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# In vitro investigation of lipid implants as a controlled release system for interleukin-18

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**Abstract**

Operating on the inductive and effective phases of an anti-tumor immune response and uncovering pivotal functions that may reduce cancer cell growth, interleukin-18 (IL-18) appears to be an attractive candidate for the sustained local adjuvant immunotherapeutic treatment of brain gliomas. The objective of this work was to develop IL-18 loaded lipid implants as a controlled delivery system. For the preparation of protein loaded triglyceride matrix material, a solid-in-oil (s/o) dispersion technique was chosen for which protein particles in the micrometer range were first prepared by co-lyophilization with polyethylene glycol (PEG). Implants of 1 mm diameter, 1.8 mm height and 1.8 mg weight were manufactured by compression of the powder mixture in a specially designed powder compacting tool. The in vitro release behavior of $^{125}$I-Bolton-Hunter-radiolabeled IL-18 was assessed in a continuous-flow system. A cell culture assay was established for the determination of bioactivity of released IL-18. Implants showed a continuous release of 10–100 ng IL-18 per day for 12 days. A progressive integrity loss was observed with ongoing release, which would be related to protein degradation during incubation. The initially released fraction proved complete retention of bioactivity, indicating that the manufacturing procedure had no detrimental effects on protein stability.


*Keywords:* Lipid implants; Protein release; Polyethylene glycol; Interleukin-18; Biological activity assay

## 1. Introduction

Malignant tumors of the CNS represent an illness where therapeutic approaches so far have only been able to achieve a short extension in life span of the patient (Wang et al., 2002). Conventional treatment includes surgical removal of the tumor, radiation therapy and chemotherapy. Despite efforts and advances in neuroradiology and neurosurgical techniques, median survival time of glioblastoma patients is less than 1 year. Those tumors tend to recur within centimeters of their original location and novel approaches are urgently needed to treat the disease.

In this context, local immunotherapy has become an area of growing interest. Tumor cells express proteins foreign to the host, which render them vulnerable to an immune response. The immune reaction can be activated by local application of cytokines; potential bioactive agents include interleukins (IL), interferons (IFN) and colony-stimulating factors (Fenstermaker and Ciesielski, 2004; Ehtesham et al., 2004). IL-18 is a cytokine, which was chosen by our group for its prospects of being used as a therapeutic agent in this regard.

First described as interferon-γ (IFN-γ) inducing factor (IGIF) in 1989 (Nakamura et al., 1989), IL-18, also called IL-1F4, is a molecule made up of a single peptide chain of 18.3–18.4 kDa produced primarily by macrophages and mononuclear blood cells from an inactive precursor. IL-18 belongs to the IL-1β cytokine superfamily and interacts with a heterodimeric receptor complex (IL-18 receptor-α and IL-18 receptor-β) to mediate a wide range of immune and non-immune biological effects (Lebel-Binay et al., 2000). In addition to stimulating IFN-γ production from macrophages, T cells and NK cells, IL-18 stimulates the production of IL-2, GM-CSF and chemokines (IL-8, MIP-1β and MCP-1) from peripheral blood mononuclear cells. IL-18 also promotes the development of Th1 cells from Th0 cells. IL-18 is able to enhance T and NK cell cytotoxicity towards

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tumor cells and exerts chemoattractive effects that have so far been demonstrated on Th1 T-lymphocytes (Komai-Koma et al., 2003), dendritic cells (Gutzmer et al., 2003) and NK cells (Ishida et al., 2004). Additionally, IL-18 is known to reduce angiogenesis in vivo, an effect which results in hypovascularization of treated tumors (Cao et al., 1999). Collectively, IL-18 activities suggest the possibility of applying this cytokine to local tumor immunotherapy.

In parallel to gene therapy studies, currently the most common technological method to deliver therapeutic factors directly to a specific brain region involves surgically implanting pump or cannula systems (Whittlesey and Shea, 2004). Apart from functional difficulties, such as the clogging of the pump system, the bioactive molecule itself sets a limitation to this approach. Interleukins are protein drugs and have short half-lives and limited stability in the aqueous reservoir of a pump system. Recent efforts thus have focussed on designing controlled release devices that enable the delivery of intact biomolecules directly to the tumor site (Chen et al., 1997).

To our knowledge, no delivery system for IL-18 has been described so far, limiting the opportunities to investigate its potential as a therapeutic agent in an animal model in vivo. With the intent to overcome this deficit, lipid implants were devised as a controlled release system for IL-18. Glycerol tripalmitate, a physiological triglyceride with interesting properties suggesting its use as matrix material for long-term release of bioactive compounds (Reithmeier et al., 2001a,b; Koennings et al., 2004) served as an alternative to polymeric release systems. Triglycerides are biocompatible and biodegradable molecules (Guse et al., 2006) that can avoid many disadvantages of their counterparts (Maschke et al., 2004). Indeed, no swelling occurs (Vogelhuber et al., 2003) and the formation of acidic degradation products, which can result in a pH shift or even covalent attachment to the protein drug (Lucke et al., 2001), are not of concern.

To plan for potential testing in an animal tumor model, the implants must be small enough to be inserted into the rat brain by stereotaxy and capable of releasing biologically active IL-18 in a controlled fashion. According to our estimations, for the rat brain a dose of 10–100 ng of IL-18 ($ED_{50}$ 3–6 ng/ml) per day and implant would likely be a valuable objective. This is based on analogous reports about growth factors with a similar $ED_{50}$ exhibiting significant effects when released from PLGA microspheres (Pean et al., 2000; Maschke et al., 2004). A method for incorporating protein into lipid matrix material was established. Particular interest was given to investigating the in vitro release performance and an assessment of the bioactivity of released protein in a cell culture assay.

## 2. Materials and methods

### 2.1. Materials

Rat recombinant IL-18 (rrIL-18), IL-2, IL-12, interferon-γ (IFN-γ), mouse recombinant IL-18 (mrIL-18), ELISA DuoSet antibodies directed against rat IFN-γ and human anti-CD28 were obtained from R&D Systems (Abingdon, UK). Rat anti-CD3 was purchased from BD Biosciences (Le Pont de Claix, France). Dynasan® 116 (glycerol tripalmitate) was obtained from Sasol (Witten, Germany). Polyethylene glycol (PEG, molecular weight 6000) and tetrahydrofurane (THF) were supplied by Fluka (Buchs, Switzerland), bovine serum albumin (fraction V, 66 kDa), gelatin, dithiothreitol (DTT), Sigmacote®, MOPS (3-[*N*-morpholino]propanesulfonic acid), EDTA and sodium azide were from Sigma–Aldrich (Saint Quentin Fallavier, France). Bolton-Hunter reagent (*N*-succinimidyl-3-(4-hydroxy-3-[$^{125}$I]-iodophenyl)propionate, monoiodinated, 250 μCi) was purchased from Perkin-Elmer Life Sciences (Courtaboeuf, France), sodium sulfate from VWR International (Fontenay sous Bois, France), PD-10 columns and Sephadex® G 25 M from Amersham Bioscience (Orsay, France).

### 2.2. $^{125}$I-labeled interleukin-18

IL-18 was radiolabeled using Bolton-Hunter reagent. Ten microgram of mrIL-18 were dissolved in 105 μl 0.1 M borate buffer pH 8.5 and added to the vial containing dried 250 μCi (9.25 MBq) Bolton-Hunter's reagent. The reaction was performed at 4 °C with gentle vortexing for 30 min. To stop the reaction, 0.5 ml of a 0.2 M glycine solution in 0.1 M borate buffer pH 8.5 were added. The mixture was incubated for another 10 min at 4 °C. Radiolabeled protein was purified by gel filtration using a Sephadex PD-10 column, which had been pre-equilibrated prior to use with 0.5% gelatin solution for 1 h at 4 °C. The reaction mixture was added to the column and the reaction vial was washed four times with 200 μl of 0.1 M borate buffer pH 8.5. Radiolabeled protein was eluted with 4 ml double distilled water. Fractions of 200 μl were collected in silanized glass tubes and counted with a gamma counter Cobra™ II Auto-Gamma (Packard Bioscience, Rungis, France). The peak fractions were pooled and used for preparation of protein loaded lipid powder. The $^{125}$I-mrIL-18 amount in the final formulation was negligible when compared to total IL-18 amounts used for implant preparation (150 μl peak fraction contained about 1 μg of $^{125}$I-mrIL-18 representing less then 5% of total IL-18 including 25 μg free rrIL-18) and, therefore, not considered when performing a bioassay on released protein.

### 2.3. Implant manufacture

IL-18 loaded lipid implants were prepared in a two-step process, which was optimized for handling small batch sizes in one single vial. In a first step, protein was co-lyophilized with PEG to obtain microparticles according to a modified method described by Morita et al. (2000a). Fig. 1 shows a schematic outline of the different steps. Two different amounts of PEG were studied (2 and 7% with regard to total implant weight) in order to investigate the possibility to tailor the release profile by varying the amount of hydrophilic excipient; theoretical protein content of matrices was 2%.

Briefly, 25 μg rrIL-18 dissolved in 48 μl MOPS buffer pH 7.2 (20 mM MOPS, 50 mM $Na_2SO4$, 0.5 mM EDTA and 0.5 mM DTT) were added to a solution of 500 μg BSA and either 525 or 1838 μg PEG 6000 in 100 μl water in a 2 ml safe-lock

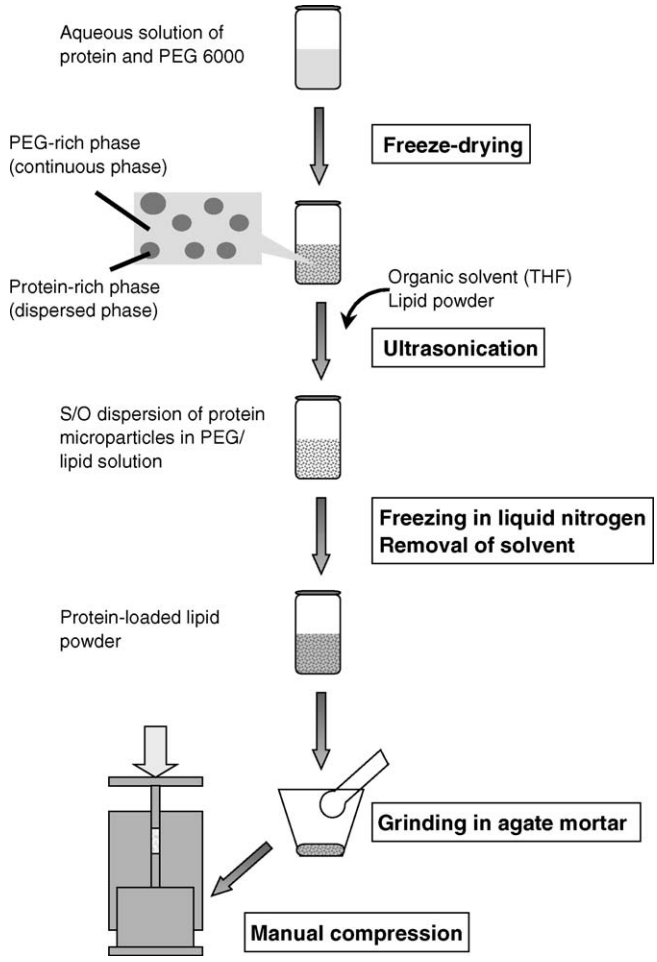


Fig. 1. Schematic representation of the manufacturing procedure for protein-loaded lipid implants.

micro test tube (Eppendorf, Wesseling-Berzdorf, Germany) cup. 150 μl of elution fraction from Bolton Hunter labeling containing radiolabeled mr$^{125}$I-IL-18 were added and the solution was rapidly frozen in liquid nitrogen before freeze-drying for 20 h. After removal of vacuum from the freeze-dryer, 250 μl of THF were added immediately to the lyophilizate to dissolve PEG. 25.2 mg (or 23.9 mg for the formulation with the higher PEG content) of powdered glycerol tripalmitate were placed in the vial. By applying ultrasound (3 s, 5 W, Microson XL2007, with a 3.2 mm microprobe, Microsonix, Servilab, Le Mans; France) the protein particles were disaggregated and dispersed in organic solvent and simultaneously triglyceride was dissolved. The mixture was placed under liquid nitrogen until the THF was completely frozen. By applying a vacuum, the organic solvent was removed, leaving behind a powder formulation. The powder was removed from the micro test tube and ground in an agate mortar.

A specially designed compression tool made of hardened steel (Vogelhuber et al., 2003) was used to compress this powder to form cylindrical implants of 1 mm diameter, 1.8 mm length and 1.8 mg weight. Compression force was controlled at 60 N for 10 s by a Perkin-Elmer hydraulic press (Perkin-Elmer, Ueberlingen, Germany). Prior to any further investigations, the lipid matrices were weighed on an analytical balance to exactly calculate the content of incorporated protein. Each implant was measured for incorporated radioactivity and the homogeneity of protein distribution was judged from radioactivity/mg ratio for all implants.

### 2.4. In vitro release

Conditions for the in vitro release study were chosen such as to minimize stability problems after release from lipid matrices. A continuous flow system, which had been previously developed for the release of proteins from microparticles by Aubert-Pouessel et al. (2002) was used for this study. The system consists of an assembly of adsorption resistant material (PEEK®, polyetheretherketone), composed of an unpacked Omega® HPLC tube (4.6 mm inner diameter × 5 mm, Upchurch Scientific, Oak Harbor, WA) fitted with 0.5 mm frits and connected with HPLC tubing at each end. The HPLC tube itself comprises an approximate volume of 1 ml and is big enough to accommodate several implants. The system allows great flexibility with regard to incubation conditions. While the tube itself can be kept at a temperature of choice (in this study 4, 20 and 37 °C) denaturation hazards for released protein are minimized by collecting eluted buffer at 4 °C in a refrigerated chamber. A continuous flow is held upright by a syringe pump (PhD 2000, Harvard Apparatus, France) supplying MOPS buffer pH 7.2 (containing 0.1% BSA, 0.02% sodium azide) to the column inlet at 1 μl min$^{-1}$. Eluent was collected for 24 h in silanized tubes. The total amount of released protein was determined by counting radioactivity (cpm) with a gamma counter Cobra™ II Auto-Gamma (Packard Bioscience, Rungis, France). Radioactive decay was considered and corrected. Samples were frozen at −20 °C until further analysis by bioassay.

### 2.5. Interleukin-18 stability

To assess the biological activity of IL-18, a bioassay allowing the quantification of γ-IFN produced by IL-18 stimulated primary rat splenocytes enriched in T-lymphocytes was developed. Briefly, spleens obtained from 9-weeks old Sprague-Dawley female rats were mechanically dissociated. Splenocytes were then isolated on a Ficoll gradient in order to eliminate debris, platelets, granulocytes and red blood cells. After differential adhesion for 1 h at 37 °C/5% $CO_2$ on plastic culture dishes to remove other unwanted adherent cells (e.g. macrophages), primary rat splenocytes enriched in T-lymphocytes were cultured in RPMI medium containing 10% fetal bovine serum (both from Biowhittake Europe, Verviers, Belgium). They were subsequently primed for activation during 24 h in the presence of 0.3 μg/ml anti-CD28 in supernatant and in a dish previously coated for 1 h at 37 °C/5% $CO_2$ with 0.5 μg/ml anti-CD3 antibodies and further washed twice with Hank's balanced salt solution. Effects of IL-18 on the production of γ-IFN by isolated splenocytes, after 48 h stimulation in the presence of low amounts of IL-12 and IL-2 (0.1 and 0.4 ng/ml, respectively), were finally determined in supernatants by using a DuoSet

ELISA system, according to the manufacturer's instructions (R&D Systems, Abingdon, UK).

## 3. Results

### 3.1. Manufacturing procedure

The manufacturing procedure described allowed an easy handling of small batch sizes for the preparation of lipid implants loaded with highly potent proteins. All preparation steps could be performed in the same vial, thus avoiding a loss of material. Fig. 1 outlines the main steps of the process. The first step aims at the formation of protein microparticles by freeze-drying an aqueous PEG–protein solution. Varying the amount of PEG present in this solution allows for control of the total PEG content of the formulation. Solid protein particles were obtained by selectively dissolving PEG with the addition of THF. Homogeneous dispersion of these particles and the dissolution of glycerol tripalmitate were achieved by shortly applying ultrasound. The formulation was frozen a second time in liquid nitrogen to induce the precipitation of dissolved lipid on dispersed protein microparticles, resulting in a very fine and homogeneous distribution of the bioactive agent within the lipid powder mixture after removal of the solvent. Radioactivity/mg was found to vary less than 1% between different implants, affirming the homogeneity of the powder formulation.

### 3.2. In vitro release characteristics

Fig. 2A and B shows release profiles of $^{125}$I-IL-18 from glycerol tripalmitate implants. Release of cytokine was investigated under the influence of different concentrations of PEG as a hydrophilic excipient. Relative amounts were 2 and 7% with regard to total implant weight (Fig. 2A and B, respectively). As many proteins show increased stability problems at elevated temperatures, the conditions in the reconstitution medium after lyophilization were previously found to play a significant role (Wang, 2000). It could be seen in preliminary stability studies that IL-18 activity decreased as a function of incubation temperature (data not shown). In order to be able to assess the ratio of bioactive/total released protein, temperatures lower than 37 °C were included in the experimental design to limit possible degradation during release.

The implants show a continuous release behavior without a burst effect (Fig. 2A and B) for the time period investigated, i.e. 3 weeks. Depending on the temperature at which the column containing the matrices was kept, different velocities could be distinguished. A higher temperature correlated with an increase in release rate. Comparing the formulations with different amounts of PEG (Fig. 2A versus 2B), an interesting effect could be seen: a slight increase in release with increased PEG content was only achieved when implants were incubated at 37 °C. In contrast, at 4 and 20 °C a higher PEG content seemed to influence protein release in a way that less IL-18 was released compared to implants with 2% of the excipient incubated at the same temperature.

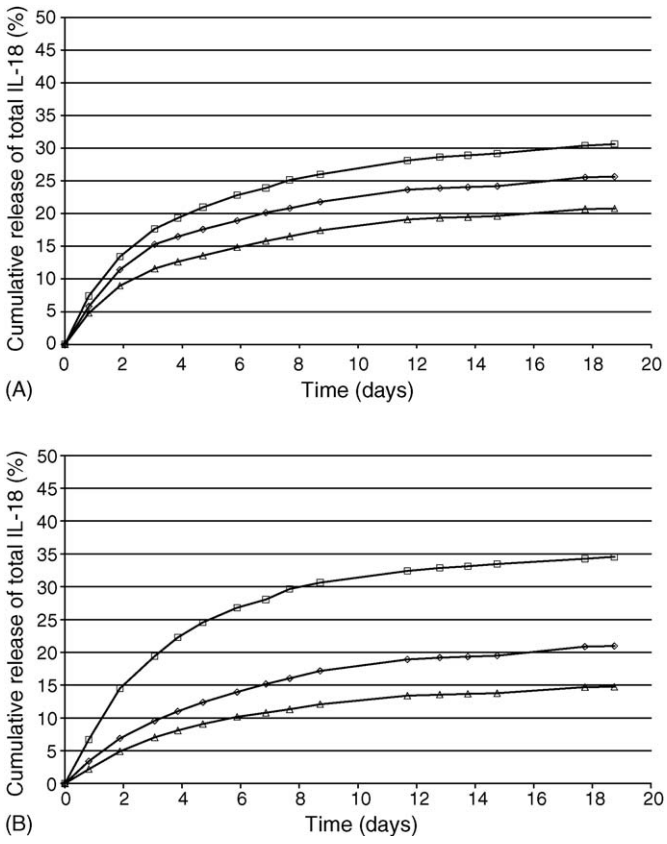


Fig. 2. Cumulative in vitro release of $^{125}$I-IL-18 from lipid implants containing 2% (A) and 7% (B) PEG 6000 incubated at different temperatures: 4 °C (△), 20 °C (◇) and 37 °C (□).

As can be seen in Fig. 3, a very good approach to the envisaged dosage regime was achieved with the preparation containing 7% PEG. Release of total IL-18 was found to be in the desired range for the first 12 days of this study: 10–100 ng of cytokine were liberated into the release medium per day. IL-18 release from the formulation containing 2% PEG fulfilled the requirements as well, however with a lower daily release rate (results not shown).

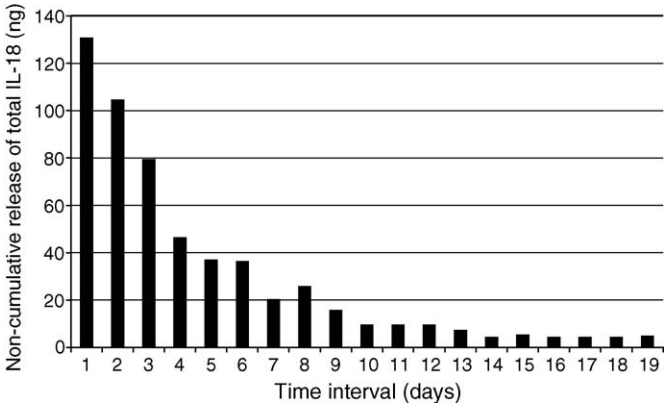


Fig. 3. In vitro release of $^{125}$I-IL-18 from implants containing 7% PEG, incubated at 37 °C in ng/day.

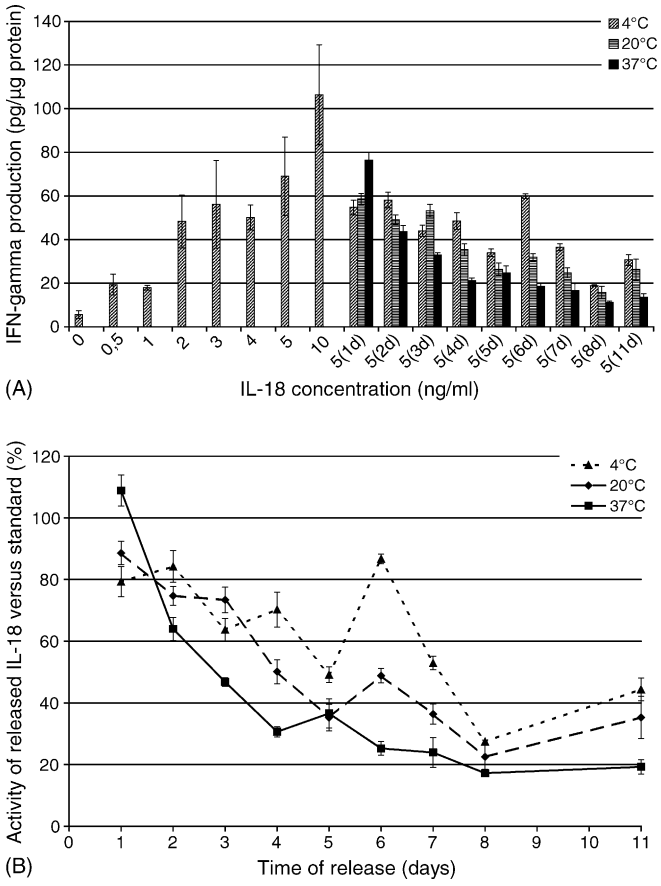


Fig. 4. Biological activity of IL-18 (A) measured as IFN-γ production of splenocytes stimulated with known concentrations of IL-18 standard (0–10 ng/ml) compared to IL-18 released from implants containing 7% PEG (theoretical concentration 5 ng/ml, day of release in brackets) and (B) expressed in % as relative amount of IFN-γ production compared to standard IL-18.

### 3.3. Biological activity of released protein

Knowing the release profile of IL-18, it was possible to assess bioactivity of the interleukin by taking an appropriate amount of sample and comparing it to a standard substance. As the best release profile was obtained with implants containing 7% PEG 6000 further analysis was made considering this condition.

Fig. 4A shows the amounts of IFN-γ that are produced by splenocytes after stimulation by IL-18. On the left hand side the response to different concentrations of standard IL-18 is depicted. On the right hand side IFN-γ production after stimulation of cells with IL-18 released from lipid implants can be found (day 1–8 and 11 of the release study). The theoretical concentration of these samples was 5 ng/ml bioactive rrIL-18 as calculated from the release of radioactively labeled $^{125}$I-mrIL-18. It can be seen that on day 1 samples seem to contain fully bioactive protein, whereas samples from later time points in the study do not induce the same amount of IFN-γ production, as compared to 5 ng/ml standard IL-18. This result is depicted in Fig. 4B as calculated percentages of released protein found in its bioactive form by referring to the standard value for 5 ng/ml.

It can be clearly seen that on day 1 (Fig. 4B), the protein was released in its bioactive form, as a response to the elution buffer was approximately 100% compared to standard. In the progression of the study, the fraction of protein that was released without a change in integrity diminished.

## 4. Discussion

### 4.1. Manufacturing procedure

To our knowledge this is the first report of triglyceride implants being small enough for an implantation into the brain of mice or rats. A diameter of 1 mm allows for the stereotactical insertion of the matrices by pushing them through a needle, thus allowing the in vivo investigation of incorporated bioactive compounds intended for cerebral application. This miniature size, however, brings about a significant challenge in the manufacturing procedure, as a fine and homogeneous distribution of the active compound within the system has to be ensured. First experiments with lysozyme – a protein model compound with similar molecular weight and isoelectric point as IL-18 – demonstrated that a simple physical mixture of lyophilized protein and lipid powder by grinding in a mortar could not guarantee a release profile in the desired range, as protein was completely released from matrices within 8 days (Wang, 2000; Koennings et al., 2003). However, if protein microparticles were incorporated by the PEG co-lyophilization approach, the continuous release for over 80 days was observed (Koennings et al., 2004), suggesting that the fineness of protein distribution within the lipid matrix influences the release profile. Therefore, this strategy was chosen for the development of IL-18 loaded implants.

Morita et al., who first described the use of co-lyophilization of proteins with polyethylene glycol for formulation purposes (Morita et al., 2000a), explain the formation of spherical protein particles by the aqueous phase separation phenomenon. When an aqueous mixture of protein and PEG is freeze-dried, two distinctive aqueous phases form, resulting in a separation of polymers in the freeze-dried product as well. The method can be applied to a wide variety of proteins.

A similar strategy for protein encapsulation has been suggested for polymer microparticles (Morita et al., 2000b; Morita et al., 2001; Castellanos et al., 2002,2003). However, until now investigations to determine the actual PEG content in the final formulation are missing although Morita et al. discuss a contribution of residual PEG amount to release profiles for microparticles prepared by s/o/w method (Morita et al., 2000b). In the case of implant formulation, the incorporation of PEG amount can be controlled, as all steps are performed in the same vial and no loss of excipient by diffusion to an outer aqueous phase (Perez et al., 2002) can occur. Different strategies are possible with regard to the fate of polyethylene glycol. Preliminary studies showed that it would be easy to remove by centrifugation of the protein particles and resuspension in new solvent before the addition of lipid matrix material (Koennings et al., 2004). Here it was allowed to remain inside the formulation as a release modifier.

### 4.2. In vitro release characteristics

The in vitro release testing of a formulation is expected to deliver results regarding performance as a controlled release carrier and retention of biological activity of the incorporated substance. When dealing with fragile compounds like protein drugs, special care has to be taken that in vitro release conditions do not exert deleterious effects upon the drug during release and storage of the samples (Perez et al., 2002; Koennings et al., 2004).

With a continuous-flow release system as described by Aubert-Pouessel et al., the risk of protein degradation after release is minimized as the buffer flow transports released protein away from the elevated temperature in the incubation chamber and eluent can be stored in a refrigerated chamber (Aubert-Pouessel et al., 2002). We chose to perform release studies in MOPS pH 7.2 buffer system, as this was recommended to be the most stable medium by the supplier (R&D Systems, Abingdon, UK). This also coincides with our own observations as IL-18 does not preserve its biological activity after incubation from 1 to 3 h at room temperature in phosphate, borate, bicarbonate and citrate buffers pH 7.4 used on their own (less than 25% recovery, data not shown). In parallel to using the best IL-18 stabilizer medium and in order to further reduce risk of unspecific adsorption, 0.1% BSA was added as a stabilizer to the release medium (Johansen et al., 1998).

The clear dependence of IL-18 release rate on temperature suggests a diffusion-controlled migration of hydrophilic substance within water filled pores in the lipid matrix. Apart from temperature, further impact factors on release rate of IL-18 from lipid matrices can be identified. Two excipients known for acting as release modifiers were added to the formulation: BSA and PEG. BSA or HSA are commonly used as diluent proteins (Nimni, 1997; Aubert-Pouessel et al., 2002; Bensadoun et al., 2003). Jiang and Schwendeman intentionally added BSA to their microparticle formulation in order to increase the release rate of their model drug by amplifying the porosity of the matrix (Jiang and Schwendeman, 2001). A ratio of 20/1 for BSA/IL-18 was chosen from experience with release of BSA alone, taking into account that a potential in vivo application requires a sufficient daily release to reach a concentration range in which IL-18 would be active at the tumor site.

PEG 6000 was left inside the formulation for similar reasons and used in two different relative amounts per implant, as presented in Fig. 2A and B (2 and 7%, respectively). It is well known for its ability to increase release rate from slowly degrading delivery systems (Lavelle et al., 1999; Kim et al., 2000; Jiang and Schwendeman, 2001) and lipid matrices (Pongjanyakul et al., 2004; Mohl and Winter, 2004) by acting as a porogen. In this study, an increase of PEG content from 2 to 7%, however, evoked only a slight increase in total amount of released protein −30 versus 35%, respectively – an effect which could only be observed at an incubation temperature of 37 °C. At lower temperatures the paradox observation of lower release rates at a higher PEG content was made. This might be explained by a slower dissolution rate of the excipient at lower temperatures and a rise in viscosity in the water filled pores of the implant and thus a hindrance of diffusion of protein—an effect which presumably is enforced in the presence of higher PEG amounts. At 37 °C dissolution of PEG may not be the limiting step thus allowing the excipient to act its part as a porogen.

Although release enhancers were present in the formulation, an incomplete release was observed. Protein aggregation following initial rehydration of particles during the release study might be a mechanism inside lipid implants leading to the formation of aggregates, which are mainly held responsible for non-release from microparticles and matrix systems due to adsorption and diffusional problems (Costantino et al., 1994; Castellanos et al., 2002; Mohl and Winter, 2004).

Considering, however, that the fineness of lysozyme distribution had a significant effect on its release behavior, as mentioned above, a possible explanation might as well be found in percolation theory (Bonny and Leuenberger, 1993): The lipid material itself is inert and protein can only be released if aqueous buffer is able to penetrate into the matrix to dissolve the protein. During this process, pores are created where protein is transferred from the solid state into solution. If the ratio of lipid material/protein is too high, an interconnecting network of hydrophilic substance is not possible and areas persist where protein is completely excluded from accession by buffer. This can result in an incomplete release of incorporated drug.

Taking this into account, it seems likely that non-release from lipid implants is caused by the lack of a continuous pore system when protein is finely distributed inside the matrix. This could be addressed by further optimizing the content of porogens in the formulation.

### 4.3. Biological activity of released protein

Being able to perform a bioassay on released protein allows for the gathering of valuable information about the quality of the manufacturing procedure as well as for the prediction of in vivo performance of the drug carrier.

The finding of complete preservation of IL-18 integrity at day 1 indicates that protein encapsulation was successfully performed without damage to the protein with the above-described manufacturing procedure. PEG is generally acknowledged as a cryoprotectant (Wang, 2000), although there are proteins for which integrity losses during co-lyophilization have been reported (Randolph, 1997). Suspending proteins in various organic solvents is considered to be possible without causing solvent-induced structural perturbations, provided they are suspended in an anhydrous state as they display less conformational flexibility than in the aqueous/organic mixtures used for microparticle preparation (Mattos and Ringe, 2001).

Although release conditions were chosen such as to minimize stability hazards for the released protein, a progressive decrease in the biological activity of IL-18 was detected over 11 days. Interestingly, the decrease in bioactivity of IL-18 is more pronounced for incubation at 37 °C followed by 20 and 4 °C. As protein degradation pathways can be enhanced at elevated temperatures, these results suggest that the mechanism leading to a loss of activity of released protein takes place during incubation rather than manufacturing. In the literature,

reports about the similar behavior of bioactive compounds during release can be found: NGF, which was released from PLGA microspheres was only entirely recognized by ELISA on day 1 (Pean et al., 1998); γ-chymotrypsin specific activity was found to decrease over release from PLGA microparticles (Castellanos et al., 2002,2003). A progressive recognition loss was also measured for GDNF formulated in PLGA microspheres, although the release medium had previously been optimized for preservation of GDNF integrity (Aubert-Pouessel et al., 2004).

A moisture induced aggregation related to the slow hydration of encapsulated proteins (Wang, 2000; Perez et al., 2002) could be responsible, indicating the need for further stabilization of the protein during release. Approaches discussed in the literature for constricting moisture-induced aggregation with regard to matrix material include an increase in polymer hydrophobicity in order to limit the amount of moisture sorbed in the matrix (Perez et al., 2002), which however also increases the risk of adsorption phenomena. A second possibility is to ensure rapid hydration of the particles, which can be achieved by the incorporation of hydrophilic additives, such as PEG (Jiang and Schwendeman, 2001; Castellanos et al., 2002,2003) or BSA (Nimni, 1997). A bell shaped aggregation versus water content dependency has been observed for several proteins, indicating that protein stability may increase again above certain water content, probably due to a dilution effect. This was especially observed in the presence of PEG (Costantino et al., 1994). Future investigations will therefore be directed towards identifying molecular events occurring during rehydration and release more closely inside the lipid matrices. A correlation between the amount of incorporated PEG and release seems desirable with regard to both the problems of incomplete release and protein stability.

## 5. Conclusions

IL-18 loaded lipid matrices have been developed, which seem fit for intracerebral implantation into rat or mouse brains by stereotaxy. A manufacturing procedure has been developed, which permits small batch sizes and prevents losses of substance due to a single-pot arrangement, therefore allowing the processing of highly potent, expensive protein drugs. The protein could be homogeneously distributed within the lipid matrix material and bioactivity upon initial liberation from the carrier was fully preserved. Further investigations are necessary to improve protein stability during release as a decrease in IL-18 bioactivity over release time suggests degradation of protein during incubation. A controlled release of protein in the desired therapeutic range of 10–100 ng/day was measured during the first 12 days of the study. An in vivo evaluation of these matrices is currently being undertaken in order to evaluate their performance in glioblastoma treatment on the basis of an animal model.

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