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Therapeutic Nanoreactors: Combining Chemistry and Biology in a Novel Triblock Copolymer Drug Delivery System

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ABSTRACT

Triblock copolymeric nanoreactors are introduced as an alternative for liposomes as encapsulating carrier for prodrug activating enzymes. Inosine–adenosine–guanosine preferring nucleoside hydrolase of Trypanosoma vivax, a potential prodrug activating enzyme, was encapsulated in nanometer-sized vesicles constructed of poly(2-methyloxazoline)-*block*-poly(dimethylsiloxane)-*block*-(2-methyloxazoline) triblock copolymers. The nanoreactor is functionalized by incorporation of bacterial porins, OmpF or Tsx, in the reactor wall. Efficient cleavage of three natural substrates and one prodrug, 2-fluoroadenosine, by the nanoreactors was demonstrated.

Currently chemotherapy is one of the major strategies to treat cancer patients. Despite its success, it is limited by several drawbacks such as low bioavailability of the chemotoxin, low drug concentrations at the tumor site, systemic toxicity, lack of specificity, and the appearance of drug-resistant tumors. To overcome these problems many different strategies have been developed including improved drug formulations (e.g., liposomes),[1] resistance modulators,[2] and antidote/toxicity modifiers.[3] Selective local enzymatic activation of prodrugs is also a possibility to increase drug concentrations in the tumor and decrease systemic toxicity,[4] thus improving the therapeutic index. Unfortunately human tumors rarely express useful activating enzymes at high concentrations. Therefore exogenous enzymes need to be used and directed to the tumor.

Directed enzyme−prodrug therapies involve two stages. In the first step the activating enzyme is directed to the tumor. In the second step the nontoxic prodrug is systemically administered and subsequently converted in high local concentrations of an anticancer drug by the enzyme at the tumor site. The targeting of the enzyme can either be mediated by antibodies, termed antibody−directed enzyme−prodrug therapy (ADEPT) or by a gene-vector, termed gene−directed enzyme−prodrug therapy (GDEPT). Both ADEPT and GDEPT suffer from the same shortcomings. First, most activating enzymes are immunogenic. Second, the efficient targeting remains an obstacle, and, finally, most prodrugs are also activated by endogenous enzymes.

Several strategies have been proposed to decrease the immunogenicity of the activating enzyme such as antibody-directed abzyme prodrug therapy (ADAPT).[5] Abzymes are catalytic antibodies that are raised against a stable transition state analogue and can be humanized to reduce immunogenicity. Their catalytic activity however is usually 1000-fold lower compared to enzymes that catalyze the same reaction.[6] Another way to avoid immunogenicity is polymer−directed enzyme−prodrug therapy (PDEPT).[7] In this approach a polymer−drug conjugate is injected first and accumulates in the tumor tissue by a mechanism called enhanced permeability and retention effect (EPR).[8] Afterward an enzyme−polymer conjugate is injected that activates the prodrug at the tumor site. The polymer shields the enzyme from its environment, thereby reducing immunogenicity. Unfortunately, syntheses of polymer−enzyme conjugates typically have a low yield and reduced activity of the enzyme.[9]

We recently proposed a novel strategy in the form of therapeutic nanoreactors to avoid immunogenicity without loss of enzyme activity.[10] In this scheme the enzyme is

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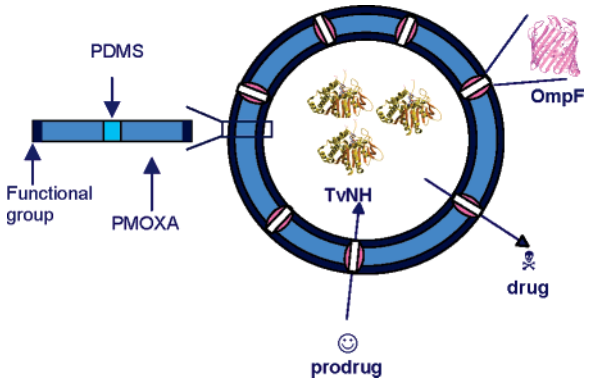

Figure 1. Schematic representation of a completely functionalized nanoreactor build up of poly(2-methyloxazoline)-*block*-poly(dimethylsiloxane)-*block*-(2-methyloxazoline) (PMOXA−PDMS−PMOXA), permeabilized by the bacterial outer membrane protein OmpF and encapsulated with *Trypanosoma vivax* nucleoside hydrolase (TvNH).

shielded from the immune system by encapsulation in liposomes. To make these liposomes functional, they were permeabilized by channel-forming proteins to allow diffusion of the substrate and product but not the enzyme through the reactor wall. Unfortunately, these reactors showed some uncontrollable characteristics, potentially leading to immunogenicity of these liposomes. Furthermore, liposome-based carriers are unstable in blood serum and need to be grafted with poly(ethylene glycol) (PEG) or other polymers to increase the average circulation time in the body.[11]

In this study we introduce a more promising kind of nanoreactor in which the reactor wall is composed of the amphiphilic ABA triblock copolymer, poly(2-methyloxazoline)-*block*-poly(dimethylsiloxane)-*block*-(2-methyloxazoline) (PMOXA−PDMS−PMOXA) (Figure 1). This copolymer has an average molecular weight of 8660 g/mol and self-assembles to form stable vesicular structures in aqueous solutions.[12] Containers with controlled mean diameters of 200 nm can be obtained by successive extrusion. Vesicles made of this triblock copolymer are more stable and less permeable, especially in dilute solutions, compared to liposomes.[13] This is due to the higher length of the hydrophobic block of the polymer, slower dynamics, and intermolecular steric stabilization. Furthermore, these vesicles are completely covered with PMOXA that has stealth properties similar to PEG. Stealth properties allow vesicles to escape clearance by the immune system because of low adsorption of plasma proteins and low hepatosplenic uptake.[14] This results in longer blood circulation times. Also, nonspecific uptake of nonpermeabilized PMOXA−PDMS−PMOXA nanocontainers by COS-7 cells and THP-1-derived macrophages is completely absent in vitro,[15] which makes these nanocontainers biocompatible and good candidates for in vivo use.

Despite a polymeric membrane thickness of 10 nm, three times wider than a biological lipid membrane, channel-forming proteins have previously been incorporated in PMOXA−PDMS−PMOXA vesicles without loss of function.[16] This is probably due to the high flexibility of the hydrophobic PDMS block and the presence of shorter

Table 1. Kinetic Parameters of TvNH for Natural Substrates and Prodrugs

	k_{cat} (s^{-1})	K_M (μM)
inosine	5.19 ± 0.08	5.37 ± 0.42
adenosine	2.6 ± 0.2	8 ± 1.8
guanosine	2.31 ± 0.11	2.33 ± 0.47
2-fluoroadenosine	1.86 ± 0.11	39.05 ± 7.99
2-chloroadenosine	1.41 ± 0.05	4.56 ± 0.91
6-methylpurine riboside	4.34 ± 0.13	<10

polymers which segregate around the protein. A recent mean field analysis also indicated that protein incorporation only causes a minor energy penalty in the polymeric membrane.[17] We permeabilized our nanoreactors by incorporation of two different bacterial outer membranes, channel forming proteins, called porines (OmpF and Tsx) in the vesicle wall. OmpF forms a 16-stranded transmembrane β-barrel that functions as a molecular sieve, allowing concentration-driven diffusion of solutes <600 Da.[18] Tsx on the other hand forms a 12-stranded β-barrel and allows specific transport of nucleosides and nucleotides.[19] Since it has a binding site for nucleosides in the interior of the channel, rapid transport of nucleosides at very low concentrations is possible compared to slow diffusion through the nonspecific porine OmpF. By permeabilizing the nanoreactors with OmpF or Tsx, small substrates can be transported across the vesicle membrane to reach the interior where they are activated by the enzyme. Subsequently products can diffuse out of the vesicle to the exterior.

As a proof of principle for our system, we chose the purine-specific nucleoside hydrolase of *Trypanosoma vivax* (TvNH) as prodrug activating enzyme. This enzyme is a member of the nucleoside hydrolase superfamily that catalyses the hydrolysis of the *N*-glycosidic bond of β-ribonucleoside forming the free nucleic base and ribose.[20] These enzymes are widely distributed in nature, but they are not present in mammals. Since TvNH is purine specific, its natural substrates are inosine, adenosine, and guanosine. Crystallographic data on the *T. vivax* enzyme showed that the ribose is tightly bound to the enzyme with all its hydroxyl groups involved in multiple stereoselective H-bonds.[21] This makes the enzyme highly specific toward the ribose moiety. The nucleic base in contrast is stacked between two tryptophanes residues and forms very few specific interactions with the enzyme. Consequently TvNH is less specific toward the nucleic base moiety. This feature makes TvNH a promising candidate for enzyme−prodrug strategies since many known chemotoxins are nucleobase analogues.[22,23] To explore this possibility, the activity of TvNH for three known prodrugs, 2-fluoroadenosine, 2-chloroadenosine, and 6-methylpurine riboside was tested (Table 1). These prodrugs are efficiently hydrolyzed to their cytotoxic base with the same efficiency as the natural substrates.

To produce permeabilized nanoreactors, a 1% (w/v) polymer−ethanol solution was mixed with water-solubilized porins, purificated according to Prilipov et al.[24] Typically 250 μL of polymer−ethanol solution was mixed with porine solution to give a final porine concentration of 0.01 or 0.1

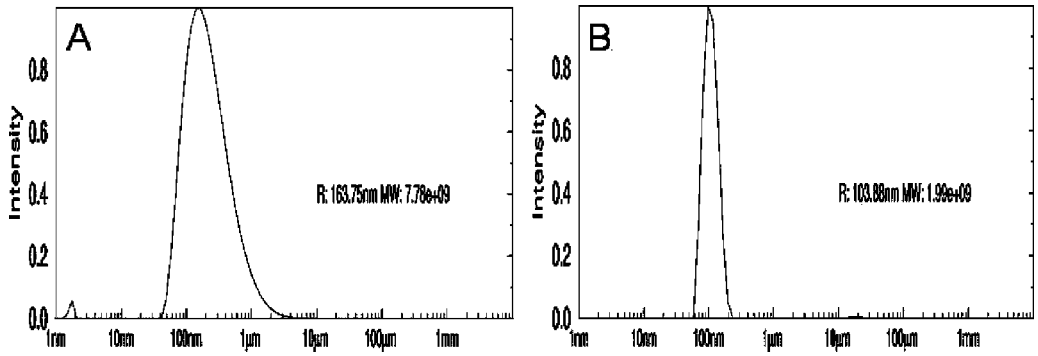

Figure 2. Analysis of the size distributions of functionalized nanoreactors by DLS before extrusion (A) and after extrusion (B). Measurements were carried out at 90° and 532 nm on a laser-spectroscatter 201 by RiNa GmbH, Berlin, Germany.

μg/μL. This results in a molecular ratio of porine to polymer of 1:100 and 1:10, respectively. Since a vesicle with a diameter of about 200 nm contains about 13000 triblock copolymer molecules, this results in 130 to 1300 porine molecules per vesicle.[25] The solution was dried to produce a lamellar polymer/porine film. This film was subsequently rehydrated for several hours under continuous stirring in 20 mM Hepes, pH 7.0, containing $\pm$50 μM of the prodrug activating enzyme TvNH. This resulted in aggregates with a mean diameter of 326 nm. After successive extrusion through a polycarbonate filter with a pore diameter of 200 nm, nanoreactors with a mean diameter of 200 nm were obtained (Figure 2). Nonencapsulated prodrug activating TvNH was removed either by trapping TvNH on a Ni−Nta affinity column (Amersham Biosciences) or by gel filtration (Sepharose 4B medium, Pharmacia). After each step the samples were analyzed by dynamic light scattering (DLS) to determine the size of the nanoreactors and the polydispersity of the sample (laser-spectroscatter 201 by RiNA GmbH, Berlin, Germany). Purification by gel filtration resulted in monodisperse nanoreactors.

Since TvNH is deactivated by detergents, it was impossible to determine the encapsulated enzyme concentration by dissolving the nanoreactor and measuring the enzyme activity. Therefore the efficiency of encapsulation was determined by comparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Known quantities of free TvNH (0.1−3 μg) were run on an SDS-PAGE together with an aliquot of nanoreactor sample (Figure 3). To ensure complete disruption of the nanoreactors, 0.5% TritonX-100 was added to the sample. The intensity of each coomassie blue stained TvNH band was measured with Intelligent Quantifier software (Bio Image Systems Inc., Jackson, MI) to determine the quantity of TvNH in the nanoreactor sample. A standard curve of band intensity versus known amount of TvNH was calculated and used to determine the quantity of TvNH in the nanoreactor sample. This leads to an estimated encapsulation efficiency of 15% and an estimated enzyme concentration of 47 μM inside each vesicle. Two protein bands are visible in the nanoreactor sample corresponding to TvNH and porine. The porine band however is running at a higher molecular weight than the purified porine, probably due to strong interaction between polymer and

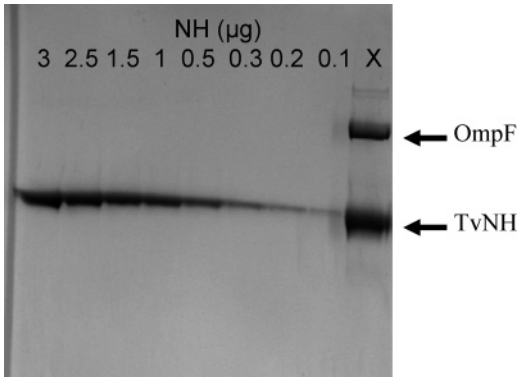

Figure 3. SDS-PAGE analysis for determination of the encapsulation efficiency of TvNH. Free TvNH (0.1−3 μg) was run together with an aliquot of nanoreactor sample (X).

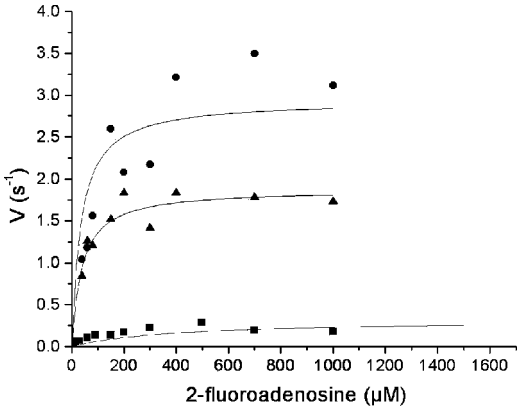

Figure 4. Product formation rate of nanoreactors permeabilized with ratios 1:100 OmpF (■) or Tsx (▲) to polymer and ratio 1:10 OmpF (●) to polymer in function of 2-fluoroadenosine concentration. The data were fitted to a hyperbolic curve.

porine. Therefore we could not use comparative SDS-PAGE analysis to determine the amount of incorporated porine.

The enzymatic activity of the nanoreactors for three natural substrates, inosine, adenosine, and guanosine, and one prodrug, 2-fluoroadenosine, was determined using a reducing sugar assay as described by Parkin.[26] Briefly, the enzymatic reaction was stopped by adding a $CuSO_4$ solution. The Cu^{2+} is reduced to Cu^{+} by the reaction product ribose. This reduced Cu reacts with neocuproin to form a complex. Color development of this complex was achieved by heating the solution 8 min at 95 °C and the optical density at 450 nm

Table 2. Kinetic Properties of Nanoreactors Permeabilized with OmpF or Tsx

		inosine	adenosine	guanosine	2-fluoroadenosine
(TvNH) free	k_{cat} (s^{-1})	5.19 ± 0.08	2.6 ± 0.2	2.31 ± 0.11	1.86 ± 0.11
	K_M (μM)	5.37 ± 0.42	8 ± 1.8	2.33 ± 0.47	39.05 ± 7.99
1/100 OmpF nanorectors	$(k_{cat})_{app}$ (s^{-1})	2.28 ± 0.05	2.15 ± 0.12	4.26 ± 0.19	0.32 ± 0.05
	$(K_M)_{app}$ (μM)	356 ± 23	602 ± 71	582 ± 57	396 ± 169
1/10 OmpF nanoreactors	$(k_{cat})_{app}$ (s^{-1})	37.46 ± 1.88	12.13 ± 0.76	52.77 ± 3.10	2.94 ± 0.34
	$(K_M)_{app}$ (μM)	84 ± 15	41 ± 12	125 ± 23	35 ± 19
1/100 Tsx nanoreactors	$(k_{cat})_{app}$ (s^{-1})	24.09 ± 1.48	19.02 ± 1.47	21.48 ± 1.12	1.88 ± 0.10
	$(K_M)_{app}$ (μM)	80 ± 18	24 ± 17	100 ± 18	38 ± 10

was measured. A standard curve with known ribose concentration was used to determine the extinction coefficient under the assay conditions. First of all the activity of nonpermeabilized nanoreactors with encapsulated TvNH was measured, data not shown. These nanoreactors showed no activity at all which is an improvement compared to the liposomal reactors. For the permeabilized nanoreactors, the rate of product formation, V, was determined for various substrate concentrations (0−1000 μM) and for porine ratios 1:100 and 1:10 OmpF and 1:100 Tsx. These rates were fitted to a hyperbolic curve to determine apparent kinetic constants (Figure 4 and Table 2). For fitting purposes, 10 individual points were measured per experiment. The $(K_M)_{app}$ value corresponds to the exterior substrate concentration at which the rate of product formation (V) is half its maximal value (V_{max}). V_{max} divided by the total enzyme concentration equals $(k_{cat})_{app}$.

In all cases we saw that $(K_M)_{app} > K_{M,enzyme}$. This observation indicates that the substrate concentration is higher outside the nanoreactor as compared to inside. Furthermore, the activity of the nanoreactors is a function of the porine-to-polymer ratio in the reactor wall. When this ratio increases 10 times (from 1:100 to 1:10) for OmpF permeabilized nanoreactors, the $(K_M)_{app}$ and $(k_{cat})_{app}$ also increase approximately 10 times. This observation is also seen when Tsx, a nucleoside-specific transporter, is used. All data indicate that the $(k_{cat})_{app}$ value is correlated to the amount and the nature of the porine used. It thus appears that the activity of the nanoreactors is limited by transport at low porine concentrations. At higher porine concentrations or when the specific transporter Tsx is used, the $(k_{cat})_{app}$ value of the nanoreactors is higher than that of the free enzyme. This might indicate that under these conditions, the activity of the nanoreactors is no longer limited by transport. The exact reason for this exceptionally high activity of the nanoreactors is as yet not understood.

In this study we propose a new therapeutic tool based on nanoreactors that are composed of PMOXA−PDMS−PMOXA triblock copolymers. These nanoreactors were functionalized by encapsulating the prodrug activating enzyme TvNH and permeabilising the reactor wall with bacterial membrane porines. We demonstrated that these nanoreactors can efficiently hydrolyze different substrates including the prodrug 2-fluoroadenosine, resulting in the release of the cytotoxic molecule, 2-fluoroadenine. To further optimize these nanoreactors, it is important to know the different diffusion rates through OmpF and Tsx so the interior substrate concentration can be determined. Therefore diffusion constants for the different substrates will be measured in the future. A second step will be the targeting of the nanoreactors to different tumors by using camel antibodies,[27] ligands, or adhesins.

Evidently, these nanoreactors are flexible systems that can be used with different enzyme−substrate combinations and targeted to different tumor tissues or organs. Depending on the functionalities of the nanoreactors, they could be applied in fields other than cancer therapy such as gene, RNAi or drug delivery, diagnostics and in vivo imaging.

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