Polymer Nanoparticles

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Nanoparticles with In Vivo Anticancer Activity from Polymer Prodrug Amphiphiles Prepared by Living Radical Polymerization**

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Drug-loaded polymer nanoparticles are a promising approach to the treatment of severe diseases, such as cancer, infections, and neurodegenerative disorders.[1] These nanoconstructs are obtained by the encapsulation of a drug during self-assembly of amphiphilic copolymers in aqueous solution.[2] In the field of cancer, although this approach has led to numerous encouraging results and proofs of concept in vitro,[3] important limitations, which may explain the lower number of successful in vivo studies, still remain. The "burst release", in which a large fraction of chemotherapeutic agent is quickly released post-administration, can be harmful to patients. Poorly soluble drugs exhibit a high tendency to crystallization upon encapsulation. Finally, maximum achievable drug loadings are generally only a few percent, thus the use of a large amount of nanocarrier is required and this can lead to prohibitive toxicity in vivo.

To overcome these obstacles, inspiration can be taken from the prodrug approach, whereby the drug is covalently linked to a (macro)molecule. The inactive prodrug is metabolized in vivo into an active metabolite.[4] This strategy improves drug solubility, prolongs in vivo circulation, and reduces adverse effects, the last feature being of paramount importance in many chemotherapy treatments. In the particular case of polymeric prodrugs, the standard approach is to link hydrophobic drugs to a preformed hydrophilic polymer (e.g., poly(ethylene glycol),[5] poly(*N*-(2-hydroxypropyl)-methacrylamide),[6] poly(L-glutamic acid),[7] dextran,[8] cyclodextrins,[9] or poly(methacryloyloxyethyl phosphorylcholine)[10]) to give fully water-soluble conjugates or small-size aggregates.[11] Likewise, prodrugs prepared through conjugation to amphiphilic copolymers have alleviated some of the drawbacks of nanoparticles.[12]

Herein, we report the design of a new class of efficient anticancer nanocarriers with high drug payloads. These nanocarriers are made of well-defined polymer–drug conjugate amphiphiles obtained by nitroxide-mediated polymerization (NMP),[13] and comprise a hydrophobic polymer block and a hydrophilic drug tail. Like atom-transfer radical polymerization (ATRP)[14] and reversible addition–fragmentation chain transfer (RAFT),[15] NMP is a living radical polymerization (LRP) technique that enables well-defined macromolecular architectures to be synthesized and that has recently been successfully applied in the area of bioconjugation.[16] The new strategy we propose relies on the controlled growth of a hydrophobic oligomer from an anticancer drug-bearing macroalkoxyamine initiator, to position one chemotherapeutic at the extremity of each polymer chain (Scheme 1). Because of the amphiphilic nature of the resulting drug-polymer conjugates, they spontaneously self-assemble in aqueous solution to form stable, narrowly-dispersed nanoparticles that show significant anticancer activity both in vitro, on various cancer cell lines, and in vivo, on tumor-bearing mice. For the first time, nanoparticles with in vivo anticancer activity have been obtained from the self-assembly of hydrophilic drug/hydrophobic polymer prodrugs. This study also opens up the possibility of further applications for LRP techniques in the biomedical field, as this general method could be extended to many other hydrophilic drug/hydrophobic polymer combinations.

Scheme 1. Strategy to achieve well-defined polymer–drug conjugate nanoparticles by nitroxide-mediated polymerization (NMP).

Polyisoprene (PI) has been chosen as hydrophobic polymer for its interesting properties such as chemical[17] and enzymatic[18] degradability, as well as its biocompatibility[19] and its structural similarity with natural polyisoprenoids. Isoprene is the basic structural motif of naturally occurring, biocompatible terpenes (e.g., coenzyme Q10, retinol, vitamin E, etc.). We therefore believed that synthetic PI of controlled structure may also have interesting biomedical applications, especially as a nanocarrier. The anticancer drug gemcitabine (Gem), a nucleoside analogue with demonstrated activity against a wide range of solid tumors (e.g.,

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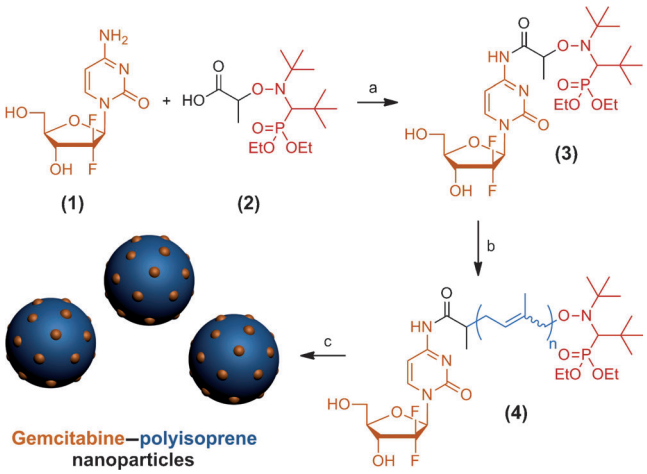
colon, lung, pancreatic, breast, bladder, and ovarian cancers),[20] was selected as a drug model. Nucleoside analogues are a class of therapeutic agents with significant anticancer or antiviral properties, but also with serious limitations that often restrict their use, such as short plasma half-life, rapid metabolism, the induction of resistance, and the advent of severe side effects.[21] The employed strategy is expected to protect Gem from rapid deamination by deoxycytidine deaminase,[22] thus leading to greater in vivo anticancer activity than that of the free Gem.

Gem–PI conjugates were prepared by NMP of isoprene under SG1 control,[23] utilizing Gem-functional alkoxyamine initiator **3**, which was obtained by coupling unprotected Gem (**1**) to the AMA–SG1 alkoxyamine[24] (**2**) under PyBOP-linkage chemistry (Scheme 2). Polymerization of isoprene in the presence of **3** allowed for the preparation of a small library of Gem–PI conjugates (**4a–d**, Table 1), of controlled molar mass and with a terminal gemcitabine functionality attached to the polymer chain by a hydrolysable amide linkage.

A similar pathway employing a TBDMS-protected Gem derivative was also investigated but led to 20–30% loss of Gem during the deprotection step (see the Supporting Information). The alternative strategy of attaching Gem to a previously formed polymer was rejected owing to the difficulty of purifying the mixture of products, byproducts, and unreacted PI that would result. By contrast, the "grafting from" approach, employed here, yields a mixture of the functionalized polymer, solvent, and unreacted monomer; the latter two can be removed by evaporation. Gem–PI conjugates with number-average molar mass (M_n) between 840 and 2510 g mol^{-1} and dispersities ($Đ$) of 1.3–1.4 were prepared (Table 1). The presence of gemcitabine was confirmed through proton and fluorine NMR spectroscopy, as well as by ESI-MS, and its distribution across polymer chains of all molar masses was confirmed by size exclusion chromatography (SEC) with UV detection (see the Supporting Information).



Scheme 2. Design of gemcitabine–polyisoprene conjugate nanoparticles. Reaction conditions: a) PyBOP, DIPEA, DMF, 25 °C, 24 h. b) Isoprene, pyridine, 115 °C, 2–16 h. c) Nanoprecipitation (THF/H_2O, 1:2). DIPEA = diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, PyBOP = benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate.

Table 1: Characterization of gemcitabine–polyisoprene conjugates and nanoparticles.

4	M_n[a] [g mol^{-1}]	$Đ$[a]	size[b] [nm]	PSD[b]	ζ[c] [mV]	% Gem[d] [wt.%]
4a	840	1.35	159 ± 4	0.10 ± 1	−77 ± 3	31.2
4b	1190	1.29	137 ± 3	0.10 ± 2	−70 ± 5	22.1
4c	1560	1.28	133 ± 4	0.11 ± 2	−66 ± 5	16.9
4d	2510	1.40	138 ± 1	0.11 ± 1	−68 ± 4	10.5

[a] Determined by SEC, calibrated with PS standards and converted into PI by using Mark-Houwink-Sakurada parameters.[23] [b] Determined by DLS. [c] Zeta potential. [d] %Gem = $MW_{Gem}/M_{n,PI}$.

Owing to the amphiphilic nature of the Gem–PI conjugates, the corresponding nanoparticles were prepared by self-assembly from a THF solution of Gem-PI in water, with subsequent evaporation of THF in vacuo. The resulting suspensions (2.5 mg mL^{-1}) contained nanoparticles of 130–160 nm in diameter, which is in the suitable window for drug delivery through intravenous administration, with narrow particle-size distributions (PSD = ca. 0.1) and remarkable colloidal stability of up to one month. Some dependence of nanoparticle size on molar mass was observed, with the PI having the lowest molar mass giving consistently larger nanoparticles than the other samples (see the Supporting Information, Figure S9a and b). Surface charges of the particles ranged from −66 to −77 mV, thus indicating significant electrostatic stabilization of the nanoparticles, and were correlated with the nanoparticle sizes (see the Supporting Information, Figure S9c and d). Nanoparticle sizes, size distributions, and zeta potentials were reproducible, with three independent preparations giving nearly identical results for each sample. A small library of nonfunctionalized PI nanoparticles with similar macromolecular and colloidal characteristics was also prepared under identical experimental conditions (see the Supporting Information, **5a–e** in Tables S2 and S6, and Figure S5,). Gem–PI and PI nanoparticles were further characterized by cryogenic transmission electron microscopy (Figure 1) and showed spherical morphologies and colloidal characteristics that are in good agreement with dynamic light scattering (DLS) data. Importantly, this approach allowed the production of nanoparticles

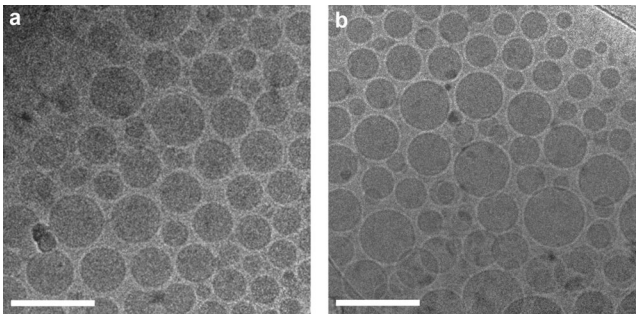


Figure 1. Cryogenic transmission electron microscopy of Gem–PI **4d** (a) and PI **5d** (b) nanoparticles. Scale bars = 200 nm.

with very high drug contents, ranging from 10.5 wt. % (**4d**) to 31.2 wt. % (**4a**; Table 1). This result is a significant improvement on classic drug-loaded nanoparticles, which usually carry drug payloads of only a few percent.

The Gem–PI conjugate nanoparticles were then tested for their in vitro activity by measuring the half maximal inhibitory concentration (IC_{50}) of cell proliferation on four cancer cell lines: 1) murine leukemia (L1210), 2) human leukemia (CCRF-CEM), 3) human pancreatic cancer (MiaPaCa-2), and 4) human lung carcinoma (A549). Gem–PI nanoparticles **4a**–**4d** showed significant anticancer activity on all tested cell lines (Table 2), whereas a control series of nonfunctionalized

Table 2: Anticancer activity of gemcitabine–polyisoprene conjugate nanoparticles after 72 h of incubation (expressed as $IC_{50} \pm$ SD in nM).[a]

Cell line	**4a**	**4b**	**4c**	**4d**	Gem
MiaPaCa-2	810 ± 82	568 ± 53	169 ± 7	186 ± 11	36 ± 4
L1210	659 ± 5	358 ± 9	330 ± 18	252 ± 8	14 ± 1
CCRF-CEM	232 ± 20	144 ± 1	84 ± 2	91 ± 3	6 ± 1
A549	303 ± 8	216 ± 7	104 ± 5	87 ± 1	13 ± 1

[a] Determined by cell viability assay (MTT test).

PI nanoparticles of similar molar masses were inactive (see the Supporting Information, Table S7). As expected owing to their prodrug nature, all nanoparticles showed lower cytotoxicity than free Gem while their IC_{50} values remained in the nanomolar range. Interestingly, as the controlled chain growth performed by the NMP process allowed for the fine-tuning of the polymer chain length, a preliminary structure–activity relationship, which is of high importance in the rational design of a drug delivery system, can be extracted from these results. For all tested cell lines, the higher the M_n of the Gem–PI conjugate, the greater the anticancer activity of the corresponding nanoparticles. This trend may be correlated with the surface hydrophobicity of the Gem–PI nanoparticles (which can be readily adjusted due to the employed LRP process), leading to a higher rate of endocytosis owing to opsonin adsorption when the PI chain length is increased.[26]

The in vivo anticancer activity of these novel Gem–PI polymer prodrug nanoparticles was then investigated against the human pancreatic (MiaPaCa-2) carcinoma xenograft model in mice after intravenous injections (on days 0, 4, 8, and 12) of Gem at 7 mg kg^{-1}, Gem–PI nanoparticles (at 7 mg kg^{-1} Gem-equivalent dose), or nonfunctionalized PI nanoparticles of similar chain lengths (Figure 2a). Untreated mice (saline 0.9 %) exhibited a rapid tumor growth, with

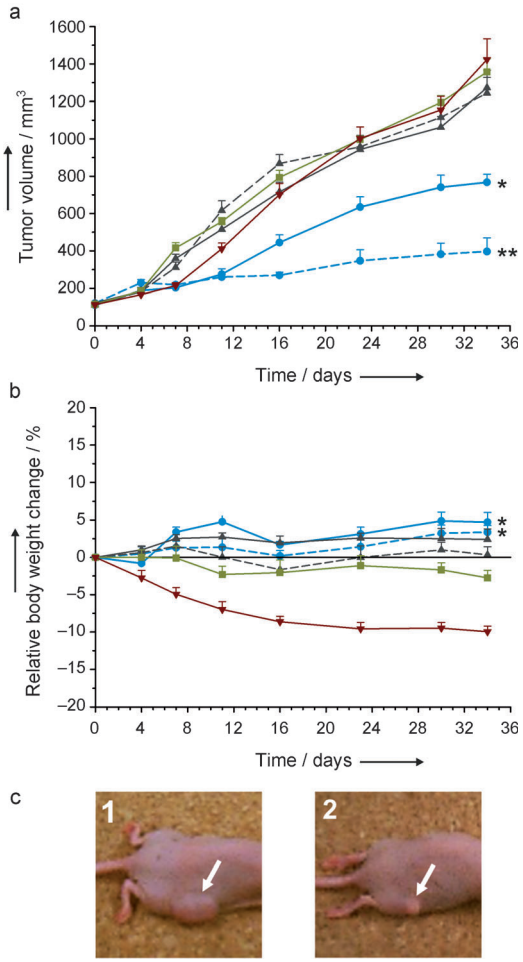


Figure 2. In vivo anticancer activity of gemcitabine (–▼–, 7 mg kg^{-1}), polyisoprene–gemcitabine nanoparticles (**4b** –●–; **4d** –●–; 7 mg kg^{-1} Gem-equivalent dose), control (saline 0.9 % –■–), and polyisoprene nanoparticles (**5a** –▲–; **5d** –▲–; same dose of polymer as **4d**) after intravenous treatment (on days 0, 4, 8, and 12) of mice bearing MiaPaCa-2 subcutaneous tumors: a) tumor progression as a function of time and b) relative body weight change as a function of time. The values are the mean ± SD ($n = 6$). Statistical differences between Gem- and Gem–PI-treated groups (**4b** or **4d**) with confidence levels of > 95 % (Student's *t*-test with Bonferroni correction for multiple comparisons) are marked by * ($p < 0.025$) or ** ($p < 0.005$). c) White arrows point to the position of the implanted tumor on a representative mouse at the end point for the Gem-treated group (1) and Gem–PI **4d** treated group (2).

tumor volumes of approximately 1350 mm^3 at day 34. Mice treated with Gem or nonfunctionalized PI nanoparticles (**5a**, **5e**) showed a similar pattern, with equivalent tumor volumes at the end of the treatment, thus demonstrating the absence of anticancer activity of Gem in this model. In contrast, treatment of mice with Gem–PI nanoparticles **4b** at the equivalent Gem dose led to a considerable decrease in the tumor

progression. Even higher anticancer activity was obtained with Gem–PI nanoparticles **4d**, with tumor growth inhibition as high as 72% (compared to 46% for **4b**) and a tumor growth that plateaued after 23 days of treatment. As already observed in the cell culture experiments, nanoparticles obtained from the higher molar mass Gem-PI conjugate **4d** demonstrated greater in vivo anticancer activity than their lower molar mass counterparts (**4b**). This finding is of crucial importance as it shows that in vitro cytotoxicity trends for the polymer–drug conjugates can be extrapolated to in vivo experiments.

The relative body-weight loss was also monitored throughout the treatment (Figure 2b). Importantly, Gem-treated mice exhibited significant weight loss (approximately −10%) compared to those that received the control injections; this result highlights the toxicity that arises from the free-drug treatment. By contrast, the Gem–PI-treated mice maintained or slightly increased their body weight (approximately +3%), a result that supports both the efficient anticancer activity of Gem–PI polymer prodrug nanoparticles and the disappearance of Gem-related adverse effects.

In summary, the design of a new class of drug–polymer conjugate prodrug nanoparticles has been reported. The strategy involves the controlled growth of PI by NMP from a gemcitabine-functionalized macroalkoxyamine initiator based on the SG1 control agent (a nitroxide that is non-cytotoxic even at very high doses),[27] and the self-assembly of the resulting amphiphilic species into stable, narrowly dispersed nanoparticles of 130–160 nm in diameter. These nanoconstructs exhibit efficient anticancer activity both in vitro, on various cancer cell lines, and in vivo, on human pancreatic carcinoma-bearing mice, while suppressing the inherent toxicity of the employed chemotherapeutics. The synthetic pathway, which can be applied to virtually any LRP technique, is robust and very versatile as it only requires: 1) the use of hydrophilic/polar drugs; 2) suitable drug functionalization, and 3) the controlled growth of a hydrophobic polymer. As a consequence, this method is easy to carry out and can allow for the design of a broad range of drug–polymer conjugates with high drug payloads, thus leading to nanoparticles with more potent biological activities. Furthermore, the use of a controlled/living radical process allows for fine-tuning of the polymer chain length and therefore for the determination of structure–activity relationships to design optimized drug delivery systems.

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