubated in media containing EL4 lymphoma cells. A DY-615-labeled polymer carrier D$_2$-SS-P$_{NH2}$-Dyo was incubated in RPMI medium containing EL4 cells for 6, 12 and 24 h. Changes in the molecular weight and distribution of the labeled carriers were determined after extraction of the carrier degradation products from the cell lysate. Fluorescent labeling with DY-615 dye allowed GPC evaluation using a UV detector at 550 nm. At this

### Table 1

<table>
<thead>
<tr>
<th>Polymer or Conjugate</th>
<th>Dendrimer (type, generation)</th>
<th>Biodegradable spacer$^a$</th>
<th>$M_w$ (g/mol)</th>
<th>$M_n/M_w$</th>
<th>$R_h$ (nm)</th>
<th>Dox (wt%)</th>
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</thead>
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<tr>
<td>P-Dox</td>
<td></td>
<td></td>
<td>32,500</td>
<td>1.86</td>
<td>4.3</td>
<td>9.8</td>
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<td>D$_{NH2}$, G2</td>
<td></td>
<td>196,000</td>
<td>1.78</td>
<td>12.6</td>
<td>–</td>
</tr>
<tr>
<td>D$<em>2$-P$</em>{NH2}$</td>
<td>D$_{NH2}$, G4</td>
<td></td>
<td>711,000</td>
<td>2.05</td>
<td>27.9</td>
<td>–</td>
</tr>
<tr>
<td>D$<em>2$-P$</em>{NH2}$-F1$^b$</td>
<td>D$_{NH2}$, G4</td>
<td></td>
<td>587,000</td>
<td>1.45</td>
<td>21.4</td>
<td>–</td>
</tr>
<tr>
<td>D$<em>2$-P$</em>{NH2}$-F2$^b$</td>
<td>D$_{NH2}$, G4</td>
<td></td>
<td>1,023,000</td>
<td>1.63</td>
<td>39.3</td>
<td>–</td>
</tr>
<tr>
<td>D$_2$-P-Dox</td>
<td>D$_{NH2}$, G2</td>
<td></td>
<td>202,000</td>
<td>1.72</td>
<td>13.1</td>
<td>9.6</td>
</tr>
<tr>
<td>D$_2$-P-Dox-1</td>
<td>D$_{NH2}$, G4</td>
<td></td>
<td>601,000</td>
<td>1.41</td>
<td>24.4</td>
<td>10.0</td>
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<tr>
<td>D$_2$-P-Dox-2</td>
<td>D$_{NH2}$, G4</td>
<td></td>
<td>1,079,000</td>
<td>1.66</td>
<td>42.5</td>
<td>9.7</td>
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<tr>
<td>D$<em>2$-SS-P$</em>{NH2}$</td>
<td>D$_{NH2}$, G4</td>
<td>S=–</td>
<td>178,000</td>
<td>1.64</td>
<td>12.3</td>
<td>–</td>
</tr>
<tr>
<td>D$_2$-SS-P-Dox</td>
<td>D$_{NH2}$, G4</td>
<td>S=–</td>
<td>201,000</td>
<td>1.75</td>
<td>13.2</td>
<td>10.2</td>
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<tr>
<td>D$<em>2$-GFLG-P$</em>{NH2}$</td>
<td>D$_{NH2}$, G2</td>
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<td>1.70</td>
<td>13.8</td>
<td>–</td>
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<tr>
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<td>D$_{NH2}$, G2</td>
<td>GFLG</td>
<td>215,000</td>
<td>1.75</td>
<td>14.2</td>
<td>9.2</td>
</tr>
</tbody>
</table>

$^a$ GFLG sequence degradable by lysosomal enzymes or reductively degradable S-S bridge.

$^b$ polymer prepared by gel fractionation of polymer D$_2$-P$_{NH2}$.
wavelength, any possible interference of the polymer signal with a signal of the cell compartment peptides was avoided.

To show the stability of the star HMW polymer carrier in the RPMI medium and during the process of separating the polymers from the cell lysate, two control experiments were performed. Star polymer D2-SS-PHy-Dyo was incubated in the RPMI medium for 24 h to verify the stability of the polymer in the medium. In the second experiment, D2-SS-PHy-Dyo was mixed with a suspension of cancer cells just before cell lysis to prove potential degradation of the polymer during cell lysis and polymer extraction. No evidence of the degradation of the polymer D2-SS-PHy-Dyo was observed in either the RPMI medium or during cell lysis and extraction. Therefore, the degradation of the star polymer carriers after incubation with cells can be ascribed to the intracellular degradation of polymers inside EL4 lymphoma cancer cells.

GPC chromatograms of the star polymer D2-SS-PHy-Dyo incubated with cancer cells showed that, after 6 h, the polymer carrier is already partly degraded to a polymer with lower $M_w$ (Fig. 2). Degradation was more pronounced after 12 h and 24 h of incubation when the distribution curve obtained for the polymer degradation products resembled that of the DY-615-labeled linear polymer PHy-Dyo that was used as a control, indicating efficient intracellular degradation of the star polymer carrier containing disulfide linkages.

Similar experiments were performed with star polymer D2-GFLG-PHy-Dyo containing the oligopeptide GFLG sequence tailored as a substrate for lysosomal enzymes. Regrettably, GPC chromatograms showed no significant change in the distribution profiles of the polymer after 6 or 12 h of incubation with EL4 cancer cells. The first signs of degradation were noticeable after 24 h of incubation. Degradation was more pronounced after 48 h of incubation when the polymer degradation products exhibited distributions that were significantly shifted to the lower $M_w$ (data not shown). As in the previous case, control experiments in the RPMI medium and during...
the process of polymer separation from the cell lysate did not show any evidence of polymer D₂-GFLG-P₃D–Dyo degradation. Small changes in the molecular weight distribution of the star polymer D₂-GFLG–P₃D–Dyo during incubation is evidence of the small susceptibility of this star conjugate to enzymatic intracellular degradation. These results show that star conjugates with grafts connected to the dendritic core via a disulphide spacer is more suitable for the preparation of polymer–drug conjugates undergoing intracellular degradation and for providing polymer products that are excretable from the body by glomerular filtration.

3.5. Blood clearance, excretion in urine and tumor accumulation studies

Results of blood clearance, urine excretion, and tumor and muscle accumulation experiments should demonstrate the fundamental differences in the biodistribution of water-soluble polymer drug conjugates that differ in molecular weight and the detailed structure of the polymer drug carrier (linear or star polymers with different Mₘ). Blood clearance of star HMW polymer conjugates D₂-P-Dox, D₄-P-Dox-1 and D₄-P-Dox-2 differing in molecular weight were compared with that of the linear hydrazone conjugate P-Dox and free Dox·HCl. As previously described, the size of a polymer carrier significantly influences the rate of polymer elimination from blood circulation.

Blood clearance of the star conjugates D₂-P-Dox, D₄-P-Dox-1 and D₄-P-Dox-2 was much slower than that of linear conjugate P-Dox (Fig. 3), which could be more easily removed from circulation by glomerular filtration. These results are in agreement with the higher cumulative amount of the conjugate P-Dox in urine (collected within 72 h after drug administration) than that of all star conjugates (Fig. 3). Free Dox·HCl was rapidly cleared from the blood, at a rate that was significantly faster than all the polymer conjugates. Moreover, the star polymer conjugates D₂-P-Dox-1 and D₂-P-Dox-2, with higher molecular weights (601,000 and 1,079,000 g/mol), circulated in the blood stream for a longer period than the star polymer conjugate D₂-P-Dox, which has a lower molecular weight (202,000 g/mol), thus demonstrating the effect of molecular weight and low polydispersity of the conjugates on the blood clearance. Uniform star conjugates with very high molecular weights contain lower portions of low-molecular-weight polymer chains, which, if present in high polydispersity conjugates such as the branched and grafted conjugates studied earlier, could be easily removed from the organism by glomerular filtration. This would influence the results of biodistribution studies and, consequently, the efficiency of tumor accumulation and anti-tumor activity.

In agreement with the results of blood clearance and urine excretion measurements, the longer circulating conjugates D₂-P-Dox, D₂-P-Dox-1 and D₄-P-Dox-2 accumulated more efficiently in the tumor tissue than the smaller and more easily excretable linear conjugate P-Dox and the free Dox·HCl. In the case of all HMW conjugates, the Dox concentrations culminated between 12 and 48 h after i.v. injection of the conjugates, reaching almost 50 μg Dox per g (~15% of Dox dose/g) of the tumor for the star conjugates D₂-P-Dox and D₄-P-Dox-1.

Interestingly, the star polymers D₂-P-Dox and D₄-P-Dox-1, with molecular weights from 200,000 g/mol to 601,000 g/mol, displayed more efficient accumulation in the tumor than the star polymer D₂-P-Dox-2, which has a molecular weight exceeding 1,000,000 g/mol. These results show that there is an optimal molecular weight for HMW polymer carriers to be accumulated in the solid tumor by the EPR effect. In the case of star conjugates based on HPMA copolymers, it seems that this optimum value is in the range between 200,000 and 600,000 g/mol. A higher molecular weight is accompanied with a higher hydrodynamic radius and results in decreased extravasation and lower tumor accumulation.

Moreover, after 96 h, the Dox content in the tumor was more than ten times higher for the star conjugates D₂-P-Dox and D₄-P-Dox-1 than that found for the linear conjugate. Of course, tumor accumu-

![Fig. 3. Blood clearance, tumor and muscle accumulation and cumulative urine elimination of free Dox·HCl, linear conjugate P-Dox and star conjugates D₂-P-Dox, D₂-P-Dox-1 and D₄-P-Dox-2 in mice bearing EL4 T-cell lymphoma, i.v. injection; Blood, tumor and muscle: (C — ) Dox·HCl, (■ — ) linear conjugate P-Dox, (▲ — ) star conjugate D₂-P-Dox; (○ — ) star conjugate D₂-P-Dox-1; (● • • ) star conjugate D₄-P-Dox-2.](image)
comparison with the linear conjugate P-Dox and almost one hundred times higher in comparison with the free drug. In contrast, accumulation of the star and linear polymer conjugates in the muscle were significantly lower than that found for tumor tissue. In all cases, the drug concentration decreased rapidly to less than 2 μg of Dox/g of muscle after 48 h and less than 1 μg of Dox/g of muscle after 72 h. No significant accumulation of polymer conjugates was observed in muscle.

The tumor-to-blood ratio for all polymer conjugates increased with time, demonstrating that the conjugates passively accumulate within a tumor mass due to the EPR effect (Fig. 4). The highest tumor-to-blood ratio we have found for the star polymers D2-P-Dox and D4-P-Dox-1 was at a maximum of 35 at 96 h after the administration of the polymer. In the case of free Dox·HCl, we have observed a tumor-to-blood ratio close to 1 within the whole period of time, which shows the random distribution of free Dox in the body and the significance of the absence of specific tumor accumulation. The highest tumor-to-muscle ratios obtained for star polymers D2-P-Dox and D4-P-Dox-1 were nearly one hundred times higher than the ratio obtained for the free drug, which clearly demonstrates the efficacy of passive targeting using these HMW star polymer drug carriers and their potential as efficient carriers for solid tumor drug delivery.

3.6. Anti-tumor activities of polymer conjugates in the EL4 lymphoma model

3.6.1. The effect of molecular weight

The anti-tumor activities of four star copolymers with molecular weights ranging from 201,000 g/mol to 1,079,000 g/mol were compared using a well-defined model of experimental malignant tumor, mouse EL4 T cell lymphoma, inoculated in conventional mice with an intact/unimpaired immune system. It was observed that 50% tumor, mouse EL4 T cell lymphoma, inoculated in conventional mice with a tumor-to-blood ratio we have found for the star polymers Dox-2.

A slightly higher percentage of long-term survivors (LTS) (i.e., mice surviving more than 60 days without showing signs of tumor progression) was achieved with treatment with conjugates D2-P-Dox-1 (65%) and D2-P-Dox (65%), and approximately 30% survived with conjugates D4-SS-P-Dox and D4-P-Dox-2 if the dose of the injected polymer–drug conjugate was 10 mg Dox eq./kg (Fig. 6). Fig. 6 also clearly documents the involvement of the EPR effect [14], with the efficiency of treatment with HMW conjugates superior to that of the linear conjugate. Treatment with the linear conjugate P-Dox (Mn = 27,000 g/mol) given at the dose of 10 mg Dox eq./kg produced only 10% LTS. Treatment with free Dox·HCl resulted in very short prolongation of survival of treated mice without LTS animal observation. A lower therapeutic effect was achieved with the conjugate D4-P-Dox-2 with Mn = 1,079,000 g/mol and is in good agreement with the observed lower tumor accumulation (see Fig. 3). This was most likely caused by the limited extravasation of the conjugate with an extremely high Mw. Based on such results, we conclude that a Mn of HPMA copolymers of 200,000 to 600,000 g/mol is optimal for polymer carriers designed for the efficient passive targeting to solid tumors. However, molecular weight is only one of the factors that may influence the rate of vascular and tissue penetration of polymeric drugs.

3.6.2. The effect of intracellular cleavability

The cleavability of the disulfide linkers and conjugate degradation do not seem to substantially influence the anti-tumor effect of the star polymer conjugates. The final anti-tumor efficacy, depicted as a percentage of long-term survivors, was comparable when a lower dose of 5 mg Dox eq./kg was used and slightly better with a non-cleavable form of the polymeric drug when a higher dose (i.e., 10 mg Dox eq./kg) was used (Figs. 5 and 6).

Similar experiments performed with the biodegradable star conjugate D2-GFLG-P-Dox containing enzymatically cleavable GFLG sequences (Fig. S13) showed an impressive treatment efficacy of up to 100% long-term survivors in a group of mice treated with a dose of 10 mg Dox eq./kg, and an efficacy of 60% LTS was observed in a group treated with a dose of 5 mg Dox eq./kg.

3.6.3. Therapy-dependent anti-tumor resistance

All of the doses employed so far (25 mg Dox eq./kg, 15 mg Dox eq./kg, 10 mg Dox eq./kg, 5 mg Dox eq./kg) induce therapy-
dependent anti-tumor resistance (TD-ATR) after re-inoculation of cured mice (LTS) with a lethal dose of cancer cells used for the establishment of primary solid tumor. Such re-transplanted mice were kept under treatment-free regimes, which means that they were not receiving any therapeutic drugs and that their survival reflects the involvement of the immune defense mechanisms that were activated during primary treatment with the tested drugs. Fig. 7 shows the percentage of surviving re-transplanted tumor-free mice that were cured in the primary treatment with conjugates containing either 5 mg Dox eq./kg (Fig. 7A) or 10 mg Dox eq./kg (Fig. 7B). The number of tumor-resistant mice surviving re-transplantation was 20% for a group of mice cured with 10 mg Dox eq./kg of conjugate D2-P-Dox in a treatment foregoing re-transplantation and 100% for mice cured in the previous treatment with 5 mg Dox eq./kg of conjugates D2-P-Dox and D2-SS-P-Dox. These results suggest the existence of a long-term treatment-induced immunity against experimental tumors. These results confirm the general rule we have already defined in our previous publications [29]. We have regularly observed that fast/better responses to the primary treatment induce worse anti-tumor resistance. This is compatible with the idea of “immunogenic cancer cell death” formulated in recent years [30–33], which states that when killed or dying cancer cells are exposed to particular drugs, the attention of the dendritic cells (DC) of the immune system is triggered by translocation of intracellular chaperons such as calreticulin (CRT). Simultaneously, molecules such as alarmin (HMGB 1 protein) are released to trigger the maturation of DC and consequently effective anti-tumor immune response. If cancer cells (i.e., antigens) are rapidly removed in the immune system, the host does not have enough time to be activated and is thus unable to beat the second cancer cell attack modeled by re-transplantation of the mice cured from the primary cancer attack. In the future, immunostimulating macromolecular therapeutics may be used in clinical practice, and the decision by the oncologist of how fast the tumor mass needs to be removed to profit from an potential anti-tumor response will be an enormous responsibility and require significant experience.

In the present study, we have shown that high molecular-weight macromolecular drugs (nanomedicines) that simultaneously target tumor cells and stimulate components of the immune system are able to effectively eradicate large tumors in almost all treated animals and represent a complementary approach based on the association between immunotherapy and chemotherapy. Immune-based approaches that recruit the host anti-tumor immune response to the therapeutic effort are particularly attractive strategies for improving the clinical outcomes of malignant diseases.

4. Conclusion

In this study, we describe the synthesis, characterization, drug release and detailed biological evaluation of new biodegradable HMW polymer–doxorubicin conjugates based on star HPMA polymer carriers designed for solid tumor treatment. The star conjugates were prepared by grafting the semitelechelic HPMA copolymer onto 2nd or 4th generation PAMAM dendrimers. The conjugates differ in the structure of the spacers between the polymer grafts and dendrimer core, which enables the controlled degradation of the carrier after incubation with EL4 lymphoma cells. Controlled synthesis results in conjugates with a well-defined structure and enables the preparation of conjugates with a broad range of molecular weights from 200,000 g/mol to more than 1,000,000 g/mol and a low polydispersity. The high molecular weight of the conjugates predetermines their accumulation and delivery into solid tumors due to the EPR effect. Controlled Dox release was achieved with a hydrazone bond-containing spacer, predestinating the conjugate to pH-controlled intratumoral or intracellular hydrolysis and drug release. The degradability of the carrier was possible with reductively or enzymatically degradable spacers with high potential for intracellular degradation. The star conjugates showed significantly prolonged blood clearance and enhanced tumor accumulation compared with the linear conjugate or free Dox when injected into mice bearing EL4 lymphoma. Treatment of mice bearing tumor models of EL4 T-cell lymphoma with a single dose of the star conjugate administrated in a therapeutic regime resulted in an excellent therapeutic efficacy that was superior to that obtained by treatment with previously developed and tested polymer conjugates. Also, we have shown that a HMW polymer drugs (nanomedicines) that simultaneously target tumor cells and stimulate components of the immune system are able to effectively eradicate large tumors in almost all treated animals and represent a complementary approach based on association between immunotherapy and chemotherapy. We can also conclude that a HMW polymer copolymers of 200,000 to 600,000 g/mol seems to be optimal for

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Fig. 6. The anti-tumor efficacy of star polymer conjugates-treated EL4 T-cell lymphoma-bearing C57BL/6 mice were transplanted s.c. with 1×10⁵ EL4 T-cells and treated with (○−−−) Dox.HCl (■−−−) P-Dox, (●−−−) D2-P-Dox, (▲−−−) D4-P-Dox-1, (Δ−−−) D4-P-Dox-2 or (−−−) D2-SS-P-Dox using i.v. drug administration on day 8. Control mice (−) were left untreated. Mice from primary treatment with: (●−−−) D2-P-Dox, (▲−−−) D4-P-Dox-1, (Δ−−−) D4-P-Dox-2 or (−−−) D2-SS-P-Dox. Control mice (−) were left untreated. Primary treatment with dose 1×5 mg Dox eq./kg (A) and 1×10 mg Dox eq./kg (B).

Fig. 7. The survival mice after re-transplantation of lethal dose of tumor cells into star conjugate-treated EL4 T-cell lymphoma-bearing C57BL/6 mice. The surviving mice after primary treatment (70 days) were re-transplanted with 1×10⁵ EL4 T-cells and left untreated. Mice from primary treatment with: (●−−−) D2-P-Dox, (▲−−−) D4-P-Dox-1, (Δ−−−) D4-P-Dox-2 or (−−−) D2-SS-P-Dox. Control mice (−) were left untreated. Primary treatment with dose 1×5 mg Dox eq./kg (A) and 1×10 mg Dox eq./kg (B).
polymer carriers designed for the efficient passive targeting to solid tumors. The precisely defined structure, controlled molecular weight, drug release and degradation profiles of the polymer-grafted dendrimer conjugates suggest that they may have the potential to be efficient anticancer nanomedicines.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jconrel.2011.06.015.

References

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