Poly(ethylene glycol)-Coated Hexadecylcyanoacrylate Nanospheres Display a Combined Effect for Brain Tumor Targeting

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ABSTRACT

The aim of the present study was to evaluate the tumor accumulation of radiolabeled long-circulating poly(ethylene glycol) (PEG)-coated hexadecylcyanoacrylate nanospheres and non-PEG-coated hexadecylcyanoacrylate nanospheres (used as control), after intravenous injection in Fischer rats bearing intracerebrally well established 9L gliosarcoma. Both types of nanospheres showed an accumulation with a retention effect in the 9L tumor. However, long-circulating nanospheres concentrated 3.1 times higher in the gliosarcoma, compared with non-PEG-coated nanospheres. The tumor-to-brain ratio of pegylated nanospheres was found to be 11, which was in accordance with the ratios reported for other carriers tested for brain tumor targeting such as long-circulating liposomes or labels for magnetic resonance imaging. In addition, a 4- to 8-fold higher accumulation of the PEG-coated carriers was observed in normal brain regions, when compared with control nanospheres. Using a simplified pharmacokinetic model, two different mechanisms were proposed to explain this higher concentration of PEG-coated nanospheres in a tumoral brain. 1) in the 9L tumor, the preferential accumulation of pegylated nanospheres was attributable to their slower plasma clearance, relative to control nanospheres. Diffusion/convection was the proposed mechanism for extravasation of the nanospheres in the 9L interstitium, across the altered blood-brain barrier. 2) In addition, PEG-coated nanospheres displayed an affinity with the brain endothelial cells (normal brain region), which may not be considered as the result of a simple diffusion/convection process. The exact underlying mechanism of such affinity deserves further investigation, since it was observed to be as important as specific interactions described for immunoliposomes with the blood-brain barrier.

Clinical studies have shown that many brain tumors, especially primary tumors, are among the most resistant to chemotherapy, presumably due to the presence of a tight blood-brain barrier (Tamai and Tsuji, 1996). However, the blood-brain barrier can be selectively disrupted at the tumor site, which opens the possibility for carrier-mediated chemotherapy with more active compounds than the widely used nitrosoureas.

Indeed, the vasculature of gliomas, like that of other solid tumors, may present some peculiarities, which all form the basis of an increased microvascular permeability. Some of these peculiarities include open endothelial gaps (interendothelial junctions and transendothelial channels; diameter of about 0.3 μm), fenestrations (maximum channel width of 5.5 nm), and cytoplasmic vesicles such as caveolae (diameter of 50–70 nm) and vesicular vacuolar organelles (diameter of 108 ± 32 nm). An increase in vessel wall thickness has also been reported, which was attributed to endothelial cell hyperplasia, reflecting an increase in noneselective transendothelial transport. All these characteristics, which may be due in major part to the secretion of the vascular permeability factor (vascular endothelial growth factor) and which can lead to a loss of blood-brain barrier function in the case of cerebral malignancies, would be beneficial for transvascular transport of drugs (for a review, see Vajkoczy and Menger, 2000).

Another challenge lies in the fact that, despite being highly vascularized (Vajkoczy and Menger, 2000), brain tumors are exposed to subtherapeutic drug concentrations, as many...
compounds possess a short plasma half-life, giving them slim chances to circulate through the tumor vascular bed and to permeate in the interstitium.

An emerging approach for improving systemic chemotherapy is the use of surface-modified carriers. Coating the carriers with hydrophilic polymers such as polyethylene glycol (PEG) gives them a form of steric barrier against interactions with plasma proteins, such as opsonins and lipoproteins. As a result, PEG-coated carriers evade capture by the mononuclear phagocyte system (MPS), resulting in a longer circulation time in the blood compartment (Storm et al., 1995; Perachia et al., 1999). This prolonged plasma half-life will increase the chances for the sterically stabilized carriers to passively extravasate in the tumor interstitium, across the leaky and hyperpermeable blood-brain barrier. This approach should also permit the selective delivery of chemotherapeutics at the pathological site and thus to enhance its effectiveness, while sparing the other tissues from drug exposure (Gabizon, 1995).

This strategy has been conducted in many solid tumors, including glioma; for example, intracerebral implanted 9L tumors appeared hyperintense relative to brain on T1-weighted magnetic resonance images, after intravenous injection of long-circulating dextran-coated iron oxide particles to rats (Moore et al., 2000). In much the same fashion, Sine-rex, another long-circulating label for magnetic resonance imaging, was successfully used for the detection of brain tumors in patients (Enochs et al., 1999).

As far as tumor therapy is concerned, a study related that the use of long-circulating liposomes as cytotoxic drug carriers resulted in enhanced drug exposure and improved therapeutic activity, when compared with the nonencapsulated drug used on the same animal brain tumor model (T-749 in syngeneic Fischer rats) (Siegal et al., 1995). These results were very much in line with those reported in a rat 9L gliosarcoma tumor (Sharma et al., 1997). Finally, in patients, the radiolabeled Stealth pegylated carrier Caelyx (liposomal doxorubicin) showed a high intratumoral accumulation in glioblastoma and in metastatic brain tumors (Koukourakis et al., 2000). Caelyx also made possible a long-term stabilization in patients with recurrent malignant glioma (Fabel et al., 2001).

As an alternative to sterically stabilized liposomes or labeled ferrous nanoparticles, this work proposes new polymeric nanoparticles as carriers for cytotoxic drugs against glioma. The advantage of nanoparticles over liposomes arises from the possibility of controlling drug release depending on the polymer used. Moreover, nanoparticles are known to be more stable in the bloodstream, due to reduced interactions and exchanges with blood components (Lasic, 1998). Finally, cyanoacrylate nanoparticles were found to be able to reverse P-glycoprotein-mediated multidrug resistance by a specific mechanism (Colin de Verdière et al., 1997).

The above characteristics make long-circulating poly(cyanoacrylate) nanoparticles a potentially advantageous delivery system for brain tumor chemotherapy. Hence, this study aimed to evaluate the ability of these nanoparticles to accumulate in a rodent cerebral gliosarcoma model. Moreover, a pharmacokinetic model was proposed, to understand the mechanisms involved in the uptake of PEG-coated nanoparticles in both normal and tumoral brains.

### Materials and Methods

**Materials.** Control nanospheres and sterically stabilized nanospheres were prepared with poly(hexadecylcyanoacrylate) (PHDCA) and poly(MePEG<sub>2000</sub>/cyanoacrylate-co-hexadecylcyanoacrylate) 1:4 (PEG-PHDCAl, respectively. These polymers and copolymers were synthesized as described before (Brigger et al., 2000). Their <sup>14C</sup>-radiolabeled counterparts were prepared at the Commissariat à l’Energie Atomique (Saclay, France) according to the previously described protocols (Perachia et al., 1999; Brigger et al., 2000). <sup>14C</sup>PHDCA had a specific activity of 5.8 μCi/mg, whereas the activity of <sup>13C</sup>PEG-PHDCAl was 1.6 μCi/mg. Pluronic F88, a nontoxic tensio-active agent used in the preparation of the pegylated nanospheres, was purchased from BASF Corp. (Worcester, MA). Glucose 5% (Laboratoires Aguettant, Lyon, France) was used as the dispersion medium for the purified nanospheres. The hydrophilic tracer <sup>3H</sup> sucrose (specific activity, 16.1 μCi/mg; radioactive concentration, 1.0 μCi/ml) was obtained from Amersham Pharmacia Biotech (Les Ulis, France). All the solvents and other reagents used were of analytical grade.

For the nanosphere stability study, Fischer rat plasma was purchased from Iffa Credo (L’Arbresles, France). The 9L gliosarcoma cell line (Weizsaecker et al., 1981) was kindly offered by Dr. V. W. van Beusechem (Division of Gene Therapy, Department of Medical Oncology, Vrije Universiteit Medical Center Amsterdam, Amsterdam, The Netherlands). Cell culture medium and additional products were purchased from Invitrogen (Cergy Pontoise, France).

**Biodegradable Cyanoacrylate Nanosphere Preparation and Characterization.** Biodegradable <sup>14C</sup>PHDCA and <sup>14C</sup>PEG-PHDCAl nanospheres were obtained by the method of nanoprecipitation (Fessi et al., 1989). Typically, for the control nanospheres, <sup>14C</sup>PHDCA (18.1 mg corresponding to 105 μCi) and PHDCA (281.9 mg; isotopic dilution, 1:15.6) were dissolved under slight warming in acetone (15 ml). This organic phase was then rapidly poured through a syringe in milliQ water (30 ml) (Millipore), under magnetic stirring. Nanosphere precipitation occurred instantaneously. Directly afterward, acetone was evaporated under reduced pressure. The colloidal suspension was purified by ultracentrifugation (145,000g, 1.5 h, 4°C; Beckman L7-55 ultracentrifuge; Beckman Coulter, Inc.), and the resulting nanosphere pellet was suspended in a solution of 5% glucose (12.0 ml), to obtain the following relation: 25 mg of polymer in each nanosphere, was purchased from BASF Corp. (Worcester, MA). Glucose 5% (Laboratoires Aguettant, Lyon, France) was used as the dispersion medium (Fessi et al., 1989). Typically, for the control nanospheres, <sup>14C</sup>PHDCA (18.1 mg corresponding to 105 μCi) and PHDCA (281.9 mg; isotopic dilution, 1:15.6) were dissolved under slight warming in acetone (15 ml). This organic phase was then rapidly poured through a syringe in milliQ water (30 ml) (Millipore), under magnetic stirring. Nanosphere precipitation occurred instantaneously. Directly afterward, acetone was evaporated under reduced pressure. The colloidal suspension was purified by ultracentrifugation (145,000g, 1.5 h, 4°C; Beckman L7-55 ultracentrifuge; Beckman Coulter, Inc.), and the resulting nanosphere pellet was suspended in a solution of 5% glucose (12.0 ml), to obtain the following relation: 25 mg of radiolabeled nanospheres/8.75 mg of radiolabeled counterparts was prepared at the Commissariat à l’Energie Atomique (Saclay, France) according to the previously described protocols (Perachia et al., 1999; Brigger et al., 2000). <sup>14C</sup>PHDCA had a specific activity of 5.8 μCi/mg, whereas the activity of <sup>13C</sup>PEG-PHDCAl was 1.5 μCi/mg. Pluronic F88, a nontoxic tensio-active agent used in the preparation of the pegylated nanospheres, was purchased from BASF Corp. (Worcester, MA). Glucose 5% (Laboratoires Aguettant, Lyon, France) was used as the dispersion medium for the purified nanospheres. The hydrophilic tracer <sup>3H</sup> sucrose (specific activity, 16.1 μCi/mg; radioactive concentration, 1.0 μCi/ml) was obtained from Amersham Pharmacia Biotech (Les Ulis, France). All the solvents and other reagents used were of analytical grade.

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of PEG-coated nanospheres, −20 mV. In this latter case, the tensio-active Pluronic F68 may also have contributed to raising the surface charge.

Finally, it was important for the validation of the biodistribution and pharmacokinetic studies to check whether the 14C label remained stably bound to purified PHDCA or PEG-PHDCA carriers during in vivo experiments. To answer this question, 14C-radiolabeled PHDCA or PEG-PHDCA nanospheres were incubated in vitro, in Fischer rat plasma, at 37°C (experiment realized in triplicate). The nanospheres/plasma ratio was the same as that obtained in vivo (see below). At predetermined time intervals (5 min, 30 min, 4 h, and 8 h), an aliquot of the plasma medium was withdrawn and ultrafiltered (Ultrafree-MC, 100-kDa cutoff; Millipore) at 10,000 g during 20 min. The radioactivity of the ultrafiltrate, containing soluble polymer or degradation products, was then measured by liquid scintillation counting. The results were expressed as a percentage of the total measured radioactivity in the plasma medium.

Animals. Male CD 344 Fischer rats, weighing 250 to 300 g, were purchased from Charles River Laboratories (L’Arbresles, France). The animals were housed in the Animal Experiment Unit of the Institut Gustave-Roussy and had free access to food and water. Experiments were carried out in compliance with the conditions established by the European Community (Directive n°86/609/CEE).

Brain Tumor Model. 9L gliosarcoma cells were cultured in a 5% CO2 incubator at 37°C, in Dulbecco’s modified Eagle’s medium with Glutamax, supplemented with 10% inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (1.5 μg/ml). A minimum of 2 weeks of in vitro culture was allowed before in vivo engraftment of the 9L cells. The rats were anesthetized by an intraperitoneal administration of xylazine (11 mg/kg)-ketamine (73 mg/kg), and a small incision was made in the right hemisphere, at the following coordinates: 2 mm lateral from the midline, 2 mm anterior to bregma, and 3 mm deep. Control animals received an injection of an equal volume of 0.9% NaCl, without tumor cells. Preliminary experiments with intravenously injected Blue Evans revealed that the blood-brain barrier was locally disrupted at the pathological site 7 days after tumor implantation.

Tissue Distribution Study. The tissue distribution study was performed 7 days after intracerebral inoculation of tumor cells or NaCl (control group) in rats. Tumor-bearing or control animals received an intravenous bolus injection (450 μl) of [14C]PHDCA or [14C]PEG-PHDCA nanospheres (400 μl, 3.5 μCi, 40 mg/kg), with [14C]sucrose (50 μl, 15 μCi) added in the dispersion medium of the nanospheres (5% glucose). After 3 min (one tumor-bearing rat), 5 min (four tumor-bearing rats), 30 min, and 4 h (four control and four tumor-bearing rats), animals were anesthetized by intraperitoneal administration of xylazine (11 mg/kg) and ketamine (73 mg/kg). An aliquot of the blood sample was centrifuged to separate the cellular components from the plasma fraction. The radioactivity contained in the blood, plasma, and, if possible, the urine was finally analyzed by routine liquid scintillation counting.

Various organs including liver, lung, spleen, kidney, and muscle were dissected and weighed, and their activity was measured by liquid scintillation after dissolution with Soluène 350 (PerkinElmer Life Sciences, B.V. Groningen, The Netherlands). The brain and cerebellum were also removed for analysis and the following regions were dissected: the visible 9L tumor mass or the intracerebral injection site (control animals), the 2- to 4-mm brain area surrounding the tumor or injection site, and the matching contralateral brain region.

In the calculation of the radioactivity distribution, muscle and blood volume were assumed to be, respectively, 45 and 6.5% of the total body weight (Ritschel, 1987). For blood partition calculation, the plasma volume was estimated to be 45% of the total blood volume (Ritschel, 1987).

Pharmacokinetic Modelization and Tumor Uptake Rate. To understand how the nanospheres accumulated in the normal or tumor brain, a pharmacokinetic model was built up. A simplified two-compartment open model allowed separation of normal or tumoral brain uptake from biodistribution and excretion phenomena. Thus, the pharmacokinetic model consisted of a central compartment (blood pool) and the normal or tumoral brain. The normal (k1) or tumoral (k1′) brain uptake rates as well as the rate constant “blood elimination” (ke), which included all processes that lead to the removal of the carrier from the blood, such as excretion, phagocytosis, and biodistribution into organs and tissues other than the brain, were assumed to be unidirectional. k1, k1′, and ke (min−1) were the arithmetic means obtained for each rat at the time points mentioned earlier (see Tissue Distribution Study). Qblood and Qbrain were the amount of nanospheres (μg/organ weight (g)) present in the bloodstream and in the brain, respectively. Hence, the pharmacokinetic model represented in Fig. 1 could be described by the following equations:

\[
\frac{dQ_{\text{blood}}}{dt} = -(k1 + ke)Q_{\text{blood}} \quad (1)
\]

\[
Q_{\text{blood}} = Q_0 \cdot e^{-k1t + ke} \quad \text{and} \quad (k1 + ke) = \frac{1}{t} \cdot \ln \left( \frac{Q_0}{Q_{\text{blood}}} \right) \quad (2)
\]

k1 or k1′ was then calculated as follows:

\[
\frac{dQ_{\text{brain}}}{dt} = k1 \cdot Q_{\text{blood}} \quad \text{and} \quad k1 = \frac{Q_{\text{brain}}}{Q_{\text{blood}}} \cdot (k1 + ke) \quad (3)
\]

k1 was obtained by replacing (k1 + ke) with eq. 2. Thereafter, ke could be determined.

Accumulation into normal brain or tumoral brain at t∞ could be formulated as follows (from eqs. 1 and 3):

\[
Q_{\text{brain}} = k1 \cdot ke \cdot \frac{k1 + ke}{1 + K} \quad \text{with} \quad K = \frac{ke}{k1} \quad (4)
\]

The tumor uptake rate k1″ was obtained according to the same modelization, by replacing the normal or tumoral brain by the neo-plastic tissue itself (Fig. 1).

Statistical Analysis. Data were represented as means ± S.E. Sets of data were then compared with the nonparametric Mann-Whitney U test. Differences were considered significant at p < 0.05.
Results

Plasma Stability of PHDCA and PEG-PHDCa Nanospheres. It is now well established that cyanoacrylate nanoparticles are biodegradable. Their degradation mechanism involves an enzymatic degradation with hydrolysis of the ester function, leading to the formation of the soluble cyanoacrylic acid and the corresponding alcohols (i.e., hexadecanol for PHDCA and PEG-PHDCa, as well as polyethylene glycol for PEG-PHDCa) (Lenaerts et al., 1984). In addition, the biodegradation rate of poly(cyanoacrylate) depends on the length of the alkyl chain (Couvreur et al., 1979), with hexadecyl being more slowly degraded compared with isohexyl and isobutyl. As shown in Fig. 2, representing the biodegradation profile of the poly(hexadecylcyanoacrylate) nanospheres in vitro (Fischer rat plasma), PEG-coated nanospheres were more rapidly degraded when compared with noncoated nanospheres. Since PEG-coating inhibits protein adsorption including enzymes, a rapid partial (7%) solubilization rather than an enzymatic degradation was likely to occur within the first 30 min. In our case, this solubilized fraction could correspond to the more PEG-containing and hydrophilic oligomers present in the PEG-PHDCa nanosphere dispersion (Brigger et al., 2000). After 30 min, the PEG-PHDCa nanosphere biodegradation rate (probably by an enzymatic reaction) became quite similar to that of PHDCA nanospheres.

However, the amount of soluble released polymers measured in vitro could be correlated with in vivo renal elimination determined precisely in a previous study (Calvo et al., 2002). Moreover, when the solubilized nanospheres were injected intravenously, a very low level of radioactivity was recovered in the plasma as soon as 1 min after administration (Peracchia et al., 1999). Thus, the measured radioactivity in vivo was likely to represent the biodistribution and pharmacokinetics of the insoluble cyanoacrylate polymers only.

Tissue Distribution Study. The biodistribution of [14C]PHDCA and [14C]PEG-PHDCa nanospheres in the blood, the plasma, and the main organs of 9L tumor-bearing rats is presented in Table 1. No significant difference in biodistribution was observed between 9L tumor-bearing rats and control animals (data not shown) for both types of nanospheres. Control PHDCA nanospheres had a very short plasma residence time, with a rapid and massive uptake by the MPS, especially the liver and the spleen. On the other hand, sterically stabilized PEG-PHDCa nanospheres displayed a longer circulation time. Uptake of the pegylated nanoparticles by the MPS also took place, but it was both delayed and less important than for control nanospheres, except for the lungs. For the blood partition of PHDCA and PEG-PHDCa nanospheres, it can be seen in Table 1 that all of the 14C radioactivity was contained in the plasma fraction, which suggested that none of the circulating radioactivity was associated with blood cells.

The brain distribution in 9L tumor-bearing rats, shown in Fig. 3, A and B, revealed that both types of nanospheres accumulated preferentially in the gliosarcoma, rather than in the peritumoral brain or the healthy contralateral hemisphere. However, this accumulation was 3.1 times greater for the PEG-PHDCa nanospheres (p < 0.05), with a maximal intratumoral concentration reaching 25 (±5) μg/g when compared with 8 (±3) μg/g for the control particles [mean gliosarcoma weight 45 (±30) mg]. At the same time, no accumulation of PHDCA or PEG-PHDCa nanospheres was observed at the intracerebral injection site of the control animals, when compared with the normal brain areas (Fig. 3, C and D). Hence, 240 min after intravenous injection, the tumor/injection site ratio for the long-circulating PEG-PHDCa nanospheres had a value of 11. In addition, an interesting result was the always higher accumulation of the pegylated nanospheres in the different brain regions, regardless of the animal group (control or tumor-bearing rats). Indeed, PEG-

### Table 1

<table>
<thead>
<tr>
<th>Biodistribution of [14C]PHDCA and [14C]PEG-PHDCa nanospheres in the main organs at 3 min, 5 min, 30 min, and 4 h after intravenous injection of the carriers to 9L tumor-bearing Fischer rats</th>
<th>Mean % injected dose/g of tissue</th>
<th>S.D. % injected dose/g of tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>[14C]PHDCA</strong> Nanospheres</td>
<td><strong>[14C]PEG-PHDCa</strong> Nanospheres</td>
<td></td>
</tr>
<tr>
<td>3 min</td>
<td>5 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Blood</td>
<td>1.11</td>
<td>0.48</td>
</tr>
<tr>
<td>Plasma</td>
<td>2.07</td>
<td>1.04</td>
</tr>
<tr>
<td>Liver</td>
<td>10.91</td>
<td>6.62</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.50</td>
<td>0.58</td>
</tr>
<tr>
<td>Lung</td>
<td>0.18</td>
<td>0.08</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.82</td>
<td>0.79</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Liver</td>
<td>9.39</td>
<td>8.92</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.70</td>
<td>1.21</td>
</tr>
<tr>
<td>Lung</td>
<td>0.11</td>
<td>0.32</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.44</td>
<td>2.63</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.02</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* p < 0.05, ** p < 0.01, *** p < 0.001 (nonparametric Mann-Whitney U test).
PHDCA nanospheres were more concentrated than PHDCA nanospheres not only in the hyperpermeable neoplastic tissue (ratio of 3.1), but also in parts of the brain, with a normal blood-brain barrier (see below for sucrose biodistribution), such as the contralateral hemisphere, as well as the stereotactic injection site and its adjacent tissue in control animals (ratio PEG-PHDCA/PHDCA nanospheres of 4–8, p < 0.05). Finally, even if their plasma concentration was lower than their tumor concentration, both types of colloidal carriers were retained for at least 4 h in the gliosarcoma interstitium.

As reported in a previous study (Rapoport et al., 1980), the hydrophilic tracer [3H]sucrose is very slightly permeant at the normal blood-brain barrier (see below for sucrose biodistribution), such as the contralateral hemisphere, as well as the stereotactic injection site and its adjacent tissue in control animals (ratio PEG-PHDCA/PHDCA nanospheres of 4–8, p < 0.05). Finally, even if their plasma concentration was lower than their tumor concentration, both types of colloidal carriers were retained for at least 4 h in the 9L gliosarcoma interstitium.

As reported in a previous study (Rapoport et al., 1980), the hydrophilic tracer [3H]sucrose is very slightly permeant at the normal blood-brain barrier and is not metabolized when administered intravenously to rats. Consequently, it was added to the dispersion medium of both [14C]PHDCA and [14C]PEG-PHDCA nanospheres to assert the state of the blood-brain barrier alteration (Fig. 4). The biodistribution profiles of sucrose in the main organs were, for all purposes except brain tumors, independent of the type of nanospheres dispersed ([14C]PHDCA or [14C]PEG-PHDCA), as well as the group the rats belonged to: tumor-bearing or control group (data not shown). In the main organs, this compound featured a biodistribution profile which was essentially reduced to the blood compartment and the kidney; high concentrations were also found in the urine (data not shown). Plasma concentrations dropped rapidly to 0.3%/g of organ after 30 min and to 0.01%/g of organ after 240 min, due to substantial elimination.

In contrast, in the brain, notable differences in sucrose concentrations between the tumor (9L tumor-bearing animals; Fig. 4, A and B) and the intracerebral injection site (control animals) (Fig. 4, C and D) were obtained when the marker was dispersed in the external medium of both nanosphere preparations. Indeed, 7 days after tumor engraftment, the 9L gliosarcoma and, to a lesser extent, the peritumoral brain region were hyperpermeable to sucrose, indicating a selective disruption of the blood-brain barrier at the pathological site with a slight edematous surrounding brain. No blood-brain barrier permeabilization was observed at this time at the intracerebral injection site of the control group, which means that the blood-brain barrier alteration was only due to the presence of cancerous cells and not to the stereotactic procedure itself. Moreover, as for the main organs, the brain sucrose profile was identical in each animal group with PHDCA and PEG-PHDCA nanospheres. Thus, the observed higher concentration of the pegylated nanospheres in all the analyzed brain regions could not be explained by a specific toxicity of these carriers on the blood-brain barrier (i.e., barrier permeabilization due to the
amphiphilic properties of PEG-PHDCA nanospheres), as compared with PHDCA nanospheres. Finally, sucrose intra-tumoral t\text{max} was around 5 min. Thus, contrary to the PHDCA and PEG-PHDCA nanospheres, this hydrophilic tracer was not retained in the tumor interstitium.

Pharmacokinetic Modelization and Tumor Uptake Rate. Based on the biodistribution study, the question remains whether the higher concentration of PEG-coated nanospheres observed in all the brain regions could be attributed to their long-circulating properties and/or to an affinity with brain tissue and tumor. Therefore, to gain insight into how the pegylated nanospheres accumulated more easily in the brain when compared with control nanospheres, a simplified two-compartment open pharmacokinetic model was developed (as described earlier and represented in Fig. 1). Table 2 shows the experimental values of the rate constants \( k_1 \) and \( k_1 \) upon which our model was based. First, \( k_{\text{PHDCA}} \) and \( k_{\text{PEG-PHDCA}} \), when calculated for the tumor-bearing group (case A) or the control group (case B), were always approximately 1,000 to 10,000 times higher than their respective \( k_1 \) (case A) or \( k_1 \) (case B), indicating that the rest of the body was predominantly responsible for nanosphere blood clearance and excretion. Moreover, even if PEG-PHDCA nanospheres were degraded and excreted faster, \( k_{\text{PHDCA}} \) was about 2 to 7 times higher than \( k_{\text{PEG-PHDCA}} \). The control nanospheres were captured more rapidly and massively by the MPS than the PEG-coated nanospheres, resulting in a shorter circulation time. Then, each type of nanospheres featured \( k_1 \) values that were approximately equivalent in both cases (A and B). This was not the case for the \( k_{\text{PEG-PHDCA}} \) and \( k_{\text{PHDCA}} \) values, which were 2- to 7-fold higher in a tumoral brain (\( k_1 \) ratio for case A), when compared with a normal brain (\( k_1 \) ratio for case B). This result agrees with the fact that the nanospheres more easily cross the blood-brain barrier in the presence of a 9L gliosarcoma, by extravasation across its altered and leaky blood-brain barrier. Finally, the \( k_{\text{PEG-PHDCA}}/k_{\text{PHDCA}} \) ratios were greater than 2 in both cases, showing an affinity of the pegylated nanospheres for the brain with a normal or an altered blood-brain barrier.
TABLE 2
Nanosphere rate constants obtained with the simplified two-compartment open pharmacokinetic model

<table>
<thead>
<tr>
<th>Case A</th>
<th>PEG-PHDA Nanospheres</th>
<th>PHDCA Nanospheres</th>
<th>k1'</th>
<th>ke</th>
<th>k1'</th>
<th>ke</th>
</tr>
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<tr>
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<td>1.11E-04</td>
<td>8.36E-02</td>
<td>7.67E-05</td>
<td>5.58E-01</td>
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<th>Case B</th>
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<th>ke</th>
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<td>7.63E-06</td>
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E-04, scientific notation for $10^{-4}$.

cells ($k_1$ or $k1'$), as compared with the control PHDCA nanospheres.

Finally, when replacing the tumoral brain by the tumor itself and using the same modelization (Fig. 1), the mean tumor uptake rates $k1''$ were $5.0 \times 10^{-6}$ min$^{-1}$ and $6.5 \times 10^{-6}$ min$^{-1}$ for PHDCA and PEG-PHDA nanospheres, respectively. The fact that the mean rate constants $k1''$, which express the extravasation of the carriers in the tumor interstitium, were approximately identical suggested that the intratumoral accumulation mechanism was the same for control or pegylated nanospheres.

**Discussion**

The successful transvascular delivery of optimal quantities of blood-borne agents (particles or molecules) into tumors is critical to the effectiveness of chemotherapy. Now, as a result of structural and functional anomalies, the blood vessels in a tumor are generally leaky. This can be taken advantage of when targeting tumors passively with long-circulating particulate carriers (Gerlowski and Jain, 1986; Yuan et al., 1995).

As expected, PHDCA nanospheres had a very short circulation time in vivo, mainly due to a rapid and massive uptake by the MPS (Storm et al., 1995). This result was also mirrored by mean rate constants $k_1$, which expressed elimination from the blood compartment either by capture by organs or by urinary excretion, which comprised between $9.95 \times 10^{-2}$ and $3.13 \times 10^{-1}$ min$^{-1}$. These mean values were about 3 to 7 times higher than those of the stericly stabilized PEG-PHDA nanospheres. However, mean $k_1$ for the PHDCA and PEG-PHDA values were still 500 to 2000 times higher than the rate constants expressing brain accumulation ($k_1'$, normal brain and $k1'$, tumoral brain), which demonstrated that, although biodegradable pegylated nanospheres are engineered to evade the MPS, there was still a significant amount of clearance via this route. This is the common drawback for brain tumor targeting with intravascularly administered compounds: the body acts as a sink, and only a small fraction of administered compound actually reaches the tumor (Groothuis, 2000). However, the longer circulation time of the PEG-PHDA nanospheres, as compared with PHDCA ones, allowed us to enhance importantly the fraction of carrier redirected to non-MPS sites, for instance, brain tumors.

The delivery of carriers to brain tumors may be affected not only by their blood clearance by MPS or by excretion, but also by physiological barriers limiting tumor accumulation (Yuan et al., 1994; Jain, 1998, 2001).

Nevertheless, PHDCA and PEG-PHDA nanospheres were able to selectively extravasate across the blood-brain barrier in the 9L gliosarcoma interstitium. However, this effect was significantly increased for the pegylated nanospheres. Their maximal tumor-to-brain ratio, measured 4 h after intravenous injection, was found to be 11. This ratio was quite consistent with the results obtained 2 h after intravenous injection of radiolabeled Caelyx to patients, where the carrier accumulation was 13 to 19 times higher in the glioblastoma and 7 to 13 times higher in the metastatic lesions compared with normal brain (Koukourakis et al., 2000). In the same way, Vescan liposomes, characterized by an improved plasma stability, showed at best a tumor-to-brain ratio of 7.5 in patients (Khalifa et al., 1997). For a final comparison, tumor uptake of radiolabeled long-circulating dextran-coated iron oxide nanoparticles exceeded the uptake in any of the normal brain tissues by a factor of 10, 24 h after their intravenous injection (Moore et al., 2000).

The PHDCA or PEG-PHDA nanosphere accumulation in the 9L gliosarcoma correlated well with the alteration of the blood-brain barrier at the tumor site, as unequivocally evidenced by the measurement of the hydrophilic tracer sucrose penetration. Like many solid tumors (Gerlowski and Jain, 1986; Yuan et al., 1995), 7 days after intracerebral engraftment, 9L tumor vessels were thus hyperpermeable, when compared with normal blood vessels, even to blood-borne particles with a diameter approximating 200 nm. This value was 2 times higher than the pore cutoff size reported for the U87 human glioblastoma implanted intracerebrally in mice (7–100 nm) (Hobbs et al., 1998).

As far as the mechanism for tumor accumulation in the 9L gliosarcoma was concerned, our simplified pharmacokinetic model allowed the calculation of $k1''$ for the PHDCA and PEG-PHDA nanospheres, which is the constant rate governing the passage from the bloodstream to the malignant...
tissue. Because the obtained $k_{1}'$ values were approximately the same for both types of nanospheres (PHDCA nanospheres, $5 \times 10^{-6}$ min$^{-1}$ and PEG-PHDCA nanospheres, $6.5 \times 10^{-6}$ min$^{-1}$), this suggested that the intratumoral accumulation mechanism was the same, probably by diffusion/convection across the altered and hyperpermeable tumoral endothelium (Yuan, 1998). Thus, the higher accumulation of the pegylated nanospheres in the gliosarcoma was mostly due to their longer blood circulation time, which gave them more chances to extravasate in the interstitium.

Finally, both carrier systems displayed a retention effect in the glioma, even if their plasma concentration was lower than that in the tumor. On the contrary, such retention was not observed for the hydrophilic tracer sucrose, which diffused out of the tumor during the time of analysis (4 h). Under physiological conditions, components of the interstitial fluid can either reenter the bloodstream directly via the postcapillary venules, or they can enter the lymphatic system, depending on their size. Smaller molecules are better suited to reenter the veins, whereas larger molecules tend to enter the lymphatic system (Seymour, 1992). Hence, the observed extravasation and retention of the nanospheres in the 9L tumor interstitium accounted for the leakiness and the coexisting lack of a lymphatic system in the brain. This phenomenon was described as the EPR effect: enhanced permeability and retention effect (Maeda, 2001). Conversely, because sucrose displayed a small molecular size as well as a rapid elimination by renal clearance, this molecule could diffuse out of the tumor in the bloodstream, following gradient concentration equilibrium.

An interesting result was the 4- to 8-fold higher concentration of the more hydrophilic PEG-coated nanospheres observed in normal brain regions (contralateral hemisphere in the tumor-bearing and control groups: stereotactic injection site and surrounding tissue in the control group), when compared with the PHDCA nanospheres. This higher accumulation could neither be explained by residual radioactivity in blood due to their longer circulation time, since a transcatheter perfusion with saline was systematically performed, nor by a specific blood-brain barrier permeabilization due to a toxic surfactant effect of the amphiphilic PEG-coated carriers. Indeed, in the last case, an identical and negligible brain sucrose penetration was observed for both types of nanospheres, indicating the same state of the blood-brain barrier.

Our simplified pharmacokinetic model supposed that the concentration of the nanospheres in the brain depended on their blood clearance, especially by MPS organs, represented by $k_e$, and on their absorption in the brain, expressed by $k_{1}'$ [$Q_{\text{brain}} = Q_f/(1 + k_e/k_{1}')$. This points out that the accumulation in the brain was improved when using a long-circulating carrier with reduced MPS clearance. Besides, PEG-coated carriers displayed a 2.5 times higher mean $k_1'$ than did PHDCA nanospheres, suggesting that PEG-coated carriers had a stronger affinity for the blood-brain barrier than did their nonpegylated counterparts. A specific interaction of the PEG or Pluronic coating with the blood-brain barrier endothelial cells could explain this result. The mean concentration of the PEG-PHDCA carriers reached 0.027% injected dose/g of brain tissue at 30 min (Fig. 3D), which was quite in line with the accumulation obtained for long-circulating OX26 monoclonal antibody immunoliposomes directed to the rat transferrin receptor, which featured an average brain delivery of 0.030% injected dose/g of tissue 1 h after their intravenous injection (Huwyler et al., 1996).

However, the exact uptake mechanism across the normal blood-brain barrier could not be explained for the pegylated nanospheres. Among possible mechanisms of transport to the brain are transcytosis or inhibition of an efflux system, especially P-glycoprotein (Batrakova et al., 1999; Kreuter, 2001). Another hypothesis could be the adsorption of apolipoprotein E on the PEG-coated nanospheres. This overcoating with apolipoprotein E could mimic low-density lipoprotein particles, leading to an uptake of the nanospheres by an endocytic process, after interaction with the low-density lipoprotein receptor (Kreuter, 2001). Finally, adhesion of the nanospheres to the vessel walls of the brain, rather than a real uptake across the blood-brain barrier, is also possible.

This point deserves further investigations, but it seems that the density of the hydrophilic coating has an importance for brain targeting: it should be a compromise between the long-circulating properties of the carrier and appropriate surface characteristics to still permit interaction with brain endothelial cells and macrophages (Hong et al., 1999; Calvo et al., 2001). For example, the same PEG-coated liposomes as previously reported, but without the grafted monoclonal antibody OX26, displayed a greatly increased plasma half-life, when compared with conventional liposomes, OX26 PEG-immunoliposomes, and also PEG-PHDC nanospheres; however, the brain uptake of these pegylated liposomes was zero (Huwyler et al., 1996).

In conclusion, although PHDCA and PEG-PHDC nanospheres were cleared by the MPS organs, their accumulation, probably by diffusion/convection across the altered and leaky blood-brain barrier, and retention in the 9L brain tumor was found to be possible. In addition, due to their higher concentration in the malignant tissue and their lower uptake by MPS organs, the sterically stabilized PEG-coated nanospheres showed a biodistribution profile that was better suited for brain tumor targeting than that of the control PHDCA nanospheres. Finally, contrary to the long-circulating pegylated liposomes from Huwyler et al. (1996), our sterically stabilized PEG-PHDC nanospheres displayed an affinity for brain regions protected by a normal blood-brain barrier. This can be of benefit for the treatment of infiltrating brain tumors.

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References


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