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Sustained release carrier for adenosine triphosphate as signaling molecule

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Abstract

Adenosine triphosphate (ATP) is a molecule with a fascinating variety of intracellular and extracellular biological functions that go far beyond energy metabolism. Due to its limited passive diffusion through biological membranes, controlled release systems may allow to interact with ATP-mediated extracellular processes. In this study, two release systems were explored to evaluate the capacity for either long-term or short-term release: (i) Poly[(*rac*-lactide)-*co*-glycolide] (PLGA) implant rods were capable of ATP release over days to weeks, depending on the PLGA molecular weight and end-group capping, but were also associated with partial hydrolytic degradation of ATP to ADP and AMP, but not adenosine. (ii) Thermosensitive methylcellulose hydrogels with a gelation occurring at body temperature allowed combining adjustable loading levels and the capacity for injection, with injection forces less than 50 N even for small 27G needles. Finally, a first *in vitro* study illustrated purinergic-triggered response of primary murine microglia to ATP released from hydrogels, demonstrating the potential relevance for biomedical applications.

Keywords: Adenosine triphosphate, methyl cellulose hydrogel, PLGA implant rods, controlled release carrier, microglia cells

Introduction

Adenosine triphosphate (ATP) is a molecule with a fascinating variety of biological functions. ATP is generally known to be a key substance in energy metabolism of animals and plants, where it experiences a typically very rapid turnover of formation, consumption, and recycling. When present in extracellular space, it was shown that ATP can activate a variety of purinergic P2Y and P2X receptors on different cell types, thereby participating in cellular communication [1]. Intra- and extracellular ATP signalling spreads throughout the body in both physiological and pathophysiological conditions and includes e.g. synaptic transmission in some central neurons, pain sensing and sensitization of peripheral neurons [2], mechanosensing and paracrine communication e.g. by subendothelial fibroblasts in the intestine [3], the sensing of taste [4], the regulation of the assembly, stability and enzymatic activity of the proteasome [5], or the function of insulin-producing pancreatic cells [6]. Extracellular ATP can also trigger a directional cell movement (chemotaxis) along ATP concentration gradient towards the site of lesion *in vivo*, e.g. of microglia cells as immune cells of the CNS (central nervous system).

ATP is an interesting molecule also from the perspective of its physicochemical properties. Being a small hydrophilic compound, ATP is composed of a purine base, a sugar, and a triphosphate group. It has a high aqueous solubility, small hydrodynamic radius, and a high diffusivity in aqueous environment. The high local concentration of negative charge is basis of its capacity to store energy for catalysis of biochemical processes. At the same time, repulsive forces make it sensitive also for hydrolysis under e.g. acidic conditions. It should be noted that ATP actions are subject of a discrete spatio-temporal control. Intra- and extracellular biological actions can be separated due to the limited passive diffusion of ATP across biological membranes, which may allow to control ATP mediated extracellular processes by controlled release systems.

So far, ATP release systems have only rarely been studied. Along with the emerging recognition of ATP as a neurotransmitter [7], polypyrrole films were suggested first in the 1990s for

electrochemically stimulated release of biologically active ions such as ATP [8] [9] [10] [11] [12]. Recently, nanoparticles from crosslinked chitosan oligosaccharides were suggested as ATP carrier in order to assay the phosphate metabolism for diagnosis of liver disease [13]. Another nanoparticle-based approach involved mesoporous silica nanoparticles, in which ATP solution could be soaked and trapped by pore closure with cadmium sulphide [14] or poly(amido amine) dendrimers [15]. As capping of mesopores occured via disulfide bonds, disulfide-reducing agents were used for stimuli-induced ATP release. In this technique, ATP was incompletely released in a timeframe of minutes to two hours after stimulation. Although some of the dendrimer capped particles showed a trend towards sustained release, this model was not compatible with the aims of the present study, e.g., due to the required addition of reducing agents that also could alter cell functions.

Therefore, here, medically relevant injectable ATP sustained release systems based on compatible polymers should be explored. For their application, two possible scenarios should be distinguished: the timeframe of hours as most relevant for mechanistic cell studies *in vitro*, and the timeframe of days to weeks as possibly useful for future *in vivo* applications. The short term release system should provide high flexibility of released doses in a fixed, limited time frame of few hours as defined e.g. by the cell culture experiment. Here, polymer matrices with a hydrophilic environment like hydrogels may be suitable. Hydrogels from methylcellulose were selected as model system for thermosensitive materials that undergo sol-gel transition when heated to physiological temperature [16]. Relevant injection forces should be ensured for its applicability. A long term release as desired for *in vivo* applications may best be achieved by rather hydrophobic polymer carriers with low water uptake acting as diffusion barriers. Therefore, small diameter implant rods from different poly[(*rac*-lactide)-*co*-glycolide] (PLGA) materials were selected as a clinically established carrier technology for subcutaneous placement and long-term release over > 1 week. Considering acidic microenvironments found in PLGA matrices, the capacity of this polymer to provide ATP stability in addition to long-term release should be explored here as well. In particular, a degradation of ATP

to adenosine interacting with different cellular pathways should be excluded. As a perspective, a cell response to the extracellular ATP should be shown *in vitro*.

2. Materials and Methods

2.1 Materials

The used methyl cellulose was Methocel A15 USP/NF (Colorcon, Dartford Kent, UK) with a viscosity of 16 mPa·s and a methoxyl content of 29.6%. Poly[*rac*-lactide)-*co*-glycolide) 50:50 [PLGA] was from Boehringer Ingelheim (Ingelheim, Germany) and included (i) PLGA-COOH 6k (RG 502H; inherent viscosity [i.v.] of 0.1% polymer in $CHCl_3$ 0.18 dl·g^{-1}; weight average molecular weight M_w 6.1 kDa; polydispersity PD 3.2), (ii) PLGA-COOH 17k (503H; i.v. 0.36 dl·g^{-1}; M_w 17.3 kDa; PD 3.3), and (iii) ethyl ester endcapped PLGA-Et 11k (Resomer RG 502; i.v. 0.22 dlg^{-1}; M_w 10.7 kDa; PD 1.6). Adenosine triphosphate (ATP; 99% purity), adenosine diphosphate (ADP; 97%), adenosine monophosphate (AMP; 99%), and adenosine (99%) were from Sigma-Aldrich, Taufkirchen, Germany. All other chemicals were of analytical grade or higher.

2.2 Preparation of ATP loaded PLGA implant rods

ATP loaded PLGA implant rods were prepared by a solvent extrusion process [17]. Prior to use, ATP was sieved through 90 µm metal filters (Newark Wire Cloth Company, Clifton, NJ, USA) for destruction of aggregates. PLGA was dissolved at several defined concentrations (w/w) in acetone under vigorous vortexing to obtain 1 g of polymer solution, e.g., 50% (wt/wt) PLGA-COOH 6k containing 500 mg of polymer and 500 mg of solvent. In this viscous solution, sieved ATP was dispersed and provided different percent loading (w/w) related to the dry polymer mass. The obtained suspensions were extruded with a syringe and needle at a rate of ~100 µl·min^{-1} in silicon tubing of 0.5 mm inner diameter (Th. Geyer GmbH & Co. KG, Renningen, Germany). For solvent removal, filled tubings were placed on a rotating mixer for 2 d at room temperature and subsequently in a vacuum oven at 40 °C for 2 d. Finally, the implant rods were isolated by cutting the tubing.

2.3 Drug release from implant rods

ATP release from PLGA implants (0.5 mm diameter, 5 mm length) was determined under aseptic conditions in 1.5 ml Eppendorf test tubes containing 500 µl of 0.02% (w/v) Tween 80 in PBS buffer pH 7.4 (150 mM NaCl, 5.8 mM $NaH_2PO_4 \cdot 2H_2O$, 5.8 mM $Na_2HPO_4 \cdot 12H_2O$). For optimal mixing, the test tubes were tipped on their sides on a horizontal shaker (Certomat® IS) at 60 rpm at 37 °C. For sampling, 400 µl of medium were withdrawn and replaced by fresh buffer.

2.4 Preparation and characterization of ATP loaded hydrogels from methylcellulose

Solutions of methylcellulose of different concentrations, e.g. 7.5 wt.%, were prepared by stirring at 4 °C in PBS buffer pH 7.4 or ATP solutions in PBS under aseptic conditions until a homogeneous viscous solution was obtained. These were additionally filtered through 5 µm syringe filters in order to exclude any gel clots.

The gelation temperature was determined by the vial inversion method that involves stepwise heating of samples in glass tubes in a water bath and visual control of the capability to flow upon inversion. Additionally, samples were analyzed in the oscillation mode with a Mars II rheometer with a DC 60/2° Ti measuring assembly (Haake, Karlsruhe, Germany). Samples were heated from 15 to 70 °C with a heating rate of 0.25 $K \cdot min^{-1}$ and the crossover point of the storage modulus G' and the loss modulus G'' was determined (RheoWin Datamanager software).

The kinematic viscosity and density of samples were analyzed with an Ubbelohde capillary viscosimeter (PSV1, Lauda, Lauda-Königshofen, Germany) and a DMA 4500 density meter (Anton Paar, Graz, Austria), respectively, at 20.0 °C. The corresponding dynamic viscosity η was calculated.

The handling of the hydrogels in syringes was tested by measuring injection forces with a Zwicki Z2.5 tensile tester (Zwick GmbH & Co. KG, Ulm, Germany) with a 200 N load cell at a pre-load of 0.5 N. Hydrogels were equilibrated at 20.0 °C in disposable 1 ml syringes (Omnifix-F, B. Braun; Ø of plunger 4.7 mm) and immediately injected into air at ambient conditions through 27G x 1" needles (Sterican, B. Braun; inner diameter ~230 µm) at a feed rate of 100 $mm \cdot min^{-1}$.

2.5 Drug release from methylcellulose implants

Hydrogels were injected into ~8 mm pieces of Cellmax implant membrane tubing (Spectrum). Both ends of the tubing were sealed with hot tweezers and gelation was allowed at 37 °C for 45 min. Then, samples were incubated in microtiter plates with 500 µl of PBS buffer pH 7.4 in a shaker at 40 rpm at 37 °C. For sampling, 250 µl of medium were withdrawn and replaced by fresh PBS.

2.6 Scanning electron microscopy (SEM)

Implant rods were cryofractured prior to analysis. SEM analysis of Pt/Pd sputtered samples was conducted with an Gemini Supra™ 40 VP SEM (Carl Zeiss NTS GmbH, Oberkochen, Germany) using the secondary electron detector, backscattered electron (BSE) detector (ATP visualization in implants), and energy dispersive X-ray (EDX) detector (distribution of elements in implants). EDX signals were imported in Origin® 8 software and smoothed curves were plotted using the adjacent-averaging function of the software.

2.7 Quantification of ATP by HPLC

HPLC analysis (Agilent 1200 instrument; Agilent, Böblingen, Germany) was performed on a 125-4 RP-18 column (LiChroCART® 125-4, LiChrospher® 100, 5 µm; Merck, Darmstadt, Germany) with detection at 260 nm. The eluent contained 215 mM KH_2PO_4, 2.3 mM tetrabutylammonium hydrogen sulphate (TBAHS, >99%) to form ion pairs with anionic nucleoside phosphates, 4 % (v/v) acetonitrile, and 0.4% (v/v) of 1 M KOH [18]. Ten microliters of samples were injected and separated in an isocratic method at 1 $ml \cdot min^{-1}$ at 30 °C.

2.8 Cell studies with murine microglia cells

Microglial cultures were prepared from cerebral cortex of 1-3 day old mice (ethical approval No. T0014/08) and cultured in DMEM supplemented with 10 vol.% fetal calf serum, 2 mM L-glutamine, 100 $units \cdot ml^{-1}$ penicillin and 100 $\mu g \cdot ml^{-1}$ streptomycin (PAA Laboratories, Cölbe, Germany) [19]. After establishment of an astrocytic monolayer, medium was additionally supplemented with 30% L929-conditioned DMEM (M-CSF-secreting mouse fibroblast cell line) to stimulate microglia proliferation. After separating microglia from the underlying astrocytic layer by shaking for one

hour at 100 rpm, cells were seeded on glass coverslips at a density of 5 x 10^4/cover slip. Cultures were used for experiments one day after plating. After washing, 500 µl HBSS medium (blank), ATP standards in HBSS, or ATP loaded hydrogels in Cellmax implant tubing were added and incubated for 60 min. Subsequently, the cell culture supernatants were analyzed by HPLC.

3. Results

3.1 PLGA implant rods as long-term depots

For ATP delivery over an extended period of time, small diameter PLGA implant rods were prepared by a solvent extrusion process of viscous PLGA solutions in acetone with subsequent solvent removal. Since ATP is not soluble in acetone or other suitable solvents of PLGA, sieved ATP powder was dispersed in the polymer solution. Implant cross-sections were studied by SEM, where several spots were observed when a backscattered electron detector was used, which displays sample areas of higher average atomic number as a brighter contrast (Fig. 1A). The analysis of the elemental composition with an EDX detector (Fig. 1B) confirmed that bright spots coincided with ATP as seen by an increased signal intensity of nitrogen, phosphorus, and sodium (from ATP-Na salt) and a decreased intensity of carbon signals (mainly from PLGA). Also, as phosphate groups contain several oxygen atoms, the oxygen signal showed peaks corresponding to ATP.

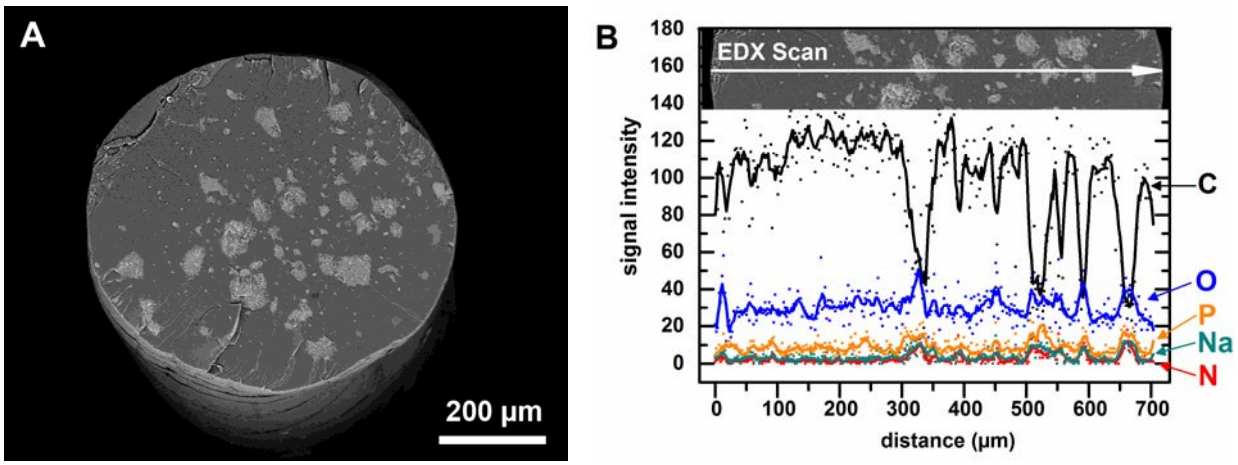

Fig. 1: SEM analysis of ATP-loaded PLGA implant rods. (A) Cross section image as obtained by the backscattered electron detector. (B) Elemental analysis of the cross section by an energy dispersive X-ray (EDX) detector scan. Displayed data represent numerous single data points as measured and overlaid smoothed curves for carbon [C], oxygen [O], phosphorus [P], sodium [Na], and nitrogen [N]. The presented data correspond to implants

prepared from 60 wt.% solutions of PLGA-Et 11k in acetone with 9.1 wt.% drug loading (loading given based on the dry sample mass).

Although relatively stable in the absence of enzymes and strong acids, ATP may undergo hydrolysis in an acidic microenvironment of degrading PLGA, possibly resulting in the removal of phosphate groups from the nucleoside to its diphosphate (ADP)´, monophosphate (AMP), or the undesired totally dephosphorylated adenosine. In order to select a PLGA of most suited properties, the impact of endgroup-capping and molecular weight on the release of ATP as well as its potentially formed degradation products were compared.

Implants from PLGA-Et 11k (Fig. 2A) showed an induction period of 15 d prior to a relevant release of ATP. Importantly, also ADP and AMP as ATP degradation products were detected, but no adenosine (to acknowledge the different molecular weights of ATP/ADP/AMP, the cumulative release data are presented in mol.%, all referring to the nucleoside core). The analogous material with one free carboxyl endgroup per polymer chain and therefore a higher hydrophilicity, PLGA-COOH 6k, provided a much faster linear release of 40 mol.% ATP and almost no degradation products over 7 days, followed by a delayed phase of basically no release (Fig. 2B). With an increase in molecular weight (PLGA-COOH 17k; Fig. 2C), linear ATP release continued over 14 days and achieved cumulative values of 53 mol.%. Based on the PLGA-COOH 17k polymer, implants with different ATP loading levels were prepared (data not shown). When decreasing the ATP loading from 9.1 wt.% to 1 wt.%, a cumulative release of 23 mol.% over 14 days was observed with good reproducibility. However, a further decrease in loading to 0.1 wt.% gave very strong deviations in the release curves of individual samples ranging from 12 to 66 mol.% cumulative release over 14 days. Out of the different studied materials, it appears that PLGA-COOH 17k based implant rods with high ATP loading may be advantageous to realize a continuous long term release of ATP.

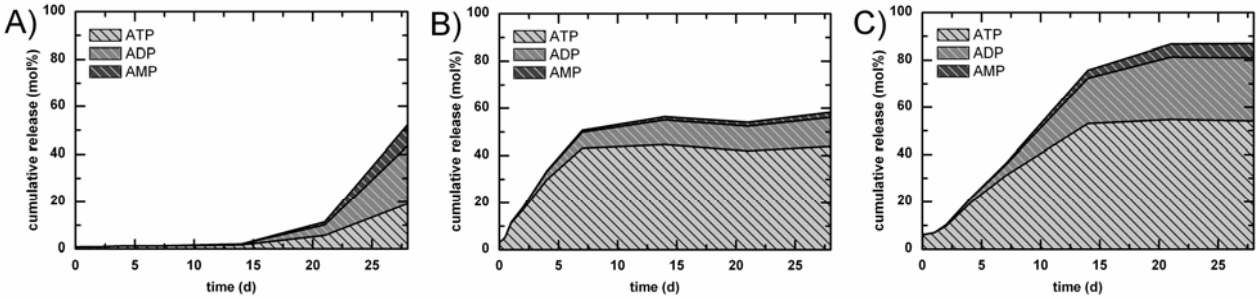

Fig. 2: Release of ATP and its degradation products ADP and AMP from rod-shaped PLGA implants. Implants were loaded with 9.1 wt.% ATP and prepared in a solvent extrusion process from 50 wt.% solutions (in acetone) of (A) PLGA-Et 11k, (B) PLGA-COOH 6k, and (C) PLGA-COOH 17k. Median of n = 3-5 samples.

3.2 Thermosensitive hydrogels as short-term ATP release system

Thermosensitive hydrogels based on methylcellulose, a well-known thermosensitive biopolymer, were employed in this study as a model for an injectable thermosensitive hydrogel matrix. In this context, the temperature of sol-gel transition as well as the viscosity at room temperature that affects the injectability needed to be characterized and adjusted.

In screening studies, methylcellulose of different molecular weights was analyzed (data not shown). The employed methylcellulose (Methocel A15) with a weight average molecular weight M_w of ~50 kDa [20] exhibited gelation in the range of body temperature depending on the methylcellulose concentration in PBS buffer (Fig. 3A). A concentration of 7.5 wt.% was selected for gelation at physiological conditions. Gelation data from the vial inversion method were confirmed by cone-plate rheological analysis using the cross-over point of the storage modulus G' and the loss modulus G'' as gelation temperature (e.g., 35.3 °C for 7.5 wt.% methylcellulose in PBS).

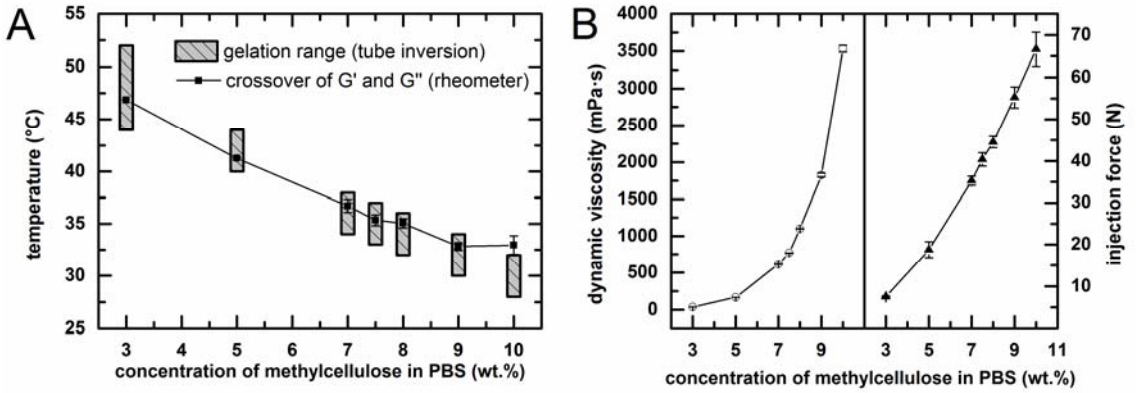

Fig. 3: Properties of methylcellulose solutions (Methocel A15) in PBS. (A) Temperature-induced gelation upon heating from room temperature as determined by either the tube inversion method (temperature range from the first signs of gelation to a non-flowing gel) or by the cross-over point of the storage modulus G' and the loss modulus G'' in rheological measurements ($n \geq 2$, mean, SD). (B) Handling study at 20 °C to evaluate sample feasibility as injectable formulation: Dynamic viscosity (capillary viscosimeter) and injection force at a feed rate of 100 mm·min^{-1} with 1 ml syringes and 27G needles.

Besides the gelation temperature, injectability as related to sample viscosity is a central requirement of a thermosensitive gel for the discussed application. The dynamic viscosity η was shown to increase in a parabolic-like fashion with increasing gel concentration, whereby η of 7.5 wt.% samples was well below 1 Pa·s (Fig. 3B). In manual handling at ambient temperature, the 7.5 wt.% methylcellulose solutions were well injectable even through small needles. Additionally, the handling with 1 ml syringes and 27G needles were characterized by recording the required injection forces at a standard plunger feed rate of 100 mm/min (Fig. 3B). For example, injection forces of ~40 N were recorded for 7.5 wt.% methylcellulose sols at 20 °C with needles as small as 27G (inner diameter ~230 µm). When increasing the inner diameter to ~365 µm (23G) corresponding to a ~2.5-fold increase in cross-sectional area, the required forces strongly decreased to 11 N for this sample (data not shown).

The ATP release from 7.5 wt% methylcellulose hydrogels was determined in a microtube assay for different initial ATP loadings ranging from 1 - 50 µg ATP per mg gel, thus addressing the need to provide a release systems with tailorable doses for systematic in vitro cell studies. The majority of ATP was released from all formulations over a time frame of 6 h (Fig. 4). Only at a very high initial loading of 50 µg ATP per mg gel, a relatively faster release in the first hour was detected. After 24 h, the release was complete in all cases.

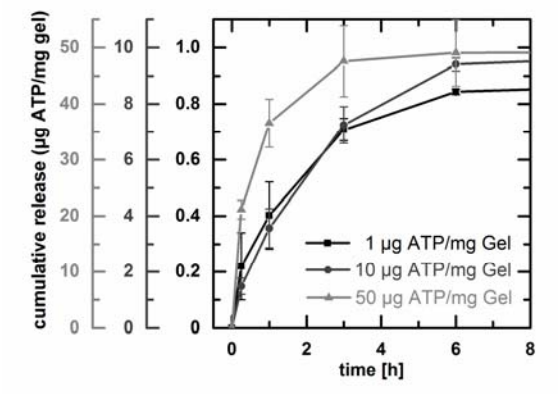

Fig. 4:
Impact of initial ATP loading on cumulative ATP release from 7.5 wt.% methylcellulose hydrogel in PBS buffer at 37 °C (n = 3, mean, SD).

3.3 Cell response to ATP release

In order to illustrate that the developed controlled release systems for ATP can induce cell responses by extracellular pathways, *in vitro* cell experiments were conducted as an outlook. Human primary microglia cells were selected for this experiment. Upon exposure to activating signals, or following the loss of signals normally present, microglial cells can shift their activation state to phenotypes of neuroprotective or neurotoxic nature [21] and thus are of high importance in the field of regenerative therapies. In addition to purinergic signalling, also adenosine receptors have to be considered since ATP can be converted by ectonucleotidases to adenosine, which is also bioactive. In case of microglia, the activity of microglia-specific, cell membrane-bound ectonucleotidases CD39 and CD73 [22] is decisive in terms of controlling ATP/adenosine concentration balance and, hence, cellular response [23, 24]. Here, the gradual ATP dephosphorylation by microglia ectoenzymes with implications on signaling cascades downstream of microglial purinergic receptors should be used as a readout of biological response. Since primary microglia that can be cultivated only over a limited period of time outside the body, the methylcellulose hydrogel carrier as a short-term release system was selected for *in vitro* tests. The hydrogel was filled into membrane tubing for easier handling. When untreated microglial cells (Fig. 5A) were studied under the microscope, a characteristic undirected movement was observed. Upon incubation with hydrogels of high ATP loading (50 $\mu g \cdot mg^{-1}$), a rapid decrease in the movement and a transfer to a spherical cell shape, i.e., toxic effects, were apparent. At lower ATP loading of gels in the range of 1-10 $\mu g \cdot mg^{-1}$, microglia remained vital.

Subsequently, two hydrogels of different ATP loading, i.e., 1 µg·mg^{-1} and 5 µg·mg^{-1} were incubated with microglia and the enzymatic conversion of released ATP was quantified. While neither ATP nor its degradation products could be detected in the cell culture medium of untreated microglia (data not shown), a substantial increase in AMP and ADP by microglia-catalysis was apparent for hydrogel samples (Fig. 5B). It should be noticed, that the obtained AMP concentration in the medium was basically identical for both implant loading levels (~ 30 µM) after incubation with microglia. Similarly, in control experiments with soluble ATP, the produced AMP was in the same range (in this case ~ 15 µM) when either 50 µM or 260 µM ATP were used.

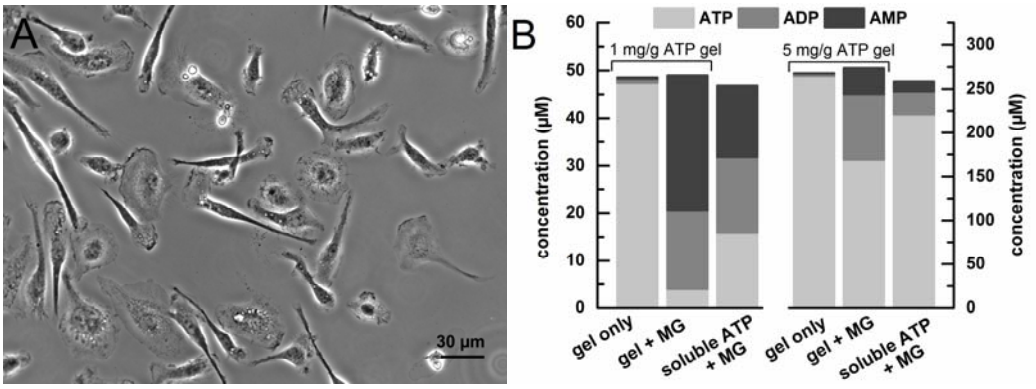

Fig. 5: (A) Contrast image of microglia (MG) in cell culture exhibiting typical mixed morphologies. (B) MG response to ATP releasing hydrogels (1 µg·mg^{-1} and 5 µg·mg^{-1} loading) *in vitro* – emergence of ADP and AMP as products of CD39 enzymatic ATP hydrolysis. After HPLC quantification of the cumulative concentration of released substances from gel samples with and without MG, soluble ATP standards (50 µM and 260 µM) were incubated at another day with fresh MG in a second set of control experiments.

4. Discussion

In this study, two scenarios of controlled ATP release have to be differentiated — the timeframe of hours as most relevant for mechanistic studies of cell behavior *in vitro*, and the timeframe of days to weeks as possibly useful for future *in vivo* studies.

PLGA implant rods were selected to release ATP over extended periods of time. When prepared by a solvent extrusion method with dispersed ATP, domains of ATP in the polymer bulk could be visualized (Fig. 1). At low ATP loadings such as 0.1 wt.%, a statistical distribution of the ATP domains either in the core or closed to the implant surface may be the reason for strong deviations

in the release of samples with low payload, as briefly mentioned. Ester-capped PLGA, particularly of low molecular weight with a large number of end-groups per mass of material like in PLGA-Et 11k, are less hydrophilic than their counterpart with free carboxyl groups. Water uptake as a precondition for drug diffusion depends on matrix hydrophilicity, which may explain the long induction period for PLGA-Et 11k compared to the rapid onset of release for PLGA-COOH 6k (Fig. 2). PLGA-COOH 17k with a higher molecular weight showed a more continuous ATP release. It may be hypothesized that the second delayed release phase particularly for PLGA-COOH 6k may result from a closure of porous diffusion pathways by swelling or self-healing phenomena [25].

A degradation of ATP to ADP and AMP, but not adenosine occurred in the PLGA matrix, which was likely due to acid-catalyzed hydrolysis by carboxyl groups of the polymer or its degradation products. The release of acidic PLGA degradation products may raise concern on the compatibility of PLGA implant rods with an *in vivo* setting. However, implant rods from PLGA were previously well accepted in various in vivo settings, even in sensitive brain tissue, although some mild reaction typical to a foreign body response were observed [26]. PLGA microparticles injected into the rat brain induced local cell response only between day 1 and day 10 [27], which probably was due to the injection-related trauma rather than the release of degradation products from high molecular weight PLGA. Similarly, animals with and without placement of PLGA sponges (3 mm cylinders) showed basically identical intensities of OX-42 immunolabelling of microglia 1 week after operation [28]. Therefore, in future *in vivo* studies of ATP releasing implants, chemotactic effects of ATP release may best be studied after ~ 1 week. The PLGA-COOH 17k based implants may be suitable for this purpose.

For short term ATP release, thermosensitive model hydrogels based on methylcellulose were selected, which are adaptable in terms of the quantities of loaded ATP and involve only few well controllable preparation steps that are compatible with handling under aseptic conditions. As expected, an increasing methylcellulose concentration allowed to decrease the gelation temperature and substantially increased the viscosity in the sol state (Fig. 3A). For ensuring practical handling

in an intraoperative scenario and considering the need to keep tissue damage by needle insertion at a minimum, injection forces were measured using very small needles (27G; outer diameter of 0.4 mm) and small diameter syringes feasible for low injection volumes. With this setup, the required injection force into air was ~40 N at 20 °C (Fig. 3B), which should well correspond to injection into a tissue as suggested by a comparative air vs. tissue injection study with viscous polymer solutions [29]. Additionally, based on the provided criteria of this study [29], all methylcellulose formulations with concentrations < 8 wt.% can be categorized as "injectable", since the required forces are < 50 N. However, at higher methylcellulose concentrations, detachment of needles may occur for syringes without Luer Lock adapters during injection, which should be avoided by selecting a suitable polymer concentration such as 7.5 wt.% (Methocel A15). This formulation also exhibited a gelation temperature slightly below body temperature as required for *in situ* gelation. No relevant hydrolytic degradation of ATP was found to occur in the hydrogel carriers. Importantly, the ATP release was scalable for a fixed hydrogel volume, i.e., different initial payloads had relatively low effects on the shape of the release curves and duration of release over 6 hours, which may match the timeframe relevant for many mechanistic cell culture studies (Fig. 4).

As an outlook for potential application in biomedical application, ATP loaded hydrogels were introduced in primary microglia cell culture as a first proof of cellular response to extracellular ATP from a controlled release system. The increase in the AMP concentration upon coincubation of microglia with the hydrogel (Fig. 5) can be justified by the action of the CD39 ectoenzyme of microglia hydrolyzing ATP to AMP. Identical values of produced AMP for gels with different ATP loading could be a hint for a saturation of the ectoenzymes in the selected setup. After completing the determination of released and converted ATP/ADP/AMP for gel samples, an equivalent dose of ATP solution was added directly to fresh P_0 culture microglia in follow-up experiments. By doing so, a rough comparison of microglia catalytic response to ATP release from gel samples and to soluble ATP was possible with slightly lower AMP concentrations being formed in case of ATP solutions. However, the lower enzymatic transformation of ATP in control samples without

hydrogels (Fig. 5) should not be over-interpreted as it may be a consequence of variations in enzymatic activity of the new microglia isolates. Overall, this set of experiments may serve as a first proof of cell response to extracellularly provided ATP from a controlled release carrier.

Conclusion

In this study, the capacity of releasing ATP by polymer-based controlled release carrier was explored either for a short-term release over hours or a long-term release over days to weeks. For PLGA-COOH, a release of ATP, but not of adenosine as its degradation product, was realized without an induction period or major burst released for extended time periods such as ≥ 2 weeks. Model hydrogels based on methylcellulose were shown to gel at body temperature, to enable variation of the release ATP quantities, and to be injectable at reasonable injection forces. In a first demonstration of potential applications, metabolic response to the extracellularly provided ATP from the release system by ectoenzymes of microglia could be shown. *In vivo* studies will have to be conducted to explore the potential of ATP carrier in biomedical applications, e.g. to direct microglia migration.

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