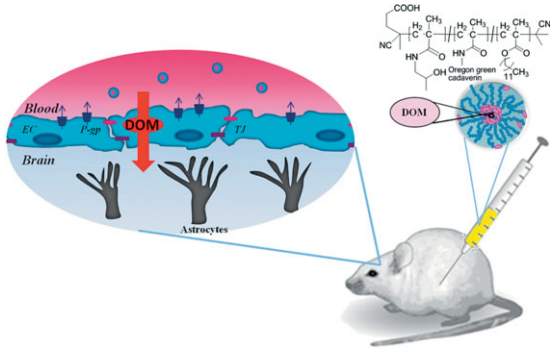
HPMA Based Amphiphilic Copolymers Mediate Central Nervous Effects of Domperidone[a]

Mirjam Hemmelmann,[b] Christiane Knoth,[b] Ulrich Schmitt,*
Mareli Allmeroth, Dorothea Moderegger, Matthias Barz, Kaloian Koynov,
Christoph Hiemke, Frank Rösch, Rudolf Zentel*

In this study we give evidence that domperidone encapsulated into amphiphilic p(HPMA)-*co*-p(laurylmethacrylate) (LMA) copolymer aggregates is able to cross the blood–brain barrier, since it affected motor behaviour in animals, which is a sensitive measure for CNS actions. Carefully designed copolymers based on the clinically approved p(HPMA) were selected and synthesized by a combination of controlled radical polymerization and post-polymerization modification. The hydrodynamic radii (R_h) of amphiphilic p(HPMA)-*co*-p(LMA) alone and loaded with domperidone were determined by fluorescence correlation spectroscopy.



Introduction

The blood–brain barrier (BBB) is a physical barrier that separates circulating blood and the central nervous system (CNS). It consists of endothelial cells around the capillaries joined together by tight junctions. The barrier efficiently restricts the exchange of solutes between the blood and the brain extracellular fluid. Functionally, it acts like a *firewall* protecting the brain against potentially harmful chemicals, but small and lipid soluble molecules may penetrate freely through the barrier via the lipid membranes of the endothelial cells. For such substances additional mechanisms protect the brain, they are trapped by specific carrier mediated efflux transporters like P-glycoprotein (P-gp) in the endothelial cells and are removed from the brain back into the bloodstream (Figure 1). A number of drugs are substrates of P-gp and therefore not applicable to treat brain diseases. One example is domperidone, a dopamine receptor antagonist, which is almost devoid of central nervous effects due to P-gp substrate properties.[1]

To overcome the limited penetration of drugs into the brain research got focused on targeted drug delivery using macromolecular carrier systems[2] to enhance the bioavailability of drugs by prolonging their blood circulation time. A number of macromolecular carriers, mostly based on poly(ethylene glycol) (PEG) and poly(*N*-(2-hydroxypropyl)-methacrylamide) (pHPMA), have already been established and entered clinical trials.[3,4]

R. Zentel, M. Hemmelmann, M. Allmeroth, M. Barz
Institute of Organic Chemistry, Johannes Gutenberg University Mainz, Duesbergweg 10-14, 55099 Mainz, Germany
Fax: +49 6131 3924778; E-mail: zentel@uni-mainz.de
U. Schmitt, C. Hiemke, C. Knoth
Department of Psychiatry and Psychotherapy, University Medical Center of the Johannes Gutenberg University Mainz, Untere Zahlbacher Str. 8, 55131 Mainz, Germany
Fax: +49 6131 176789;
E-mail: schmitt@psychiatrie.klinik.uni-mainz.de
F. Rösch, D. Moderegger
Institute of Nuclear Chemistry, University of Mainz, Fritz-Straßmann-Weg 2, 55128 Mainz, Germany
K. Koynov
Max Planck Institut for Polymer Research Mainz, Ackermannweg 10, 55128 Mainz, Germany

[a] **Supporting information** for this article is available at Wiley Online Library or from the author.
[b] Both authors contributed equally.

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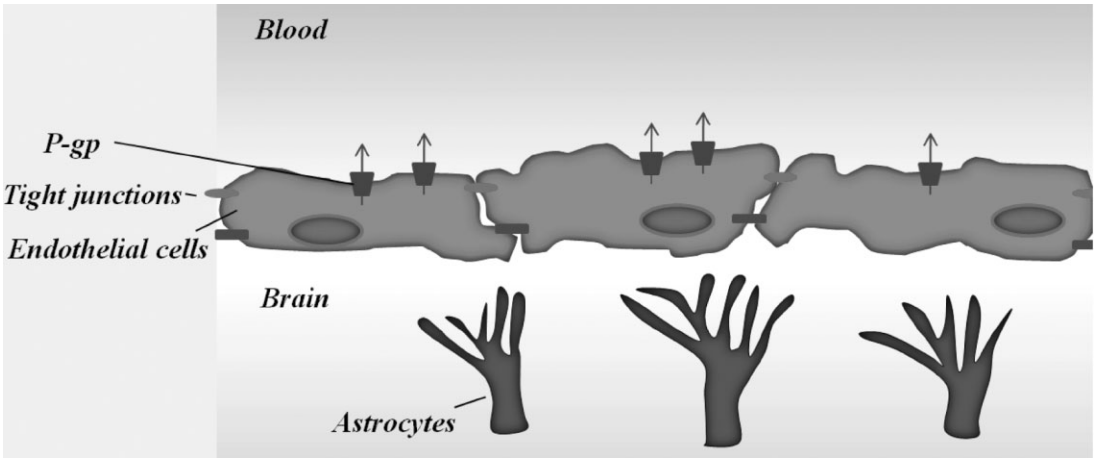


Figure 1. Schematic sketch showing the architecture of the blood–brain barrier with efflux transporters (restricted to P-gp).

Nanocarriers such as liposomes,[5] pluronic block copolymers[6] as well as poly(methyl methacrylate) (PMMA) nanoparticles[7,8] (NPs) have been developed and were tested for their potential to overcome the limits of drug delivery into the brain. Amongst others,[9] the polymer based carrier systems pluronic block copolymers[6] and poly(butylcyanoacrylate) (PBCA) NPs[7,8] have been shown to enter the brain. Although this process is not yet clearly understood it gave further insights into potential interaction mechanisms of nanocarrier mediated transport of drugs into the brain. Amphiphilic pluronic block copolymers showed increased CNS delivery in vitro and in vivo of compounds which are substrates of the efflux transporter P-gp, such as rhodamine 123, applying animal models. These copolymers are composed of a hydrophilic PEG block and a hydrophobic poly(propylene glycol) (PPG) block. It was found that the amphiphilic block copolymers possess a high affinity to membranes of brain capillary endothelial cells and inhibit P-gp by altering the membrane fluidity. Furthermore, the interaction of pluronic block copolymers with P-gp leads to conformational changes in the transporter protein which inhibits its ATPase activity. P-gp is the most prominent efflux transporter of the BBB, working under consumption of ATP as energy source.[10,11] PBCA NPs were used for in vivo delivery of the hexapeptide of dalargin into the brain. Dalargin has opioid activity and the antinociceptive effect of dalargin-loaded NPs coated with PEG-based polysorbate 80 was shown by the hot-plate test and the tail-flick test.[8] Since it was found that apoliporotein E adsorbs to the polysorbate coated NPs it was suggested that these particles mimic LDL-particles. Interaction with LDL-receptors in the BBB can lead to uptake of the particles by endocytosis.[8]

Learning from these examples, polymeric carriers which enhance penetration of the BBB need to be highly biocompatible and interact either with cell-membranes or membrane proteins enabling active transcytosis. To understand the role of the polymer and establish structure–property relations the macromolecule should be structurally and chemically well-defined. A synthetic pathway to such polymers is the combination of controlled radical polymerization such as RAFT, ATRP or NMP and post-polymerization modification.[12–14] We selected copolymers from HPMA (2-hydroxypropyl-methacrylamide), which has been under clinical approval since the 1990s and laurylmethacrylate, for which we had observed reasonable cell uptake as well as low cell toxicity recently.[14,15]

Using drugs that are unable to cross the BBB in vivo, such carrier systems can be tested for their efficiency to mediate drug delivery to the brain.[16a–c] For in vivo evaluation of CNS effects, drugs interacting with motor function are most suitable. Motor function is controlled by several neurotransmitters in the pyramidal system of the brain. Here, dopamine modulates the initiation of movement. The function can be easily quantified by the time spent in motion or the distances of horizontal movements.[17,18] When dopaminergic activity is disturbed, motor coordinating skills (in humans and in animals) get impaired. Malfunction is reflected by the neurodegenerative disease Morbus Parkinson, where movement disorders (i.e., rigor, tremor and akathisia) appear as a consequence of a loss of dopaminergic neurons. Similar motor symptoms occur as unwanted side effects under antipsychotic treatment by antagonism of dopamine D2 receptors in the brain. A well established animal model to examine central dopamine related motor functions in mice is the rotarod test.[19] It measures coordinated motor skills, i.e., the ability to balance and walk on a rotating cylinder.

For the present investigation we selected domperidone as probe drug to elucidate the possibility of transporting a drug normally not acting on brain functions across the BBB and used amphiphilic copolymers as macromolecular carrier system. The carrier, based on the clinically tested HPMA which is known to form nm-sized polymer aggregates[15] was loaded with the poorly water soluble domperidone. Polymer embedded domperidone was applied to mice and their motor coordinating skills were analysed on the rotarod. Since penetration of the antidopaminergic drug into the brain is a prerequisite for CNS actions, it was hypothesized that carrier mediated BBB penetration should be reflected by alterations in motor skills on the rotarod. Using this test system, we have previously characterized the functional role of P-gp for drugs that are substrates of P-gp.[19] In this approach, we were able to show that domperidone encapsulated in HPMA based polymers exhibits CNS activity.

Experimental Section

Materials

All chemicals and domperidone pure substance were reagent grade, obtained from Aldrich and used without further purification, unless indicated otherwise. Oregon green cadaverine was purchased from Invitrogen. All solvents were of analytical grade. Pentafluorophenol was obtained from Fluorochem (Great Britain, UK) and distilled prior to use. Dioxane and dimethylsulfoxide (DMSO) were dried and freshly distilled. 2,2′-Azobis(isobutyronitrile) (AIBN) was recrystallized from diethyl ether and stored at −20 °C. Laurylmethacrylate was distilled under reduced pressure prior to use. Motilium® (domperidone suspension, Nycomed Deutschland GmbH, Konstanz, Germany) was obtained from the hospital pharmacy.

General Synthetic Route of Statistic Copolymers

RAFT Polymerization

The synthesis of the described statistic polymers was reported previously.[15] Shortly, in a typical reaction 1.5 g of PFPMA and 0.15 g of LMA were dissolved in absolute dioxane. CTA and AIBN were added (molar ratio AIBN to CTA 1/8) and after three freeze pumping circles the reaction mixture was stirred at 65 °C over night. The reactive precursor polymer was precipitated three times in hexane, centrifuged and tried in a vacuum oven. A pink powder with a yield of 52% was obtained.

Removal of the Dithiobenzoate Endgroup

The dithiobenzoate endgroup was removed according to the procedure reported by Perrier et al.[20]

Post Polymerization Modification

The precursor polymer (280 mg) was dissolved in absolute dioxane and 5 mg of Oregon green cadaverine with triethylamine were added to the reaction mixture. After stirring for 4 h at 40 °C an excess of 2-hydroxypropylamine was added to the reaction mixture and the reaction continued over night. The final polymer was precipitated from diethyl ether twice and a yellow product was obtained (83% yield).

Preparation of Injection Solutions

A domperidone suspension (Motilium) was mixed with physiological saline for intraperitoneal (i.p.) injections. Polymer solutions: polymer alone or alternatively domperidone pure substance with polymer in a ratio of 1:2 DOM/polymer, were dissolved in DMSO. After the stock solution was vortexed for 10 s, for the mice' body weight adjusted aliquots were mixed dropwise with physiological saline in a total volume of 1 ml. Administered doses were $50\,mg \cdot kg^{-1}$ for domperidone and $100\,mg \cdot kg^{-1}$ for the polymer.

Animals

All experiments were conducted in accordance to the U.S. guide for the care and use of laboratory animals and approved by local authorities (NIH publication No. 86-23, revised 1985 and the current version of the German Law on the Protection of Animals). For the rotarod studies male FVB/N mice (25–40 g) were used. Animals were housed in groups of 2–5, having free access to food and water. A 12-h light-dark cycle was maintained at a temperature of 22 °C and a relative humidity of 60%.

Rotarod Test

Animals were trained on the rotarod (RotaRod Advanced, TSE Systems, Germany) five times a day for one week. On the following testing day mice were given an i.p. injection of either pHPMA-*co*-pLMA (Poly) ($100\,mg \cdot kg^{-1}$), domperidone (DOM) ($50\,mg \cdot kg^{-1}$), polymer-embedded DOM (PolyDOM) or saline as controls and tested 0.5 h later at five sequenced trials. Group sizes were $n = 8$–14, 42 animals in total. The test was performed by placing animals in a neutral position on the cylinder turning initially with a speed of 5 rpm. After 10 s, speed accelerated linearly up to 25 rpm within 5 s and braking down again to 0 rpm in order to change direction and accelerate in the new direction again. This cycle was repeated 11 times thus a trial lasted a maximum of 240 s. Time was taken automatically until mice fell from the cylinder. Statistical analysis was done by analysis of variance (ANOVA) followed by Students *t*-test with $p < 0.05$ using SPSS version 12.0G for Windows (SPSS Inc., Chicago, IL).

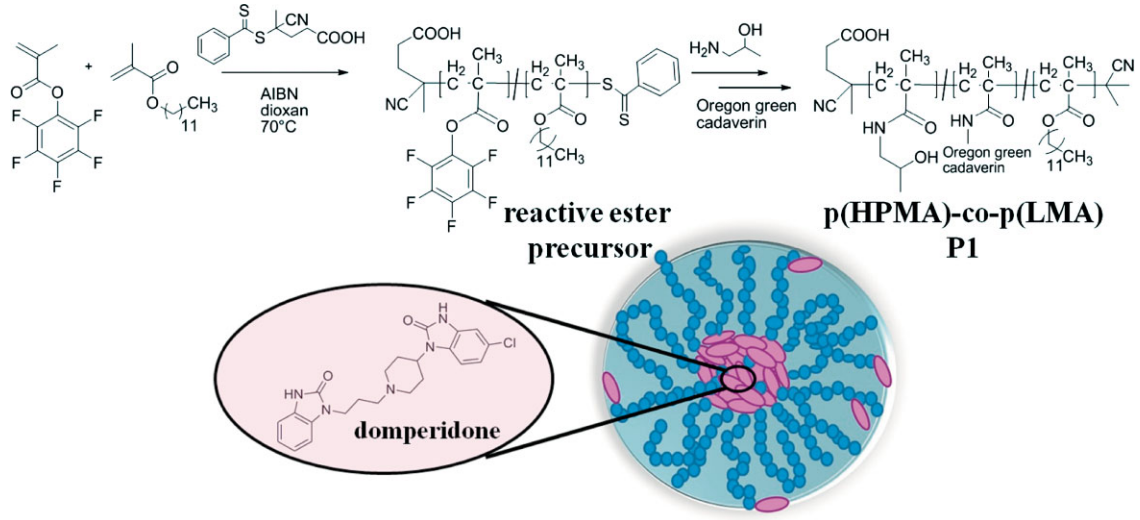
Experimental details, fluorescence correlation spectroscopy (FCS)-measurements, CMC determination, the body distribution determined by PET and the details of the experiments with the mice are shown in the Supporting Information.

Results and Discussion

Polymer Synthesis and Characterization

The amphiphilic copolymer is based on the hydrophilic clinically tested HPMA into which 10 mol-% of hydrophobic laurylmethacrylate is incorporated. It is synthesized by a combination of reversible addition–fragmentation chain transfer (RAFT) polymerization[21] of functional active ester monomers and post-polymerization modification reaction (see Supporting Information). RAFT copolymerization of pentafluorophenyl methacrylate (PFPMA) and LMA provides well characterized functional copolymers with narrow polydispersities, whose molecular weight can easily be varied. In this way two methacrylate esters are copolymerized, whereas many of the HPMA based copolymers are made by copolymerization of a methacrylamide and an acrylate ester with strongly differing copolymerization parameters. These well-defined functional precursors are transformed into amphiphilic copolymers using post-polymerization modifications (Scheme 1), whereby it is easy to introduce different functional groups such as dyes (Oregon green cadaverine 1 mol-%) or tyramine groups (3 mol-%; only **P2**) for radioactive labelling with [^{18}F]FETos[22] and in the end an excess of 2-hydroxypropylamine (Table 1).

The resulting amphiphilic copolymers with ten percent hydrophobic lauryl side chains exhibit polydispersity indices (PDIs) between 1.18 and 1.26 and a molecular weight ($\overline{M}_n$) around 14 kDa. **P1** was used for the experiment described below and **P2** for the PET-measurements described in the Supporting Information. Since the amphiphilic copolymer should function as a carrier system for the hydrophobic model drug domperidone it was important to study the aggregation behaviour of p(HPMA)-*co*-p(LMA) in aqueous solutions as well as the hydrodynamic radii of the amphiphilic copolymer alone and loaded with domperidone. The concentration dependent aggregation behaviour of the copolymer was studied using a pyrene fluorescence technique[23] in saline solution at room temperature. P(HPMA)-*co*-p(LMA) amphiphilic copolymers exhibit a critical micelle concentration (CMC) of 4.4×10^{-4} mg · ml^{-1}



Scheme 1. Synthesis of amphiphilic pHPMA-*co*-pLMA copolymers (top) with schematic sketch of its self-assembled structure in aqueous solution (bottom).

Table 1. Characterization of the polymers **P1** and **P2** given in Scheme 1.

Polymer	Mol-% LMA	Mol-% Tyr	$\overline{M}_{n\,pre}$ [kDa]	$\overline{M}_n$ [kDa]	PDI	CMC [mg·ml^{-1}]	R_h Poly[a)] [nm]	R_h PolyDOM[a)] [nm]
P1	10	–	23	14	1.26	4.4×10^{-4}	34	62
P2	10	3	22	13.5	1.18	–	–	–

LMA: laurylmethacrylate, Tyr: tyramine, $\overline{M}_{n\,pre}$: molecular weight of the precursor polymer (Scheme 1). Note that the molecular weight of the final polymers is smaller than the precursor polymers as the 2-hydroxypropylamine side group is much smaller than pentafluorophenol.[a)]Determined by FCS ($c = 0.1$ mg · ml^{-1}).

showing that these polymers form aggregates with hydrophobic cores into which drugs can be encapsulated by hydrophobic interactions. Since the aggregates are applied in doses of 100 mg · kg^{-1} (1 ml injection volume i.p. per mouse, aggregates in saline solution) it can be expected that the aggregates stay stable as they are still above the CMC even after dilution with the blood (about 2 ml per mouse with body weight of 30 g, accordant 6.6% body weight). Fluorescence correlation spectroscopy (FCS) was applied to characterize the size of the aggregates formed by the amphiphilic p(HPMA)-*co*-p(LMA) alone or polymer loaded with domperidone. The amphiphilic copolymer self assembles into aggregates with a hydrodynamic radius (R_h) of 34 nm. P(HPMA)-*co*-p(LMA) aggregates loaded with domperidone (called PolyDOM) exhibited a significant increase in R_h to 62 nm. They contained 50 wt.-% domperidone. These results clearly show that the encapsulation of domperidone into amphiphilic pHPMA-*co*-pLMA polymer aggregates making them a suitable carrier system.

Rotarod Test and in vivo Evaluation

In animal research, mice are often the model organism of choice and i.p. injection (intraperitoneal, into the abdominal cavity, see Supporting Information) is preferred in behavioural investigations with mice albeit intravenous (i.v.) injection of drugs is feasible as well. The ability of HPMA based copolymers accessing systemic circulation after i.p. injection had already been reported.[24] This is supported for our system by first positron emission tomography (PET) experiments using small animal μPET to look for the body distribution of ^{18}F labelled polymer (**P2**) whereby accumulation in the kidneys and bladder of the mice ($n = 3$) was observed (Supporting Information) which is essential due to the fact that it is necessary that the domperidone loaded aggregates first reach the blood stream via the lymphatic system before they can reach the brain. Since the polymer can only reach the kidneys and bladder via the bloodstream, i.p. administration appears to be suitable for this potential new drug carrier system.

Mouse behaviour was analysed in the rotarod test of mice either treated with saline (controls), domperidone alone or domperidone encapsulated in polymer. To rule out effects of pure polymer, mice treated only with polymer were included in the analysis. Penetration of domperidone into the brain was indicated by a marked loss of motor skills in the group of mice treated with polymer encapsulated domperidone (PolyDOM), whereas no significant motor impairment was observed after administration of the polymer (Poly) or domperidone (DOM) alone (Figure 2). Overall analysis of variance (ANOVA) revealed significant treatment effects ($F_{(4;46)} = 3.793$, $p < 0.01$), and post-hoc analysis indicated significant differences ($p < 0.05$) between controls or polymer treated mice and PolyDOM

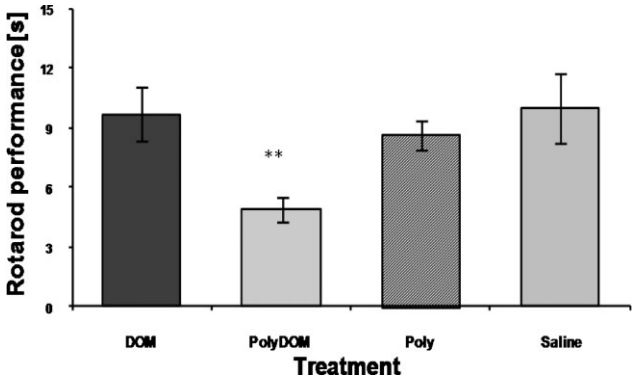


Figure 2. Rotarod performance 0.5 h after i.p. injection of pHPMA-*co*-pLMA, domperidone or PolyDOM (DOM 50 mg · kg^{-1} respectively) FVB/N mice; bars represent mean ± SEM seconds $^{**}p < 0.01$ compared with control mice, following Student's *t*-test.

treated mice. Only polymer-embedded domperidone exhibited a significant pharmacodynamic effect whereas the drug alone was obviously not able to enter the brain. Using the most sensitive animal model to evaluate in vivo drug effects we were thus able to show that encapsulation of an otherwise not CNS active drug caused behavioural changes. This can only be explained by CNS activity of the drug which required passage of the BBB.

Conclusion

The described observations taken together clearly indicated that the system worked. Encapsulation of domperidone in well-characterized copolymers obviously facilitated the passage of the drug into the brain. Furthermore, ongoing transport studies using an in vitro model of the BBB consisting of human brain microvascular endothelial cells (HBMEC) showed enhanced transport of agents encapsulated into the polymer. These findings stress the potential of the polymer.[25] The mechanisms of transport and whether the polymer itself crossed the BBB still need to be clarified. It may be assumed that polymer aggregates, carrying hydrophobic groups on their surface due to intra- and interchain interactions have affinity to cell-membranes. Because of their amphiphilic nature, similar to pluronics, interactions with cell membranes and efflux transporters is a possible way to mediate the transport of domperidone into the brain. They also might mimic or bind physiological structures leading to interactions with receptors at the BBB as it was already shown for polysorbate 80 coated PBCA NPs.[8,26]

Further studies, especially in vivo characterization of the copolymer aggregates, are required to give direct evidence that domperidone encapsulated in amphiphilic p(HPMA)-*co*-p(laurylmethacrylate) (LMA) copolymer aggregates

crosses the BBB. This was so far shown indirectly by behavioural changes in the rotarod test. Since clinically tested HPMA copolymers were used, synthesized under well-controlled conditions, it is also possible to vary the chemical properties of the carrier for further in vivo applications.

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