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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.



## 1. 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 signaling 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 an ATP concentration gradient toward 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 the basis of its capacity to store energy for catalysis of biochemical processes. At the same time, repulsive forces make it sensitive also for hydrolysis e.g. under 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 [8–12]. 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 sulfide [14] or poly(amido amine) dendrimers [15]. Since the capping of mesopores occurred via disulfide bonds, disulfide-reducing agents were used for stimuli-induced ATP release. In this technique, ATP was incompletely released

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in a timeframe of minutes to 2 h after stimulation. Although some of the dendrimer capped particles showed a trend toward 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 cell culture experiments. 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 6 k (RG 502H; inherent viscosity [i.v.] of 0.1% polymer in $CHCl_3$ 0.18 dl $\cdot g^{-1}$; weight average molecular weight $M_w$ 6.1 kDa; polydispersity PD 3.2), (ii) PLGA-COOH 17 k (503H; i.v. 0.36 dl $\cdot g^{-1}$; $M_w$ 17.3 kDa; PD 3.3), and (iii) ethyl ester endcapped PLGA-Et 11 k (Resomer RG 502; i.v. 0.22 dl $\cdot g^{-1}$; $M_w$ 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 (wt./wt.) in acetone under vigorous vortexing to obtain 1 g of polymer solution, e.g., 50% (wt./wt.) PLGA-COOH 6 k containing 500 mg of polymer and 500 mg of solvent. In this viscous solution, sieved ATP was dispersed and provided different percent loading (wt./wt.) related to the dry polymer mass. The obtained suspensions were extruded with a syringe and needle at a rate of ~100 μl $\cdot min^{-1}$ 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% (wt./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 was 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 $\eta$ 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 × 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 was 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