Assessment of stability, toxicity and immunogenicity of new polymeric nanoreactors for use in enzyme replacement therapy of MNGIE

De Vocht, Caroline; Ranquin, An; Willaert, Ronnie; Van Ginderachter, Jo; Vanhaecke, Tamara; Rogiers, Vera; Versees, Wim; Van Gelder, Patrick; Steyaert, Jan

Published in:
Journal of Controlled Release

Publication date:
2009

Document Version:
Final published version

Link to publication

Citation for published version (APA):
Assessment of stability, toxicity and immunogenicity of new polymeric nanoreactors for use in enzyme replacement therapy of MNGIE

Caroline De Vocht a,c,⁎, An Ranquin a,c, Ronnie Willaert a,c, Jo A. Van Ginderachter b,c, Tamara Vanhaecke d, Vera Rogiers d, Wim Versées a,c, Patrick Van Gelder a,c, Jan Steyaert a,c,⁎

⁎ Structural Biology Brussels, Vrije Universiteit Brussel (VUB), 1050 Brussels (Elsene), Belgium
b Laboratory of Cellular and Molecular Immunology, Vrije Universiteit Brussel (VUB), 1050 Brussels (Elsene), Belgium
b,b Laboratory of Cellular and Molecular Immunology, Vrije Universiteit Brussel (VUB), 1050 Brussels (Elsene), Belgium
c Department of Molecular and Cellular Interactions, VIB, 1050 Brussels (Elsene), Belgium
c,c Laboratory of Cellular and Molecular Immunology, Vrije Universiteit Brussel (VUB), 1050 Brussels (Elsene), Belgium
b Department of Toxicology, Vrije Universiteit Brussel (VUB), 1090 Brussels (Jette), Belgium

A R T I C L E   I N F O

Article history:
Received 23 December 2008
Accepted 30 March 2009
Available online 14 April 2009

Keywords:
Nanoreactor
Enzyme replacement therapy
MNGIE
Drug delivery
Polymeric nanoparticles

A B S T R A C T

The lack of a crucial metabolic enzyme can lead to accumulating substrate concentrations in the bloodstream and severe human enzyme deficiency diseases. Mitochondrial Neurogastrointestinal Encephalomyopathy (MNGIE) is such a fatal genetic disorder, caused by a thymidine phosphorylase deficiency. Enzyme replacement therapy is a strategy where the deficient enzyme is administered intravenously in order to decrease the toxic substrate concentrations. Such a therapy is however not very efficient due to the fast elimination of the native enzyme from the circulation. In this study we evaluate the potential of using polymeric enzyme-loaded nanoparticles to improve the delivery of therapeutic enzymes. We constructed new 200-nanometer PMOXA-PDMS-PMOXA polymeric nanoparticles that encapsulate the enzyme thymidine phosphorylase. These particles are permeabilised for substrates and products by the reconstitution of the nucleoside-specific porin Tsx in their polymeric wall. We show that the obtained ‘nanoreactors’ are enzymatically active and stable in blood serum at 37 °C. Moreover, they do not provoke cytotoxicity when incubated with hepatocytes for 4 days, nor do they induce a macrophage-mediated inflammatory response ex vivo and in vivo. All data highlight the potential of such nanoreactors for their application in enzyme replacement therapy of MNGIE.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Many known genetic deficiencies in human (like Gaucher disease [1], Pompe disease [2], Fabry disease [3]) are caused by a mutation in a gene encoding a ubiquitous enzyme, leading to the accumulation of its substrates. As a result, toxic amounts of the substrates appear in the bloodstream, disturbing the normal metabolic degradation pathways.

Mitochondrial Neurogastrointestinal Encephalomyopathy (MNGIE) is such a unique autosomal-recessive disorder associated with mitochondrial DNA alterations [4]. The disease is characterised clinically by ptosis, ophthalmoparesis, gastrointestinal dysmotility, cachexia and mitochondrial DNA alterations [4]. The disease is characterised clinically by ptosis, ophthalmoparesis, gastrointestinal dysmotility, cachexia and mitochondrial DNA alterations [4]. In 1998, Nishino et al. mapped the disease locus and identified thymidine phosphorylase (TP) as the causative gene [6]. Loss-of-function mutations in the TP gene lead to systemic accumulations of the substrates thymidine and deoxyuridine [7]. Current therapies for MNGIE are still in the developmental stage and include platelet infusion [8], gene therapy and allogeneic stem cell transplantation [9]. Unfortunately, these treatments are far from ideal and suffer from the lack of sustained TP activity and serious safety risks [10]. A more reliable and generally well-tolerated strategy to treat enzyme deficiencies is enzyme replacement therapy [11,12].

In enzyme replacement therapy the deficient enzyme is administered intravenously in its native or stabilized form [13]. The first-generation pharmaceuticals were proteins with an amino acid sequence identical to that of the native human enzyme, but suffered from fast elimination from the circulation [14]. To improve their stability, proteolytic resistance, immunogenicity and circulation half-life, therapeutic proteins can be modified by covalently linking various poly(ethylene glycol) (PEG) molecules to their surface, a technique called PEGylation [15]. Although widely used, PEGylation is a rather complicated and expensive approach [16]. In particular when the therapeutic protein is an enzyme, there is also a serious risk of a decrease or a complete loss of enzyme activity [17].

A more convenient strategy to deliver therapeutic enzymes to the body is to encapsulate them in appropriate carrier systems [18]. In a first attempt, proteins were trapped in liposomes [19]. Unfortunately, these carrier systems suffer from rapid leakage of the protein, poor stability and fast clearance by the mononuclear phagocyte system [20,21]. By grafting PEG on the liposome surface, a new generation of
long-circulating or Stealth™ liposomes was introduced [22]. However, fast drug leakage and the accelerated blood clearance [23] remain major drawbacks of these lipidic delivery systems. In order to obtain more robust membranes with controllable properties, extensive efforts were made in the last decade to design polymeric vesicles [24–27]. The use of polymeric particles in enzyme replacement therapy was demonstrated by Genta et al., in 2001 [28].

In this study, we evaluate polymeric nanometer-sized bioreactors, so-called nanoreactors, for future use in enzyme replacement therapy of MNGIE (Fig. 1). The therapeutic enzyme, thymidine phosphorylase, is encapsulated in polymeric particles constructed of the amphiphilic triblock copolymer PMOXA-PDMS-PMOXA (poly(2-methyloxazoline)-block-poly(dimethylsiloxane)-block-poly(2-methyloxazoline)) [29]. The nanoparticles are permeabilised for substrates and products by integrating bacterial channel proteins in their polymeric wall, a strategy introduced by Wolfgang Meier and co-workers [30,31]. The nucleoside-specific porin Tsx was selected as channel-forming protein for the nanoreactors, since it contains specific nucleoside binding sites [32]. In this way, the Tsx porin allows the efficient transport of the substrates (thymidine and deoxyuridine) and products (thymine and uracil) through the capsule wall. This results in reactors where the enzymatic reaction is restricted to the compartmentalised inner volume of the polymeric nanocarrier. We chose PMOXA as hydrophilic outer block for the nanoreactors because this polymer possesses protein-repellent and stealth properties, shielding the encapsulated enzyme from aggression by external agents [33,34]. Recently, other variants of this nanoreactor-technology were exploited for various biomedical applications like prodrug cancer therapy [35], prevention of heart attack [36], biosensors [37] and biotransformations [38].

Most of the aforementioned studies start from the assumption that the PMOXA-PDMS-PMOXA nanoparticles are more stable than liposomes [29] and it is often implied that those particles are non-immunogenic and inert towards macrophages [36,39–41]. However, until now systematic analysis to unequivocally support these claims is lacking. Therefore this study not only focuses on the construction of new TP containing nanoreactors, but also on the investigation of their stability and their cellular responses on hepatocytes and macrophages. Hence, this work is of high relevance for the further exploitation of the PMOXA-PDMS-PMOXA particles as drug delivery devices.

2. Materials and methods

2.1. Purification of the enzyme and the porin

The E. coli C thymidine phosphorylase (TP$_{E. coli}$) open reading frame was cloned into the HindIII–BamHI restriction sites of the pQE30 vector (Qiagen) and expressed in E. coli WK6 cells. Overnight cultures were used to inoculate baffled flasks with Terrific Broth and 50 mg/ml ampicillin. They were incubated at 37 °C while shaking until the cells reached an A$_{600}$ between 0.6 and 0.8. The medium was then cooled to 28 °C and expression of the recombinant protein was induced by adding IPTG (0.5 mM final concentration). The next morning cells were harvested and resuspended in 20 mM Tris pH 7.5 with 1 M NaCl and 10 mM imidazole and the protease inhibitors leupeptin (1 µg/ml) and 4- (2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (0.1 mg/ml) were added. After lysis with an EmulsiFlex-C5 High Pressure Homogenizer (Avestin, Inc.), the presence of an N-terminal His$_{6}$-tag allows a two-step purification scheme, consisting of a Ni-NTA affinity chromatography (Qiagen) followed by a gel filtration on a Superdex 200 16/90 column (Amersham Biosciences).

Tsx was cloned into a pGOmpF vector, which was described by Prilipov et al. [42]. In addition to the Tsx gene, an N-terminal His$_{6}$-tag and a thrombin cleavage site were also introduced. A BL21(DE3) ΔlamB ompR ΔompA strain was used for overproduction of the protein [42]. The Tsx porin was purified from the bacterial membrane by detergent extraction using octylpolyoxyethylene, followed by a Ni-NTA affinity chromatography (Qiagen) and a gel filtration on a Superdex 200 26/90 column (Amersham Biosciences).

2.2. Polymer

Poly(2-methyloxazoline)-block-poly(dimethylsiloxane)-block-poly(2-methyloxazoline) (PMOXA$_{20}$-PDMS$_{54}$-PMOXA$_{20}$) triblock copolymers were purchased from Polymer Source Inc. (Montreal, Canada) and have a molecular weight of 7484 g/mol.

---

**Fig. 1.** Schematic representation of a thymidine phosphorylase encapsulating nanoreactor. The capsule of the reactor consists of the amphiphilic triblock copolymer PMOXA-PDMS-PMOXA ($m = 20$, $n = 54$) and is permeabilised by the bacterial membrane protein Tsx. In this way, efficient transport of the substrates (thymidine and deoxyuridine) and products (thymine and uracil) through the capsule is feasible.
2.3. Preparation of TP containing nanoreactors

The thymidine phosphorylase encapsulating nanoreactors (TP-NRs) were constructed via the ethanol method as described previously [43]. Briefly, 20 mg of PMOXA-PDMS-PMOXA polymer was dissolved in 300 µl ethanol and Txs was added at the desired molecular ratio of protein to polymer (100 µg porin/10 mg polymer). This solution was added dropwise to a phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.7 mM KH2PO4, pH 7.4; analytical grade purchased from Sigma) solution containing 100 µM of *Escherichia coli* thymidine phosphorylase (TP$_{E.coli}$) and stirred for 48 h at 4 °C, resulting in self-assembled nanoreactors. If necessary, successive extrusion through a filter with pore diameter of 200 nm was performed. Since the TP$_{E.coli}$ contains an N-terminal His$_6$-tag, non-encapsulated enzyme was removed by Ni-NTA affinity chromatography. After each step, the samples were analysed by dynamic light scattering (DLS) to determine the size and polydispersity of the nanoreactors.

2.4. Size and polydispersity measurements

The size and polydispersity of the nanoreactor samples was determined via Dynamic Light Scattering (DLS) (Spectroscatcer 201, RiNA GmbH, Berlin) at 532 nm with a scattering angle of 90 °C. Ten data sets were recorded and the mean size distribution was analyzed using the CONTIN software.

2.5. Spectrophotometric assay of thymidine phosphorylase activity

The concentrations of thymidine phosphorylase and thymidine stock solutions were determined using the following extinction coefficients: $\varepsilon_{280} = 24.41$ M$^{-1}$ cm$^{-1}$ for TP$_{E.coli}$, and $\varepsilon_{267} = 9.65$ M$^{-1}$ cm$^{-1}$ for thymidine [44]. All kinetic experiments were performed in 200 mM potassium phosphate pH6.8 at 37 °C. Product formation was determined spectrophotometrically using the difference in absorption between the substrate thymidine and the product thymine ($\Delta$A$_{290}$ nm = $-1$ M$^{-1}$ cm$^{-1}$). 900 µM thymidine (Sigma) was mixed with 20 µl nanoreactors and the decrease in absorption was measured at 290 nm using a Cary 100Bio UV visible spectrophotometer (Varian).

2.6. Stability study

Stability studies were carried out by adding a certain volume of TP-NRs to a same volume of naïve mouse serum. Control dioleyl-phosphatidylcholine/egg-phosphatidylglycerol (DOPC/EPG) liposomes with encapsulated TP$_{E.coli}$ and permeabilised with the porin Txs (TP-LIPs) were prepared as described by Huysmans et al. [45] and used as a reference. In addition, also free TP$_{E.coli}$ was used as a control sample. We measured the enzymatic activity (OD/min) at the start of the incubation period for each individual sample (set as 100% value). Then, the samples were incubated at 37 °C and after 4 h of adhesion the non-adherent cells were collected by flushing the peritoneal cavity with 10 ml sucrose solution. Cell suspension was centrifuged at 1400 rpm for 8 min at 4 °C and resuspended in complete RPMI 1640 medium (supplemented with 10% heat-inactivated Fetal Calf Serum, 100 mg/ml penicillin, 100 mg/ml streptomycin, 0.03% L-glutamine, 1 mM non-essential amino-acids, 1 mM Na-pyruvate and 0.02 mM mercapto-ethanol). These thiole-colate-elicited peritoneal macrophages were plated out at 10$^6$ cells/ml and incubated at 37 °C. After 4 h of adhesion the non-adherent cells were washed away and half of the adherent cells ($>98%$ macrophages) were pre-stimulated with 100 U/ml interferon-γ (IFNγ) to prime the macrophages. The following day different concentrations of TP-NRs (50 µg polymer/ml, 100 µg polymer/ml and 500 µg polymer/ml) were added to the macrophage cultures and duplicate samples were prepared. After 24 h, 48 h and 72 h of incubation at 37 °C, the cell culture supernatants were harvested and stored at −20 °C until they were analysed for their nitric oxide and cytokine load.

2.7. Hepatocyte cell culture

Male Sprague-Dawley rats (± 250 g) were kept under controlled environmental conditions (12-hour light–dark cycle) and fed a standard diet (Animalab A04 and water ad libitum). Experiments were carried out in accordance with the regulations of the local ethical committee for animal experiments of the Vrije Universiteit Brussel (VUB).

Hepatocytes were isolated (viability > 80% as assessed by trypan blue dye exclusion) and plated at a density of 0.57 × 10$^7$ cells/cm$^2$ at 37 °C, in an atmosphere of 5% CO$_2$ and 95% air and 100% relative humidity [46]. They were cultivated in Williams medium E (GIBCO 22551) supplemented with different antibiotics (50 µg/ml kanamycin monosulfate, 10 µg/ml sodiummampicillin, 7.3 IU/ml sulfamylon-penicillin, 50 µg/ml streptomycin sulphate) and additives (2 mM L-glutamine, 5 µg/ml insulin, 7 ng/ml glucagon, 25 µg/ml hydrocortisone-sodium succinate). Cells were allowed to attach to the plastic substratum for 4 h. Then, serum-containing medium was removed and fresh, serum-free culture medium supplemented with different concentrations of nanoreactors (50 µg/ml, 100 µg/ml, 500 µg/ml and 1000 µg/ml) was added to the cultures. The medium was further renewed daily.

2.8. Macrophage cell culture

For the in vitro macrophage studies, 5 C57BL/6 mice were each administered with 3 ml thyglocylate solution intraperitoneally (ip). After 3 days, mice were euthanized and peritoneal macrophages were collected by flushing the peritoneal cavity with 10 ml sucrose solution. Cell suspension was centrifuged at 1400 rpm for 8 min at 4 °C and resuspended in complete RPMI 1640 medium (supplemented with 10% heat-inactivated Fetal Calf Serum, 100 mg/ml penicillin, 100 mg/ml streptomycin, 0.03% L-glutamine, 1 mM non-essential amino-acids, 1 mM Na-pyruvate and 0.02 mM mercapto-ethanol). These thiole-colate-elicited peritoneal macrophages were plated out at 10$^6$ cells/ml and incubated at 37 °C. After 4 h of adhesion the non-adherent cells were washed away and half of the adherent cells ($>98%$ macrophages) were pre-stimulated with 100 U/ml interferon-γ (IFNγ) to prime the macrophages. The following day different concentrations of TP-NRs (50 µg polymer/ml, 100 µg polymer/ml and 500 µg polymer/ml) were added to the macrophage cultures and duplicate samples were prepared. After 24 h, 48 h and 72 h of incubation at 37 °C, the cell culture supernatants were harvested and stored at −20 °C until they were analysed for their nitric oxide and cytokine load.

2.9. In vivo inflammatory response study

To study the inflammatory potency of the nanoparticles in vivo, naïve C57BL/6 mice (5 mice per experimental group) received a peritoneal injection of 100 µl TP-NRs (final concentration of 500 µg polymer/ml). Ip injection of PBS and a sub-lethal dose of LPS (50 µg/mouse) were used as negative and positive control, respectively. After 6 h mice were euthanized and peritoneal macrophages and blood samples were collected. Peritoneal macrophages were cultured at 37 °C and after 48 h the supernatant was analysed for the detection of the inflammatory cytokines IL-6, IL-1β and TNF-α and for NO. Similarly, the cytokines were evaluated in the serum.

2.10. LAL-assay

The level of bacterial endotoxins, more specifically LPS, in the protein or nanoreactor suspensions was determined with the *Limulus* amebocyte lysate assay (LAL-assay) according to the manufacturer instructions (Lonza, Inc.).

2.11. Toxicity assays

LDH-assay: The leakage of the enzyme lactate dehydrogenase (LDH) was measured using the Merckotest (VWR International, Leuven, Belgium) and was calculated in percentage by the ratio: (100 × LDH activity in supernatant)/(LDH activity in (supernatant + cells)) [47].
MTT-assay: The cytotoxicity in the MTT-assay was determined after isopropanol extraction by measuring the reduction of water soluble MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoli um bromide, Sigma) to water insoluble MTT formazan, at 570 nm. The results are given as relative values (%) to the negative control, while untreated control is set to be 100% viable [48].

2.12. Cytokine analysis and NO detection

Concentrations of TNF-α (Duoset ELISA Development System, R&D Systems), IL-6 and IL-1β (Pharmingen, BD Biosciences) in serum and cell supernatants were determined by sandwich ELISA as recommended by the suppliers.

Nitrite quantification in cell supernatants was assayed by a standard Griess reaction [49]. Therefore, 100 μl of cell-free supernatants were added to an equal volume of Griess reagent (0.5% sulfanilamide and 0.05% N-1 naphthylethylenediamine hydrochloride in 2.5% phosphoric acid). After a 10-minute incubation at room temperature absorbance at 540 nm was recorded. A standard curve was generated with known concentrations of NaNO₂ in culture medium. All reagents for the Griess reaction were obtained from Sigma.

2.13. Statistical analysis

Results are expressed as the mean of at least three independent experiments. For the toxicity studies, statistical analysis was performed using a one-way ANOVA test, followed by a Bonferroni correction to study differences amongst individual means.

3. Results and discussion

3.1. Nanoreactors are monodisperse and enzymatically active

In a first series of experiments, the biochemical characteristics of the nanoreactors were investigated. Following the ethanol method, monodisperse nanoreactors with a mean radius around 100 nm were obtained (Fig. 2). The monodispersity of the nanoreactors was further confirmed via Atomic Force Microscopy (AFM) (see Supplementary data for detailed description and additional figure). SDS-PAGE analysis of a TP-NR sample in comparison with known amounts of pure TP<sub>E.coli</sub> standards reveals an enzyme encapsulation efficiency of around 10% and shows a clear incorporation of the porin Tsx (see Supplementary data for detailed description and additional figure). The in vitro enzymatic activity of the TP-NRs was confirmed spectrophotometrically by measuring the decrease in absorption at 290 nm due to thymidine consumption (data not shown). TP-NRs induce, in comparison to control samples (PBS and empty particles), a clear decrease in absorption due to the conversion of thymidine to thymine. These results show that the nanoreactors possess thymidine phosphorlyase activity.

3.2. Nanoreactors are stable in vitro

A first important requirement of enzyme delivery systems to be used in vivo is their stability in physiological conditions. The stability of the permeabilised TP-NRs was therefore investigated by incubating them for several days in 50% naïve mouse serum at 37 °C. At different time points both the size distribution (by DLS) and the enzyme activity (290 nm) was measured. TP-LIPs (liposomes with encapsulated TP<sub>E.coli</sub> and permeabilised with the porin Tsx) and free TP<sub>E.coli</sub> were used as control samples.

In mouse serum, the size and dispersity of the TP-NRs stays relatively constant over time (Fig. 3). Although the polydispersity of the nanoreactor samples increases slightly, we can conclude that the nanoreactors are still intact after 4 days of incubation at 37 °C. As a negative control, detergent (TritonX-100) was added to the same sample resulting in a clear shift of the DLS pattern to lower sizes, caused by the destruction of the particles.

The enzyme activity of the TP-LIPs increases significantly during their incubation period at 37 °C. Such an increase in apparent activity of the liposome-encapsulated enzyme is expected if enzyme is leaking out of the liposomes, as previously reported [50,51]. Indeed, for the encapsulated enzymes the net rate of substrate conversion is limited by the rate of diffusion of the substrate through the porins [35]. Hence, release of the enzyme to the medium leads to the relief of diffusion barriers with concomitant higher relative rates of substrate consumption. In this context it is noteworthy that the activity values plotted in Fig. 3 represent activities relative to the value at the zero-time-point for each formulation and that hence at all times the absolute activity of the encapsulated enzyme preparations remains below the activity of the free enzyme. In contrast to the TP-LIPs, no increase in activity is observed for the TP-NRs. Thus, the enzyme is contained inside the polymeric particles without leakage. This is a clear advantage for their in vivo use because the release of a foreign enzyme will elicit an immune response. Note that the enzyme activity of the TP-NRs decreases slightly over time. After three days half of the initial activity remained. This decrease in activity cannot be due to the degradation of the enzyme, because the activity of the free enzyme in serum remains constant (Fig. 3). One possible hypothesis for this decrease in activity could be a partial obstruction of the porins by serum proteins.

3.3. Nanoreactors show very low toxicity on hepatocytes in vitro

A second important prerequisite of new enzyme delivery devices is that they may not provoke toxic effects on healthy cells and tissues. Since the liver is the predominant organ in which biotransformation of foreign compounds takes place, it is the ultimate organ to test xenobiotic-induced toxicity [52]. Although polymers made out of PMOXA-PDMS-PMOXA blocks are approved by the FDA for use in contact-lens material, the toxicity of the polymers for in vivo use is still not determined. Therefore, possible toxic effects of the TP-NRs on hepatocytes were investigated. As such, hepatocytes were isolated from male outbred Sprague-Dawley rats by a two-step collagenase perfusion method [53] and cultured in Williams medium E as described previously [46]. After 4 h of cell adhesion, serum-containing medium was removed and fresh, serum-free culture medium supplemented with different concentrations of TP-NRs (50 μg/ml, 100 μg/ml, 500 μg/ml)
100 µg/ml, 500 µg/ml and 1000 µg/ml) was added to the hepatocyte cultures. Cytotoxicity was tested by cellular morphology, membrane leakage of lactate dehydrogenase (LDH-assay) and mitochondrial function (MTT-assay) as a function of culture time. Medium alone and medium with addition of PBS were used as negative controls. SuperFasLigand™ (human recombinant; Alexis Biochemicals) in combination with cycloheximide (Sigma), two known apoptosis-inducers, acted as the positive control[54,55].

The results show no significant difference in LDH leakage between the various conditions up to 48 h of incubation (Fig. 4). Only after prolonged exposure and at higher doses of TP-NRs (96 h for the 500 µg/ml and 72 h for the 1000 µg/ml concentrations), elevated levels of LDH leakage were measured. This cytotoxic effect was confirmed by visual inspection of the morphology of the hepatocytes: after prolonged incubation with TP-NRs at high doses, the hepatocyte-morphology became apoptotic in comparison to healthy untreated cells (data not shown). However, TP-NRs at lower doses (50 µg/ml and 100 µg/ml) did not affect the viability of the hepatocytes throughout culture time. Indeed, the hepatocytes treated with TP-nanoreactors at lower concentrations (50 µg/ml and 100 µg/ml) still looked healthy after 96 h of incubation (Fig. 5). The cytotoxic effect of nanoreactors was also tested via the MTT-test. Unfortunately this assay displayed false positive results. An increase in viability was observed after incubation with TP-nanoreactors, and changes in concentration or incubation time had no significant influence on the viability. Such inconsistencies were also reported by others [56,57] and could be due to an interference of the MTT formazan with the nanoparticles. Overall, from the LDH-assay and the morphological study we can conclude that TP-NRs of concentrations up to 100 µg polymer/ml have no cytotoxic effect on hepatocytes throughout culture time. It is moreover questionable if concentrations of 100 µg/ml and higher reflect biological relevant conditions. Indeed, the TP-NRs will circulate in the bloodstream and local concentrations at liver tissue will probably never reach these levels.

### 3.4. Nanoreactors induce minor inflammatory responses on macrophages

A final important prerequisite of enzyme delivery devices for the use in enzyme replacement therapy is that they have long circulation times within the bloodstream and do not alert the immune system or induce overt inflammation [58]. An important factor in the clearance of foreign compounds from the circulation and the initiation of inflammatory responses is their recognition by the mononuclear phagocyte system, most notably macrophages [59]. Therefore, it is very important that TP-NRs are not recognized by macrophages and are immunologically inert. To investigate this, the impact of TP-NRs on the inflammatory status of macrophages was tested ex vivo and in vivo.

For ex vivo studies the effect of nanoreactors on thioglycolate-elicited peritoneal macrophages isolated from C57BL/6 mice was analysed [60]. Cells were plated in complete RPMI medium and incubated at 37 °C. After 4 h of adhesion, non-adherent cells were
Dependently elevate the amounts of NO, TNF-α (Fig. 6). Since no significant responses were found in the other primed macrophages, complete TP-NRs (NP+TP+Tsx) dose-dependently elevate the amounts of NO, TNF-α and IL-6 produced (Fig. 6). Since no significant responses were found in the other conditions (NP-TP-Tsx, NP+TP-Tsx) we can conclude that the induction of inflammatory mediators is porin-dependent. Importantly however, TP-NR-induced TNF-α levels and IL-6 levels are much lower as compared to the positive control and the free enzyme. This indicates that even on pre-activated macrophages the inflammatory effect of the nanoreactors is very limited; again highlighting that encapsulation efficiently shields the inflammatory potency of free TP.

The inflammatory potency of the nanoparticles was also investigated in vivo. Therefore, naïve C57BL/6 mice (5 mice per experimental group) received a peritoneal injection of 100 µl TP-NRs (final concentration of 500 µg polymer/ml). Intrapерitoneal injection of PBS and a sub-lethal dose of LPS (50 µg/mouse) were used as negative and positive control, respectively. After 6 h mice were euthanized and peritoneal macrophages and blood samples were collected. Peritoneal macrophages were cultured at 37 °C and after 48 h the supernatant was analysed for the detection of the inflammatory cytokines IL-6, IL-1β and TNF-α and for NO. Similarly, the cytokines were evaluated in the serum. Only low amounts of IL-6, IL-1β and TNF-α could be found in macrophage-supernatant and in serum of mice treated with TP-NRs, while high concentrations were detected in mice injected with LPS (Table 1).

Taken together, the ex vivo and in vivo data show that the effects of TP-NRs on macrophage activation and concomitant inflammation are minor. Only a slight pro-inflammatory effect was seen on strongly IFN-γ primed macrophages. Importantly, no inflammatory effect is detected in vivo. Since there is only a limited diffusion of particles from the peritoneum to the bloodstream, the level of cytokines present in the serum after intraperitoneal injection of the nanoreactors is rather an indirect indication of the potent inflammatory response. However, strong inflammatory responses in the peritoneum are usually reflected in the blood as well. Since the TP injected nanoparticles do not induce high cytokine levels in the local peritoneal macrophages nor in the serum, we can safely conclude that the nanoreactors are immunologically inert towards macrophages. Clearly, the stealthy polymers are able to protect the enzyme.

Fig. 4. LDH leakage of hepatocytes incubated with different concentrations of TP-NRs as a function of time. Medium with or without TP-NRs was renewed daily. Medium and PBS serve as negative controls, a combination of SuperFasLigand with cycloheximide is used as the positive control. The data represent at least three independent experiments. Statistical analyses were performed using a one-way ANOVA test, followed by a Bonferroni post hoc test. (⁎ p<0.05 and *** p<0.001 compared to medium and PBS control values).

Washed away, leaving almost pure (>98%) macrophages in the plate. Half of these cells were pre-stimulated with 100 U/ml of the known macrophage activating cytokine interferon-γ (IFN-γ) to prime the macrophages [61]. The following day, different concentrations of TP-NRs (50 µg polymer/ml, 100 µg polymer/ml and 500 µg polymer/ml) were added to the macrophage cultures and duplicate samples were prepared to assess the induction of inflammatory mediators. Plain polymeric nanoparticles without porin and without enzyme (NP-TP-Tsx) and nanoreactors without porin but with enzyme (NP+TP-Tsx) were used as controls and added at the same concentrations as the complete TP-NRs (NP+TP+Tsx). The free bacterial enzyme TP$_{E.~coll}$ (5 µM) was added to investigate the shielding effectiveness of encapsulation. PBS was the negative control, while the known pro-inflammatory bacterial compound lipopolysaccharide (LPS) (100 ng/ml) served as positive control. In this context it is very important to note that all the nanoparticle preparations used in this experiment need to be clean of LPS, in order to exclude LPS dependent immune responses [59]. Given that Txs is a Gram-negative bacterial outer membrane protein, the risk of LPS contamination, a potent endotoxin, is considerable. Therefore, nanoreactor samples used in this study were routinely checked by the LAL-assay to determine the LPS content. Only the samples with an endotoxin unit lower than 50 EU/ml were used. After 24 h, 48 h and 72 h the cell culture supernatants were harvested and analysed via ELISA for the presence of the inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [62]. These cytokines are produced by activated macrophages and are the principal mediators of the acute phase immune response. Another indicator for inflammatory macrophage activation is nitric oxide (NO) production [63]. The results show that none of the nanoparticles tested (NP-TP-Tsx, NP+TP-Tsx, NP+TP+Tsx) induces NO, TNF-α or IL-6 production by naïve macrophages (Fig. 6). Interestingly, the free enzyme significantly provoked the secretion of all three inflammatory mediators. This means that the encapsulation of the enzyme in polymer particles indeed protects the protein from the immune system. Additionally, these results unequivocally indicate that the polymers possess good stealth properties towards peritoneal macrophages and hence are not recognized by naïve macrophages as foreign molecules. However, on IFN-γ primed macrophages, complete TP-NRs (NP+TP+Tsx) dose-dependently elevate the amounts of NO, TNF-α and IL-6 produced (Fig. 6). Since no significant responses were found in the other

Fig. 5. Morphology of cultured hepatocytes incubated for 96 h with (A) medium as negative control, (B) FasL-CHX as positive control, (C) TP-NRs at 50 µg/ml and (D) TP-NRs at 100 µg/ml. All images were acquired with constant microscope settings (magnification = 100×).
from recognition by these immune cells. The slight inflammatory effect on IFN-γ primed macrophages in vitro is probably due to surface exposed parts of the bacterial porins that are recognized by macrophages. Previously, it was reported that the porins are mainly buried in the polymeric membrane that is two-three-fold thicker than conventional lipid bilayers [30,64]. The immunogenic response to the porins can probably be minimized by choosing thicker PMOXA side-blocks that shield the incorporated porins better from the environment. These results, demonstrating that the TP-NRs induce no acute innate immune response after intraperitoneal injection, are a first
important step towards a more profound immunologic characterisation where the nanoparticles will be injected intravenously.

4. Conclusion

The design of bioreactors based on nanometer-sized polymer vesicles permeabilised with bacterial porins was introduced by the group of Wolfgang Meier in 2001 [30,31]. Ever since, these polymeric nanoreactors are exploited for many uses and their biochemical capabilities appeared suitable for biomedical applications, such as cancer therapy, biosensors and as recently suggested by Ben-Haim et al. [41], also for enzyme replacement.

Here, we successfully constructed active thymidine phosphorylase encapsulating nanoreactors and evaluated their stability, toxicity and immunogenic properties as a first step towards their use as new enzyme delivery vehicles for enzyme replacement therapy of MNGIE. A prerequisite in the development of such new delivery particles is an understanding of their behaviour in the presence of serum and crucial cell types. We can conclude that, although a slight decrease in enzymatic activity was observed over time, the TP-NRs are stable and not leaky at 37 °C in serum for several days. Moreover, at concentrations up to 100 µg polymer/ml the TP-NRs do not affect the viability of primary hepatocytes. In addition, no stimulatory effect of TP-NRs was observed on naive macrophages, while only a minor inflammatory effect at high concentrations was detected on IFNγ-primed macrophages in vitro. No effect on inflammation was seen in vivo, upon intraperitoneal inoculation of TP-NRs, sustaining that these nanoparticles do not induce acute inflammatory responses. Altogether, these results open realistic hopes and good prospects for a better and safer enzyme replacement therapy of MNGIE. However, further investigation needs to be carried out to unravel the in vivo fate of the TP-NRs after intravenous injection and to study the in vivo efficacy of the particles as enzyme replacement vehicles.

The current study can moreover be placed in a broader perspective as it opens the possibility of evaluating and using the nanoreactor strategy in the treatment of a wide range of known enzyme deficiencies, such as ADA-deficiency, Gaucher disease and Fabry disease, amongst others. The versatility of the system also allows to encapsulate enzymes with improved properties compared to the native enzyme (e.g. from other organisms, engineered enzymes, etc) or even mixtures of different enzymes to convert the toxic substrates.

In the future, polymeric enzyme-loaded nanoreactors might become a new general approach for bringing therapeutic enzymes into the blood circulation.

Acknowledgements

Caroline De Vocht is a predoctoral fellow and is funded by a Ph.D. grant of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen). Wim Versées and Tamara Vanhaecke are postdoctoral fellows of the ‘Fonds voor Wetenschappelijk Onderzoek Vlaanderen’ (FWO) or Research Foundation Flanders. Jo Van Ginderachter is funded by a postdoctoral grant from the ‘Stichting tegen Kanker’. The authors thank Bart Defregge for the isolation of the rat hepatocytes and Benoît Stijlemans for his help with the LAL-assay.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jconrel.2009.03.020.

References


