In vivo tumor targeting of tumor necrosis factor-α-loaded stealth nanoparticles: Effect of MePEG molecular weight and particle size

Chao Fang\textsuperscript{a,b}, Bin Shi\textsuperscript{b}, Yuan-Ying Pei\textsuperscript{a,*}, Ming-Huang Hong\textsuperscript{b}, Jiang Wu\textsuperscript{b}, Hong-Zhuang Chen\textsuperscript{a}

\textsuperscript{a} Department of Pharmacology, College of Basic Medical Sciences, Shanghai Jiao Tong University, Shanghai 200025, PR China
\textsuperscript{b} Department of Pharmaceutics, School of Pharmacy, Fudan University, Shanghai 200032, PR China

\textbf{Abstract}

The aim of this study is to reveal the influence of methoxypolyethylene glycol (MePEG) molecular weight and particle size of stealth nanoparticles on their in vivo tumor targeting properties. Three sizes (80, 170 and 240 nm) of poly methoxypolyethylene glycol cyanacrylate-co-\textit{n}-hexadecyl cyanacrylate (PEG-PHDCA) nanoparticles loading recombinant human tumor necrosis factor-α (rHuTNF-α) were prepared at different MePEG molecular weights (MW = 2000, 5000 and 10,000) using double emulsion method. The opsonization in mouse serum was evaluated by Coomassie brilliant blue staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Phagocytosis was evaluated by incubating \textsuperscript{125}I-rHuTNF-α-loaded nanoparticles with mouse macrophages (RAW264.7). The pharmacokinetics, biodistribution and tumor targeting studies were performed in S-180 tumor-bearing mice. Higher MePEG molecular weight provided thicker fixed aqueous layer thickness (FALT) and smaller particle size offered higher surface MePEG density. The serum protein adsorption and phagocytic uptake were markedly decreased for the nanoparticles with higher MePEG molecular weight or smaller size. The particles (80 nm) made of PEG\textsubscript{5000}-PHDCA, possessing a thicker FALT (5.16 nm) and a shortest distance (0.87 nm) between two neighboring MePEG chains, showed the strongest capacity of decreasing protein adsorption and phagocytic uptake. These particles extended the half-life of rHuTNF-α in S-180 tumor-bearing mice by 24-fold (from 28.2 min to 11.33 h), elevated the rHuTNF-α peak concentration in S-180 tumors by 2.85-fold and increased the area under the intratumoral rHuTNF-α concentration curve by 7.44-fold. The results of the present study showed PEG-PHDCA nanoparticles with higher MePEG molecular weight and smaller particle size could achieve higher in vivo tumor targeting efficiency.

© 2005 Elsevier B.V. All rights reserved.
1. Introduction

The rapid removal of intravenously administered colloidal drug carrier systems by the mononuclear phagocytic system (MPS) has been identified as the major obstacle to the efficient targeting of colloidal carriers to target sites such as solid tumors and inflammatory regions (Dunn et al., 1997; Mosquera et al., 2000). Recently, a great deal of work has been devoted to developing 'stealth' particles, which are 'invisible' to macrophages. These stealth nanoparticles have been shown to be characterized by a prolonged half-life in the bloodstream, which increases their selective extravasation and accumulation in the pathological sites, like tumors with a leaky vasculature due to the enhanced permeability and retention effect (Brigger et al., 2002; Moghimi et al., 2001). In previous work of our lab, higher intratumoral drug accumulation and antitumor potency were achieved for the recombinant human tumor necrosis factor-α (rHuTNF-α)-loaded stealth PEG2000-PHDA nanoparticles with size of around 150 nm (Li et al., 2001a).

It is known that particle size and surface characteristics are the key for the biological fate of stealth nanoparticles (Brigger et al., 2002). Generally, a high curvature and/or a hydrophilic surface are needed, in order to reduce opsonization reactions and subsequent clearance by macrophages. Recently, the relationship between these physicochemical characteristics and their in vivo long-circulating properties and biodistribution behaviors has been discussed (Jegozaaki et al., 2003; Gref et al., 1994; Mosquera et al., 2001b), while little information about the correlation between these physicochemical parameters and their in vivo tumor targeting property has been published. Following our previous work, stealth rHuTNF-α-loaded PEG-PHDA nanoparticles with various MePEG molecular weight and particle size were fabricated. The aim of the present work is to reveal the influence of these parameters on their in vitro serum protein adsorption, phagocytic uptake, in vivo long-circulating properties and especially, their in vivo tumor targeting efficiencies. The results of this study will provide fundamental information enabling us to design a useful delivery system with long circulating half-life and selective localization in the vascular space against tumors, so further enhancing the therapeutic activity and safety of chemotherapeutic agents.

2. Materials and methods

2.1. Materials

Recombinant human tumor necrosis factor-α (rHuTNF-α, MW = 17,000) was kindly donated by Henan Yakang Pharmaceutical Co. Ltd., China. Monomethoxy polyethylene glycol (MePEG, MW = 2000, 5000), human serum albumin (HSA) and Na125I were obtained from Sigma Chemical Co. (St. Louis, MO, USA). MePEG (MW = 10,000) was donated by the Department of Polymer Material and Science of Fudan University of China. Cyanoacetic acid and poly (vinylalcohol) (PVA) (MW = 16,000, 98% hydrolyzed) were purchased from Acros Organics Co. All other reagents and solvents were of analytical grade.

Poly (methoxy(polyethylene)glycol cyanacrylate-co-n-hexadecyl cyanacrylate) (PEG-PHDA) was synthesized as previously described (Li et al., 2001b) at a MePEG to n-hexadecyl ratio of 1:5. The poly (hexadecyl cyanacrylate) (PHDA) was synthesized and used as a control.

2.2. Animals

Male Kunming strain mice (20 ± 2 g) were supplied by the Shanghai Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China). The mice were acclimatized at a temperature of 25 ± 2°C and a relative humidity of 70 ± 5% under natural light/dark conditions for one week before dosing.

2.3. Preparation of rHuTNF-α-loaded nanoparticles

rHuTNF-α was labeled with 125I using the IODO-GEN procedure. Briefly, 100 µg of protein in 60 µl 0.1 M potassium phosphate buffer (pH 7.4) was layered over a freshly prepared film of IODO-GEN (100 µg) and incubated for 10 min at 4°C in presence of 2 mCi of carrier-free Na125I. The reaction mixture was brought up to 0.5 ml volume with PBS, and the unreacted iodine was removed by gel filtration chromatography on a Sephadex G-25 PD10 column equilibrated with PBS. The specific radioactivity of the product (0.81 MBq/µg) was assessed in an autogamma (Packard Instruments, CT, USA).

Nanoparticles were prepared using the double emulsion (w1/o/w2) procedure as described elsewhere (Yang et al., 2005). Briefly, a w1/o emulsion was prepared by sonicating 0.1 ml of IODO-GEN-labeled rHuTNF-α solution (w1) containing HSA (2%, w/v) with PEG-PHDA or PHDA solution in methylene chloride in an ice bath. Thereafter, the first emulsion was poured into PVA solution and sonicated in pulsed way in an ice bath for a certain time. The double emulsion (w1/o/w2) obtained was diluted in 150 ml PVA solution (0.1%, w/v) and the organic solvent was evaporated at room temperature under reduced pressure. Finally, the nanoparticles were collected and extensively washed by two cycles of ultracentrifugation at 21,000 × g for 40 min and washed three times with water.

2.4. Characterization of rHuTNF-α-loaded nanoparticles

The amount of non-entrapped protein in aqueous phase was determined with gamma emission in the supernatant obtained after ultracentrifugation and washing of nanoparticles. The amount of rHuTNF-α present in the aqueous phase. The drug loading of the nanoparticles, given as a percentage, indicates the amount of protein encapsulated per 100 mg of nanoparticles.

The size and zeta potential of the nanoparticles were determined using dynamic light scattering and electrophoretic light scattering technique, respectively, in Nicomp 380ZLS (NICOMP Particle Sizing Systems, Santa Barbara California, USA).

For PEG-PHDA nanoparticles, the MePEG content were assayed as previously described (Zambaix et al., 2001; Brigger et al., 2000). The surface area occupied by each MePEG...
molecule on nanoparticle surface, $S_{\text{PEG}}$ (nm² per molecule), was determined as described by Peracchia et al. (1997). The average distance D between two neighboring MePEG chains was calculated as $D = \sqrt{\frac{S_{\text{PEG}}}{\pi}}$. The decrease of the $S_{\text{PEG}}$ and D represents the increase of the surface MePEG density.

The fixed aqueous layer thickness (FALT) of nanoparticles was determined by zeta potential measurements of nanoparticles in NaCl solutions of different ion concentrations. The calculation of FALT was based on the linear correlation between $\ln$ (zeta potential) and $\sqrt{C}$ (Debye-Huckel-Parameter): $\ln(\alpha) = \ln\epsilon - \frac{1}{\lambda D}$ (Sadzuka and Hirota, 1997).

2.5. SDS-PAGE analysis of protein adsorption on nanoparticles

In order to avoid the interference of rHuTNF-α and HSA, nanoparticles without rHuTNF-α and HSA were fabricated for the SDS-PAGE analysis of protein adsorption. No significant differences in particle size and zeta potential were observed between the blank nanoparticles and the rHuTNF-α-loaded ones (data not shown). The adsorption of serum proteins to the nanoparticles was studied under conditions that mimic physiological environments (Gaur et al., 2000). Nanoparticles containing constant surface areas (5 m²) were opsonised by incubation in fresh mouse serum (50%, v/v) for 30 min at 37 °C, then centrifuged (21,000 × g, 60 min) and washed with water to remove proteins not firmly adsorbed onto particle surface.

The proteins bound to nanoparticles were desorbed by PBS containing 1% SDS (Ameller et al., 2003). SDS-PAGE was performed by using the Mini Protein II electrophoretic apparatus (Bio-Rad) on an acrylamide gel consisting of 15% separating gel and 5% stacking gel under reducing conditions. The bound proteins on nanoparticles were quantified by BCA assay.

2.6. Culture of macrophages and phagocytosis assay

Mouse macrophage cell line RAW264.7 was employed to study the phagocytic uptake of nanoparticles. Cells were routinely cultured in RPMI1640 medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO₂. Cell suspension (1 ml, 4 × 10⁵ cells) and 0.5 ml tested $^{125}$I-rHuTNF-α-loaded nanoparticles (0.2 mg ml⁻¹) were added to a 5-ml centrifugation tube, and the tubes were then incubated at 37 °C for 0.5, 1, and 2 h, respectively, and shaken at 10 min intervals. After the suspending of the phagocytosis by immersion in an ice bath, the cells were separated from nanoparticles by centrifuging (1500 rpm, 5 min), discarding the supernatant, washing with 3 ml saline for three times and the radioactivity associated with the harvested cells was determined. A parallel protocol was done to evaluate the phagocytosis of probable free drug in aqueous suspensions of nanoparticles.

2.7. Pharmacokinetics, biodistribution and tumor targeting of nanoparticles in S-180 tumor-bearing mice

In order to study the pharmacokinetics and tissue distribution, nine groups of male Kunming strain mice (45 in each group) with 5-180 tumor nodules of 9-10 mm in diameter were used. The group 1 was treated with free rHuTNF-α, groups 2, 3, 4 treated with various PHDCA nanoparticles and groups 5, 6, 7, 8, 9 with various PEG-PHDC nanoparticles. For administration, free rHuTNF-α or nanoparticles were suspended in NaCl solution (0.9%, w/w)/DLE in order to obtain the required concentration. Each animal was dosed intravenously with 0.5 μg protein with a trace of $^{125}$I(130 Gy) at 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 h, respectively after administration. Blood from five mice of each group was collected from the tail vein and the radioactivity levels were measured. The pharmacokinetic parameters were calculated using the Practical Pharmacokinetic Program Version 97. The mice were sacrificed, and tissues were collected, weighed and the radioactivities were measured. The experimental design of this study was approved by the appropriate ethical committee on animal study in Fudan University.

2.8. Statistical analysis

Statistical evaluation was analyzed by using the analysis of variance (ANOVA) and Student’s t-tests.

3. Results

3.1. Characterization of nanoparticles

The basic physicochemical characteristics of the nanoparticles are summarized in Table 1. The mean diameters of PEG-PHDC nanoparticles were around 80, 170 and 240 nm, respectively. For PEG-PHDC nanoparticles, the values of zeta potentials were obviously influenced by MePEG molecular weight. In the same size range, marked decrease in the surface charge for PEG-PHDC nanoparticles occurred compared with PHDCA nanoparticles. In addition, the surface charge of PEG-PHDC nanoparticles dramatically decreased with the increase of MePEG molecular weight. The zeta potentials were also influenced by particle size. The zeta potentials of PEG1500-PHDC nanoparticles obviously decreased from −13.7 mV to nearly zero (−3.2 mV) when their particle size varied from 243 to 80 nm.

The FALTs around PEG-PHDC nanoparticles were much larger than those around PHDCA nanoparticles. For PEG-PHDC nanoparticles, the FALT increased with the increase of MePEG molecular weight but with the decrease of particle size. The values of FALT and Do (1/$\sqrt{S_{\text{PEG}}}$) decreased with the reduction of MePEG molecular weight or particle size.

The encapsulation efficiencies achieved for rHuTNF-α were obviously affected by the presence of MePEG in the PHDCA chain. In the same size range, PEG-PHDC nanoparticles showed lower entrapment efficiency compared with PHDCA ones (p<0.05). No regular changes with respect to drug loadings were found. And the differences between the drug loadings of PEG-PHDC and PHDCA nanoparticles were not remarkable (p>0.05).

3.2. Serum protein adsorption on nanoparticles

The reduction of opsonization is considered as a prerequisite for prolonged blood circulation time (Ameller et al., 2003). The quantified results for the total amount of serum proteins adsorbed as measured by BCA assay are given in Table 2. Compared to PHDCA ones, the amount of proteins adsorbed on PEG-PHDC nanoparticles were dramatically reduced.
Table 1 – Physicochemical properties of PEG-PHDCA and PHDCA nanoparticles containing rHuTNF-α/H9251 (n = 3)

<table>
<thead>
<tr>
<th>Size range</th>
<th>Nanoparticles</th>
<th>Particle size (nm)</th>
<th>Zeta potential (mV)</th>
<th>FALT (nm)</th>
<th>Entrapment efficiency (%)</th>
<th>Drug loading (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small (&lt;100 nm)</td>
<td>PEG 5000-PHDCA</td>
<td>80 ± 1 − 3.2 ± 0.1</td>
<td>5.16 ± 0.08</td>
<td>0.76 ± 0.05</td>
<td>37.6 ± 3.2</td>
<td>0.83 ± 0.04</td>
</tr>
<tr>
<td>Middle (100–200 nm)</td>
<td>PEG 2000-PHDCA</td>
<td>172 ± 2 − 14.8 ± 0.2</td>
<td>2.13 ± 0.02</td>
<td>1.17 ± 0.03</td>
<td>49.8 ± 2.7</td>
<td>0.88 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>PEG 5000-PHDCA</td>
<td>171 ± 2 − 10.6 ± 0.4</td>
<td>4.33 ± 0.04</td>
<td>1.30 ± 0.03</td>
<td>54.3 ± 3.3</td>
<td>0.87 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>PEG 10000-PHDCA</td>
<td>169 ± 1 − 3.8 ± 0.5</td>
<td>6.34 ± 0.05</td>
<td>1.25 ± 0.02</td>
<td>57.0 ± 1.8</td>
<td>0.92 ± 0.03</td>
</tr>
<tr>
<td>Large (&gt;200 nm)</td>
<td>PEG 5000-PHDCA</td>
<td>243 ± 4 − 13.7 ± 0.2</td>
<td>3.68 ± 0.05</td>
<td>2.13 ± 0.05</td>
<td>46.7 ± 2.2</td>
<td>0.86 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>PHDCA</td>
<td>242 ± 4 − 22.4 ± 0.2</td>
<td>0.28 ± 0.01</td>
<td>–</td>
<td>60.1 ± 2.9</td>
<td>0.88 ± 0.03</td>
</tr>
</tbody>
</table>

Table 2 – The adsorption of serum proteins to PEG-PHDCA and PHDCA nanoparticles incubated in murine serum and quantified by BCA assay

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Protein adsorbed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG5000-PHDCA (243 nm)</td>
<td>34</td>
</tr>
<tr>
<td>PEG5000-PHDCA (171 nm)</td>
<td>23</td>
</tr>
<tr>
<td>PEG5000-PHDCA (80 nm)</td>
<td>6</td>
</tr>
<tr>
<td>PEG2000-PHDCA (172 nm)</td>
<td>29</td>
</tr>
<tr>
<td>PEG5000-PHDCA (169 nm)</td>
<td>9</td>
</tr>
<tr>
<td>PHDCA (242 nm)</td>
<td>58</td>
</tr>
<tr>
<td>PHDCA (173 nm)</td>
<td>56</td>
</tr>
<tr>
<td>PHDCA (85 nm)</td>
<td>57</td>
</tr>
</tbody>
</table>

In addition, for PEG-PHDCA nanoparticles, the amount of serum proteins adsorbed strongly depended on the MePEG molecular weight and the particle size. The amount of adsorbed proteins significantly varied in following series: PEG10000-PHDCA (169 nm) < PEG5000-PHDCA (171 nm) < PEG2000-PHDCA (172 nm) (p < 0.05); PEG5000-PHDCA (80 nm) < PEG5000-PHDCA (171 nm) < PEG5000-PHDCA (243 nm) (p < 0.05). While, no significant difference in protein adsorption could be observed between the PHDCA nanoparticles of all sizes.

3.3. Uptake of nanoparticles by murine macrophages

Fig. 1 showed the phagocytosis uptake of nanoparticles with regard to MePEG molecular weight and particle size. Before the study of cellular uptake, the nanoparticle cytotoxicity was assessed by MTT assay. The MTT test indicated all types of nanoparticles at the concentration of 0.2 mg ml⁻¹ did not cause cytotoxicity to the macrophages within 2 h. This result ensured that, under this condition, cellular uptake of nanoparticles was not associated with their cytotoxicity.

Obviously time-dependent increased uptake was observed for all nanoparticles from 0.5 to 2 h. For PEG5000-PHDCA nanoparticles, the cellular uptake was significantly influenced by the particle size. Although the differences in phagocytosis at 0.5 h between PEG5000-PHDCA particles of all sizes (80, 171, and 243 nm) were insignificant, remarkable differences were observed after 1 and 2 h incubation, respectively. The uptake percentage varied as follows: PEG5000-PHDCA (80 nm) < PEG5000-PHDCA (171 nm) < PEG5000-PHDCA (243 nm) (p < 0.05). MePEG coating on particle surface resulted in a dramatic reduction in uptake for nanoparticles of all sizes by at least 50% at each time point. In addition, the cellular uptake of PEG-PHDCA nanoparticles in the same size range displayed MePEG molecular weight-dependent tendency. The uptake percentage varied as follows: PEG10000-PHDCA (169 nm) < PEG5000-PHDCA (171 nm) < PEG2000-PHDCA (172 nm) (p < 0.05) for every incubation period.

3.4. Pharmacokinetics of nanoparticles in S-180 tumor-bearing mice

The blood clearance curves for rHuTNF-α loaded in nanoparticles after intravenous administration are shown in Fig. 2. The PEG-PHDCA nanoparticles showed initial high blood circulating levels compared with free rHuTNF-α and PHDCA nanoparticles. Both free rHuTNF-α and PHDCA nanoparticles were quickly removed from the circulating system, and
Fig. 1 – Uptake of PEG-PHDCA and PHDCA nanoparticles by murine macrophages after 0.5, 1 and 2 h incubation at 37 °C (n = 3).

Fig. 2 – Blood clearance curves of free rHuTNF-α/H9251 and rHuTNF-α/H9251 loaded in PEG-PHDCA and PHDCA nanoparticles (n = 5).

their radioactivities in blood at 2 h after administration were very low. On the contrary, PEG-PHDC nanoparticles exhibited a markedly delayed blood clearance. It could be seen that the blood-associated radioactivity remained much higher after 24 h compared with those of free rHuTNF-α and PHDCA nanoparticles. The radioactivity-time curves for PEG-PHDC nanoparticles were fitted by two-compartment model and the pharmacokinetic parameters are shown in Table 3. The plasma elimination half-lives of PEG5000-PHDC (243 nm), PEG5000-PHDC (171 nm), PEG5000-PHDC (80 nm), PEG5000-PHDC (169 nm) nanoparticles were 4.67, 8.54, 11.33, 4.95 and 9.08 h, respectively. The radioactivity-time curves of free rHuTNF-α and PHDCA nanoparticles were fitted with one-compartment model and the pharmacokinetic parameters are displayed in Table 4. The plasma elimination half-lives of PHDC (242 nm), PHDC (173 nm), PHDCA (85 nm) nanoparticles, and free rHuTNF-α were 0.22, 0.28, 0.31 and 0.47 h, respectively. The influence of MePEG molecular weight and particle size on the elimination half-lives of rHuTNF-α loaded in PEG-PHDC nanoparticles was consistent with that on the serum protein absorption and macrophage uptake. The elimination half-lives varied as follows: PEG10000-PHDC (169 nm) > PEG5000-PHDC (171 nm) > PEG5000-PHDC (169 nm) > PEG5000-PHDC (172 nm) (p < 0.05), PEG5000-PHDC (80 nm) > PEG5000-PHDC (171 nm) > PEG5000-PHDC (243 nm) (p < 0.05).

3.5. Biodistribution and tumor targeting of nanoparticles in S-180 tumor-bearing mice

The distribution profiles of free rHuTNF-α and nanoparticles loaded with rHuTNF-α in S-180 tumor-bearing mice at 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PEG5000-PHDC (243 nm)</th>
<th>PEG5000-PHDC (271 nm)</th>
<th>PEG5000-PHDC (80 nm)</th>
<th>PEG5000-PHDC (172 nm)</th>
<th>PEG5000-PHDC (169 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1/2a (h)</td>
<td>1.22 ± 0.23</td>
<td>1.95 ± 0.35</td>
<td>1.22 ± 0.23</td>
<td>1.22 ± 0.23</td>
<td>1.37 ± 0.33</td>
</tr>
<tr>
<td>T1/2b (h)</td>
<td>4.67 ± 1.35</td>
<td>8.54 ± 2.78</td>
<td>11.33 ± 1.46</td>
<td>4.95 ± 1.85</td>
<td>9.08 ± 1.45</td>
</tr>
<tr>
<td>MRTc (h)</td>
<td>6.23 ± 1.25</td>
<td>5.52 ± 1.78</td>
<td>6.77 ± 1.42</td>
<td>4.85 ± 1.46</td>
<td>5.87 ± 1.86</td>
</tr>
<tr>
<td>AUC0-24h (h cpm/l)</td>
<td>1410.85 ± 128.66</td>
<td>2220.86 ± 187.65</td>
<td>6302.48 ± 527.34</td>
<td>1659.48 ± 87.74</td>
<td>3154.26 ± 222.87</td>
</tr>
<tr>
<td>Cle (l/h)</td>
<td>6.23 ± 0.54</td>
<td>4.21 ± 0.36</td>
<td>1.39 ± 0.47</td>
<td>5.29 ± 1.04</td>
<td>2.97 ± 0.48</td>
</tr>
</tbody>
</table>

a Distribution half-life.
b Elimination half-life.
c Mean residence time.
d Area under the plasma concentration curve.
e Clearance of rHuTNF-α from plasma.
Table 4 – Pharmacokinetic parameters of free rHuTNF-α and rHuTNF-α loaded in PHDCA nanoparticles in S-180 tumor-bearing mice (n = 5)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PHDCA (242 nm)</th>
<th>PHDCA (173 nm)</th>
<th>PHDCA (85 nm)</th>
<th>rHuTNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₁/₂a (h)</td>
<td>0.22 ± 0.06</td>
<td>0.28 ± 0.06</td>
<td>0.31 ± 0.04</td>
<td>0.47 ± 0.08</td>
</tr>
<tr>
<td>MRTb (h)</td>
<td>0.43 ± 0.04</td>
<td>0.53 ± 0.01</td>
<td>0.55 ± 0.03</td>
<td>0.63 ± 0.04</td>
</tr>
<tr>
<td>AUC₀⁻²₄ h (h cpm/l)</td>
<td>275.26 ± 26.28</td>
<td>316.25 ± 42.14</td>
<td>334.15 ± 34.32</td>
<td>388.42 ± 26.32</td>
</tr>
<tr>
<td>Cld (l/h)</td>
<td>36.01 ± 2.85</td>
<td>31.58 ± 2.67</td>
<td>27.48 ± 2.26</td>
<td>23.42 ± 2.44</td>
</tr>
</tbody>
</table>

a Plasma elimination half-life.

b Mean residence time.

c Area under the plasma concentration curve.

d Clearance of rHuTNF-α from plasma.

and 6 h after intravenous administration are shown in Fig. 3. At 1 h after administration, free rHuTNF-α and rHuTNF-α loaded in PHDCA nanoparticles were mainly distributed to the liver, spleen and lung, and then were the heart and kidney. The rHuTNF-α levels in blood and tumor tissues were very low. During this period, remarkably decreased rHuTNF-α levels in liver were observed compared with free rHuTNF-α and PHDCA nanoparticles (p < 0.05). The order of rHuTNF-α levels in liver for PEG-PHDCA nanoparticles was PEG10000-PHDCA (169 nm) < PEG5000-PHDCA (171 nm) < PEG2000-PHDCA (172 nm) (p < 0.05). PEG5000-PHDCA (80 nm) < PEG5000-PHDCA (171 nm) < PEG2000-PHDCA (243 nm) (p < 0.05). The differences between the rHuTNF-α levels in other tissues, such as spleen, lung, heart and kidney, were found to be insignificant (p > 0.05) for all rHuTNF-α formulations. The plasma levels of rHuTNF-α loaded in PEG-PHDCA nanoparticles were markedly higher than that of PHDCA nanoparticles and free rHuTNF-α at the same time point (p < 0.01). The rHuTNF-α levels in tumor tissues for PEG-PHDCA nanoparticles were a little higher than those of free rHuTNF-α and PHDCA nanoparticles (p < 0.05). At 6 h after intravenous injection, free rHuTNF-α and rHuTNF-α loaded in PHDCA nanoparticles were gradually eliminated from all tissues including the S-180 tumors. In contrast, the rHuTNF-α levels of PEG-PHDCA nanoparticles in tumors were much higher than those at 1 h (p < 0.05). The plasma levels of PEG-PHDCA nanoparticles at 6 h were still much higher than those of free rHuTNF-α and PHDCA nanoparticles (p < 0.01). And the rHuTNF-α levels in liver for PEG-PHDCA nanoparticles were still lower than those of PHDCA nanoparticles (p < 0.05).

Fig. 4 illustrated the mean observed rHuTNF-α levels in S-180 solid tumors over a 24 h period after the administration of various rHuTNF-α formulations. All the rHuTNF-α level versus time profiles exhibited gradually increased tendency from the beginning till a peak was reached. The dynamic changes of rHuTNF-α levels in tumors could be fitted with one-compartment model and the pharmacokinetic parameters are displayed in Table 5. For the evaluation of tumor targeting potency with free rHuTNF-α as a control, two indices, relative tumor tissue exposures (Rₑ = (AUC.neo)nanoparticles/(AUC.neo)free rHuTNF-α) (Gallo et al., 1989; Gupta and Hung, 1989) and the ratios of rHuTNF-α peak concentrations (Ce) in tumors (Gallo et al., 1989), were calculated and summarized in Table 6. Both AUC₀⁻²₄ h and Ceₘ₉₀ of rHuTNF-α in tumor were much higher for PEG-PHDCA.
This might be related to the increase of the FALT and thus a shift of the hydrodynamic phase of shear to greater distances from the particle surface. The zeta potential of the PEG5000-PHDCA nanoparticles decreased to nearly zero (<3.2 mV) when particle size decreased to 80 nm. This could be related to the increased FALT (5.16 nm) and surface MePEG chain density (0.87 nm). Both of them contributed to a denser MePEG-hydrated cloud "shielding" over the negatively charged surface. MePEG-D and D (\(\sqrt{\frac{S}{D}}\)) are two parameters used to evaluate surface MePEG density. In present study, they decreased with the reduction of MePEG molecular weight and particle size. This phenomenon might come from the difference of MePEG molecular weight and the procedure parameters. It has been demonstrated a grafting density close to 0.76–2.13 nm²/MePEG, which corresponds to a distance of 0.87–1.46 nm between two neighboring MePEG chains. These values are thus compatible for a long circulating carrier. Fig. 2 showed that MePEG-modified nanoparticles remained for an obviously longer time in the blood circulation after intravenous administration to S-180 tumor-bearing mice than the non-pegylated PHDCA nanoparticles.

The FALTs around PEG-PHDC nanoparticles were much thicker than those around PHDCA particles, and they increased with the increase of MePEG molecular weight (Table 3). FALT is formed around the colloidal particles by:

### Table 5 – Pharmacokinetic parameters of rHuTNFα loaded in PEG-PHDC nanoparticles after intravenous administration to S-180 tumor-bearing mice (n = 5)\(^{+}\)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PEG2000-PHDCA</th>
<th>PEG5000-PHDCA</th>
<th>PEG10000-PHDCA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(243 nm)</td>
<td>(173 nm)</td>
<td>(169 nm)</td>
</tr>
<tr>
<td>rHuTNFα-T&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>1.25 ± 0.17</td>
<td>1.38 ± 0.23</td>
<td>1.53 ± 0.25</td>
</tr>
<tr>
<td>rHuTNFα-C&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>2.47 ± 0.14</td>
<td>4.01 ± 0.41</td>
<td>8.81 ± 0.03</td>
</tr>
<tr>
<td>rHuTNFα-C&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>10.82 ± 1.32</td>
<td>14.18 ± 0.78</td>
<td>16.82 ± 1.65</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-24&lt;/sub&gt; (ng/mL)</td>
<td>10.17 ± 0.87</td>
<td>14.69 ± 1.16</td>
<td>24.88 ± 1.78</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>10.17 ± 0.87</td>
<td>14.69 ± 1.16</td>
<td>24.88 ± 1.78</td>
</tr>
</tbody>
</table>

* rHuTNFα-T<sub>1/2</sub> = elimination half-life in tumors.
* rHuTNFα-C<sub>max</sub> = peak concentration in tumors.
* rHuTNFα-C<sub>1/2</sub> = absorption half-life in tumors.
* AUC<sub>0-24</sub> = area under the rHuTNFα concentration curve.
* t<sub>1/2</sub> = maximal residence time.

### Table 6 – The nanoparticle relative tumor tissue exposures (Re) and the ratios of rHuTNFα peak concentrations (Cs) in tumors compared with free rHuTNFα (n = 5)\(^{+}\)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PEG2000-PHDCA</th>
<th>PEG5000-PHDCA</th>
<th>PEG10000-PHDCA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(243 nm)</td>
<td>(173 nm)</td>
<td>(169 nm)</td>
</tr>
<tr>
<td>Re</td>
<td>3.02 ± 0.06</td>
<td>4.53 ± 0.33</td>
<td>7.44 ± 0.24</td>
</tr>
<tr>
<td>Ce</td>
<td>1.82 ± 0.02</td>
<td>2.35 ± 0.15</td>
<td>2.85 ± 0.12</td>
</tr>
</tbody>
</table>

\(^{+}\) rHuTNFα-T<sub>1/2</sub> = absorption half-life in tumors.
interaction between the MePEG-polymer and water molecular layer prevents the attraction of opsonins, because serum proteins cannot bind to the water gathered on the surface of the particles (Sadzuka et al., 2002). It was reported that increased FALT could lead to improvements of the circulation in blood and the antitumor activity of MePEG-modified liposomes containing doxorubicin (Sadzuka et al., 2002). Thus, enlarged FALTs seem attractive in designing a MePEG-modified nanoparticulate delivery system. Usually, two MePEG regimes can be identified, ‘mushrooms’ (isolated grafts) and ‘brushes’ (extended chain conformations determined by the interaction between neighboring chains) (Needham et al., 1997). The FALT (extended chain conformations determined by the interaction can be identified, ‘mushrooms’ (isolated grafts) and ‘brushes’ (extended chain conformations determined by the interaction between neighboring chains) (Needham et al., 1997). The FALT of PEG10000-PHDCA nanoparticles increased with the decrease of particle size. This might be related to the higher MePEG chain density formed on the surface of smaller particles. It is widely expected that the preferential distribution of MePEG layers at surfaces would change from a ‘mushroom’ in the dilute, unhindered state at low surface density to an extended ‘brush’ at high surface density (Mosqueira et al., 2001a) which contributes to a thicker FALT.

Compared with PHDCA ones, the repulsion upon serum proteins was much greater for PEG-modified nanoparticles and the amount of adsorbed serum proteins displayed MePEG molecular weight and particle size dependent tendency. In the same size range, adsorbed proteins decreased as the MePEG molecular weight increased. This might be related to the increased FALT around nanoparticles. Though, in the meanwhile, the values of D increased with the MePEG molecular weight, from 1.08 nm for PEG10000-PHDCA nanoparticles to 1.25 nm for PEG2000-PHDCA nanoparticles, which might lower the protein repulsion function of MePEG chains. But the effect of increased FALT on the repulsion of proteins seemed to be dominant. On the other hand, for nanoparticles made from PEG10000-PHDCA, the amounts of adsorbed proteins were markedly decreased as particle size decreased to 80 nm. This might be related to their simultaneously increased FALT around nanoparticles. The FALT of 5.16 nm for PEG10000-PHDCA (80 nm) was smaller than the 6.34 nm for the PEG10000-PHDCA nanoparticles (169 nm), the surface MePEG density of the former (D=1.25 nm) seemed to be more optimal to reject opsonins than that of the latter (D=1.19 nm). This endowed the PEG10000-PHDCA nanoparticles (80 nm) with the strongest protein repulsion capacity, and only 6% of total proteins were adsorbed after incubation of 30 min at 37°C (Table 2).

The interaction of nanoparticles with murine macrophages (RAW264.7) was investigated with the aim of evaluating in vitro capability of nanoparticles to reduce phagocytosis. Similar to their behavior in serum proteins repulsion, the cellular uptake of PEG-PHDCA nanoparticles was significantly influenced by MePEG molecular weight and particle size. The longer the MePEG chain, the greater the uptake was reduced. Compared with the larger particles (171 and 243 nm), dramatically lower uptake of the smaller PEG10000-PHDCA nanoparticles (80 nm) was observed after 1 and 2 h incubation. The least phagocytic uptake belonged to PEG10000-PHDCA nanoparticles (80 nm) among all nanoparticle formulations after 2 h incubation at 37°C. The inhibition of uptake for PEGylated nanoparticles of higher MePEG molecular weight and smaller size might be related to their thicker FALT and higher MePEG density which decreased the interaction between particles and macrophages.

Prolonged circulation is generally considered as the most important requirement for therapeutic application of a stealth delivery system. Our experimental results showed that various PEG-PHDCA nanoparticles could extend the elimination half-life of rHuTNF-α in S-180 tumor-bearing mice to about 10–24-fold that of free rHuTNF-α, which has a half-life of only 28.2 min. The value order of the extended in vivo circulating time of PEGylated nanoparticles was consistent with their different degree of in vitro decreased serum protein adsorption and phagocytic uptake. Among them, PEG5000-PHDCA (80 nm) nanoparticles earned the longest circulation time. PEG-PHDCA nanoparticles obviously changed the rHuTNF-α biodistribution in mice, in particular, decreased accumulation in liver and increased accumulation in tumor. The dynamic pattern of rHuTNF-α levels in tumors varied with the MePEG molecular weight and particle size. The PEG10000-PHDCA (80 nm), PEG10000-PHDCA (80 nm) and PEG10000-PHDCA (171 nm) nanoparticles spent longer time (4.50, 3.79 and 3.68 h, respectively) to attain their rHuTNF-α peak concentrations in tumors. Their peak concentrations were higher than the PEG10000-PHDCA (243 nm) and PEG5000-PHDCA (172 nm) nanoparticles, whose peak times were only 2.40 and 2.75 h, respectively. Because free rHuTNF-α and PHDCA nanoparticles were quickly eliminated from blood compartment, the values

**Fig. 5** - The linear-ship between the half-life and the AUCh, Cmax and Tmax of rHuTNF-α in S-180 tumors.
of both peak concentrations and peak time in tumors were very small. The differences in peak time and peak concentration in tumors for the MePEG-modified nanoparticles might be related to their different in vivo circulating time. A longer circulating time could increase the opportunity for stealth particles to gradually extravasate through the leaky vasculature into the tumor tissues. Thus, more rHuTNF-α was accumulated, the peak concentration was increased and the peak time was delayed. In fact, the linear-ships between the elimination half-life in blood and the AUC<sub>0-24h</sub> was increased and the peak time in S-180 tumors were fairly good (Fig. 5).

With free rHuTNF-α as a control, the relative tumor tissue exposures (Re) and the ratios of rHuTNF-α peak concentrations (Ce) were much higher than one, showing better tumor targeting efficiencies. Increased exposure and high peak concentration levels in tumors are desirable and would suggest an improved therapeutic outcome in tumor-bearing animals treated with these stealth particles (Gallo et al., 1989). The Re and Ce of PEG5000-PHDCA nanoparticles (80 nm) were 7.44 and 2.85, respectively, showing the best tumor targeting efficiency and the potentially strongest therapeutic capacity. The PHDCA nanoparticles of all sizes had no tumor targeting property, because of their quick elimination form blood compartment.

5. Conclusion

In summary, MePEG molecular weight and particle size had obviously influence on nanoparticle in vitro serum protein absorption, macrophage uptake, in vivo long circulating and tumor targeting properties. These effects came from the alterations of FACT and surface MePEG density. Higher MePEG molecular weight provided thicker FALT and smaller particle size offered higher MePEG chain density. These changes on the PEGylated nanoparticles decreased serum protein adsorption and phagocytic uptake, extended circulating time in blood and most importantly, increased drug accumulation in tumors. The antitumor study of the PEG-PHDC nanoparticles with various MePEG molecular weight and particle size is now in progress in our lab. The information provided in this study would be valuable for the development of long circulating nanoparticles for the aim of tumor targeting therapy.

Acknowledgements

The work was supported by the Science and Technology Development Foundation of Shanghai, China (No. 0243nm067) and Graduate Innovation Foundation of Fudan University (No. CQF301801). We thank Professor Jian-hua Zhu (Department of Radiopharmacy, Fudan University) for assistance in the radiolabelling and measurement.

REFERENCES


