One of the most important goals in drug delivery is to carry drug molecules to their target as selectively and efficiently as possible. To reach this objective, our research was started in different directions in collaboration of different research groups and institutions. The results will be presented focusing on the following investigated subjects:

- second order interactions between model polymer (linear poly(N-isopropylacrylamide) – PNIPA) and small molecules in the solution state
- effect of additives and external stimuli on PNIPA gel
- host-guest interaction in the solution and solid state
- synthesis and use of hyperbranched polyglycerol (HbPG)
- drug release

Second order interactions between model polymer (linear poly(N-isopropylacrylamide) – PNIPA) and small molecules in the solution state

One of the major factors which influences the properties and potential applications of carriers is their interactions with their loading. As a first step, interactions between a thermoresponsive drug carrier polymer, poly(N-isopropylacrylamide) (PNIPA) and small aromatic probe molecules: phenol, dopamine and indole derivatives including tryptophan were studied by solution-state NMR spectroscopy. These substances represent structural elements often found in pharmaceutically relevant compounds. Phenol and dopamine were chosen to test the NMR methods (from previous studies their behavior in gels was known). Firstly, the NMR methods which are commonly used in the field of biomolecular binding studies were tested on these model systems. To study the effect of temperature on binding and the significance of coil-to-globule transition, ^{1}H relaxation times (T_1 and T_2), one- and two-dimensional Nuclear Overhauser Effect spectroscopy (NOESY) and diffusion ordered spectroscopy (DOSY) measurements were carried out in D_2O. In the case of phenol all the above mention methods detected the existence of strong host-guest interaction, while in case of dopamine the absence of interaction was observed. T_1 relaxation time measurement was found as the most sensitive and time efficient method to detect secondary interactions.

As a next step indole and some of its water soluble derivatives were investigated by relaxation measurements. The indole ring is an important part of biologically active natural products, it can be found in several plants and animals. Indole interacts with the polymer only above the temperature of the coil-to-globule transition, which is shifted to lower temperature (22.5 °C instead of 33.4 °C). The two substituted indoles, 5-hydroxyindole and 5-aminoindole

showed similar behavior to indole, however there was detected a weaker interaction with the polymer. The LCST was shifted to lower temperature in the presence of the two substituted indoles too, but it was higher than in the case of the PNIPA-indole system. Although the two indoles showed different behavior according to UV-VIS measurements, they behaved similarly at 20 and 40 °C shown by NMR relaxation studies. Amino and hydroxyl groups differently change the hydrophilic – hydrophobic character of the polymer when bound, resulting in different LCST values, however the strength and quality of the interaction between the indole core and the polymer is similar in case of both molecules at these two temperatures. Adding an H-bond donor and acceptor group such as hydroxyl or amino, thus making the small molecule more hydrophilic promoting an interaction with the polymer below the transition temperature. Hydroxyl- and aminoindole have two functional groups to interact with NIPA. Two dimensional and one dimensional selective NEOSY experiments showed that both molecules behave similarly. No special interaction was detected between polymer and the small molecules, the polymer surrounds the small molecules and also some water was found in this "inclusion" (indole behaves similarly). Most probably the small molecules are separated from each other thus the crystallization will be hindered in dried polymer. Tryptophan was interacting weakly with the polymer and only above the LCST. Spin-lattice relaxation times (T_1) showed that only the indole ring interacts with the polymer, while the aliphatic amino acid part behaved similarly to polymer-free solutions. The absence of interaction between the amino acid part and the polymer explains the smaller effect of tryptophan on the LCST of PNIPA. Interaction between PNIPA and tryptophan were also studied in two organic solvents: (dimethyl sulfoxide)-d6 and methanol-d4). In the two organic solvents where the phenomenon of coil-to-globule transition does not exist, no difference can be seen between chemical shifts and T_1, and T_2 relaxation times of tryptophan in polymer-containing and polymer-free solutions, which implies the role of water in the binding process.

Our methodological conclusions in the investigation of secondary interactions between small molecules and macromolecules are the following:

1. In case of "strong" secondary interactions the commonly used techniques (1D - 2D NOESY, ROESY and diffusion ordered spectroscopy (DOSY) measurements gave fast and exact results. The interacting functional groups could be identified.

2. In case of "weak" secondary interactions only the relaxation measurements were found to be reliable techniques to give satisfying results. The interacting functional groups may be identified in some cases.

Effect of additives and external stimuli on PNIPA gel

The PNIPA gels are produced by using bifunctional cross-linker molecules. By the proportion of the cross-linker molecules the physico-chemical properties of the gel can be modulated. Effect of the cross-link density on the loading and the release properties was studied with phenol and ibuprophen probe molecules. The gel samples were swollen in a solution containing the active substances, then the solvent was evaporated. As the thermogravimetric, differential scanning calorimetry and X-ray diffraction suggested the size of the crystallites depends on the cross-link density in case of ibuprophen while in case of phenol no cristallyzation can be detected. The cross-link density has also a decisive influence on the release profiles. Release from the polymeric matrix was found approximately the same in case of ibuprofen while the release of phenol was strongly dependent on the cross-link density.

Dry and swollen (from the equilibrium state) lyophilized PNIPA networks with different cross-link densities were investigated by positron annihilation spectroscopy (PAS). Positrons are sensing free volumes (space with low electron density) for a few Angstrom – while the large holes in the lyophilized samples are invisible by PAS. The distribution of free volumes seems to be independent from the cross-link density, but the answer of the network is different to the vapor uptake from the air. Distribution of the free volumes changes faster with decreasing cross-link density.

The swollen PNIPA gel has a weak mechanical stability, thus mechanical stress caused by the volume phase transition can damage the integrity of the gel. Composite systems were designed to increase the mechanical stability of the gels retaining the responsive properties. Reduced graphene oxide (RGO) containing composite hydrogels, based on poly(N-isopropylacrylamide) (PNIPA) were prepared by two different methods: i) by incorporating RGO directly into the polymer matrix; ii) applying a post-synthesis reduction of the graphene-oxide (GO) already incorporated into the polymer. Results from microscopic (small angle neutron scattering, differential scanning calorimetry, ^{1}H NMR spectroscopy, thermogravimetry) and macroscopic (kinetic and equilibrium swelling properties and mechanical testing) measurements show that the dispersity of the nanoparticles as well as their interaction with the polymer chains are influenced by their surface chemistry. Incorporation of nanoparticles limits the shrinkage and slows down the kinetics of the thermal response. Both thermogravimetric and solid-state ^{1}H NMR measurements confirmed strong polymer – nanoparticle interaction when hydrophilic GO was used in the synthesis. In these

cases, the slow thermal response may be explained by the decrease of the free volume inside the nanocomposite matrix caused by a hypernodal structure. Our results imply that both the chemistry and the concentration of the incorporated graphene derivatives influence the thermal responsivity of PNIPA.

The thermal response of graphene oxide (GO) – and carbon nanotube (CNT) – poly(N-isopropylacrylamide) composite systems was investigated (the effect of the nanoparticle filler content, both on the nanoscale and the macroscopic level) in a systematic study. While the equilibrium swelling properties of the different nanocomposites are only slightly influenced, the kinetics of the response of the swelling medium following an abrupt temperature increase from 20 to 40 or 50 °C can vary within wide limits depending on the type or the amount of nanoparticle loading, as well as on the temperature difference.

Beside of the increased mechanical stability of PNIPA gels by graphene derivatives, the effect of cross-link density on the size of API crystals was also investigated in more detail. Although the cross-link density was varied between 50-150, the difference in size of the crystals was shown to be only about 20 %. As the positron annihilation measurements revealed, the size of free volumes (space between polymer chains) shows also 20 % difference depending on the cross-link density. Most probably the distribution of the cross-link is not homogeneous, the synthetic method needs further improvements.

Host-guest interaction in the solution and solid state

Despite having a hydrophilic-hydrophobic transition, API's with high logP value are not concentrated in the PNIPA polymer globules even above the lower critical solution temperature. Morphology of API's with high logP value is a critical parameter from the solubility point of view. The amorphous form has a higher solubility in body fluids, thus better bioavailability. Different types of potential drug carrier materials were tested.

Cyclodextrines (monomer and polymer) increased the solubility of poorly or even insoluble API's forming inclusion complexes (confirmed by NOE experiments) in water. Curcumin is a water insoluble natural molecule with antioxidant properties and a possible medical use. Cyclodextrines are able to form inclusion complexes with hydrophobic molecules, so the complexes become soluble in water. Their complex formation with curcumin has already been known, but the quantitative spectrophotometric investigation is hindered by the change of optical properties caused by complexation. Maximal solubilization efficiency of α-, β-, and γ-hydroxypropyl cyclodextrines and their polymers was determined by ^{1}H NMR. γ-HP-CD was proved as the best solubilizer (approximately two CD enclose one

curcumin molecule). Proximity of the aromatic rings and the CD was found by NOESY and ROESY experiments.

These materials were also tested for water soluble drugs as well. In this case no inclusion complex was formed in the solution state, however secondary interactions could be revealed in the solid state. CD's act not only as plasticizer for poly(vinyl alcohol) fibers produced by electrospinning method, but the first results revealed a multistep release of API. Electrospun nanofibers from PVA were prepared by using polysorbate 80 (PS80) and hydroxypropyl-β-cyclodextrin (HP-β-CD) as additives. Atomic force microscopy (AFM) revealed the viscoelastic nature of the fibrous samples. At relatively low forces mostly elastic deformation was observed, while at higher loads plasticity predominated. The use of polysorbate led to about two times stiffer and less plastic fibers than the addition of cyclodextrin. The ^{1}H–$^{1}3C$ nuclear magnetic resonance (NMR) cross-polarization build-up curves pointed out that cyclodextrin acts as an inner, while polysorbate acts as an outer plasticizer. Due to its "liquid-like" behavior, it can migrate in the polymer-matrix, which results in the less plastic behavior of this formulation. Positron annihilation lifetime spectroscopy (PALS) measurements also confirmed the enhanced mobility of the polysorbate and the molecular packing enhancer properties of the cyclodextrin. Solid-state methods suggested amorphous precipitation of the active ingredient in the course of the electrospinning process. Furthermore, the nature of the amorphous systems was verified by NMR spectroscopy, which revealed that the use of the examined additives enable the development of molecularly dispersed systems of different homogeneities.

By summarizing our results, we can conclude that even a relative weak H-bond between macromolecules and small active molecules can hinder the crystallization of the active ingredients. Amorphous form of active pharmaceutical ingredients (API's) containing hydroxyl, amine and heterocyclic NH groups can be stabilized with a well-chosen macromolecular matrix. Using solution state NMR measurements, this behavior can be predicted as the example of tryptophan shows. No interaction can be detected between poly(N-isopropylacrylamide) (PNIPA) and tryptophan by either optical spectroscopy or thermal analysis techniques. However drying of PNIPA gel swollen in aqueous tryptophan solution resulted in amorphous structure of the amino acid.

Synthesis and use of hyperbranched polyglycerol (HbPG)

Hyperbranched polyglycerol (HbPG) is a polyether polyol, which contains a large number of secondary and primary hydroxyl groups. Besides of outstanding water solubility and approved biocompatibility (blood compatible, non-immunogenic, non-toxic), the simple and modular synthesis of HbPG is also a great advantage of this polymer. This branched polymer is also recommended to replace of polyethylene glycol (PEG) in several application fields. The main advantage of HbPG, compared to the linear PEG, besides the known disadvantages of the linear polymer, is that its physical or chemical properties can be further tuned by derivatization through the branched structure and the high number of functionalities.

Phthalimide monofunctional hyperbranched polyglycerol was synthesized by ring opening multibranching polymerization of glycidol. Our results confirmed that the applied new synthetic method is suitable for well-defined monofunctional macromolecules in the 1-6 kDa molar mass range, with high degree of branching determined by various independent methods. The transformation of phthalimide functional group to a primary amine by hydrazinolysis was also confirmed. The modification of monoamine HbPG to produce carboxylic, maleimide and chloroacetamide functionalities were performed quantitatively, proved by 2D NMR spectroscopic analysis. The resulting monofunctional HbPG may apply for increasing water solubility, stability and/or biocompatibility of bioactive molecules, such as proteins, drugs or dyes, and other materials e.g. catalysts which can be linked to the developed functional groups of the polymer. Because these polymers are viscous liquids, we started a research to study the possibility of producing solid pharmaceutical form for release investigations.

Hyperbranched polyglycerol (HbPG) was also investigated as affinity based stabilizer for various poorly soluble drugs in solid dispersions (for synthetic details see the next point). 100 mg of caffeine, theophylline, ibuprofen sodium or diclofenac sodium (with increasing capacity to form hydrogen bonds, respectively) were incorporated in HPMC free films containing 0, 50, 100 or 200 mg HbPG as adjuvant. The molecular interactions were tested with FT-IR, while XRPD was used to study the texture of the films. The results revealed that caffeine and theophylline exhibited no affinity for HbPG which caused a repulsion of the drug from the film texture. In contrast, ibuprofen sodium and diclofenac sodium showed high affinity to HbPG, thus were stabilized in amorphous form which was resulted in an improved dissolution rate. To conclude, HbPG may be a promising stabilizer of drugs with strong hydrogen donating capacity, while may decrease the stability of drugs with low ability of hydrogen bond formation.

HbPG-PTHF-HbPG forms micelle in water with the diameter of 10-20 nm depending on the size and chain length of components. Solubility of curcumin can be increased by at least two orders. The majority of curcumin is enclosed in the polyether core but 4-8 % in the HbPG shell. This latter part was found more mobile by NMR measurements, which give us the hope to construct of a non-monotonous release system.

Drug release

Polymer networks/hydrogels are promising matrices for regulated drug delivery but there are several factors which are less taken into account. As matrix material the poly(N-isopropyl acrylamide) while as drug indole derivatives (indole, 5-aminoindole and 5-hydroxyindole) were chosen. The gel has been swollen in solution of the indoles and dried out. Our previous results suggested amorphous structure of the indole derivatives which conjecture was proved by XRD and solid-state NMR measurements. The strong interaction between the polymer and the drug molecules was previously investigated by DSC, solution-state NMR (relaxation and NOESY measurements) and UV-VIS spectroscopy. The strong interaction slows down the release of drug molecules but these interactions can not only hinder but prevent the release process. Direct correlation was found between the strength of secondary interactions and release rate and ratio. Indole forms the strongest interaction with PNIPA, its release rate is the slowest and only 10-15 % of the dug released within six hours. 5-aminoindole forms the weakest interaction and its release ratio is 100 % (5-hydroxyindol forms medium strong interaction, 70%). On the one hand, these results are consistent and show the importance of investigation and better understanding of drug-matrix interaction. On the other hand the release rate of amino- and hydroxyindole was found approximately the same. This led us to widen our focus and investigating the role of water as swelling medium of the gel and medium for the release process of drug. Release ratio of the indols from swollen gels were found 100 % in all cases (with different rate). Drying of the drug containng gel can produce inclusions which have slower drug release rate.

Intensive study has been carried out to investigate the loading of drug molecules in micelles. As model drug indole derivatives with different logP, as micelle HbPG-PTHF-HbPG (hyperbranched polyglycerol-polytetrahydrofuran triblock polimer) were chosen. Drugs with low logP value were added directly to the aqueous solution of micelle, while water insoluble drugs were added in a small amount acetone or methanol. The volatile solvent was removed by bubbling nitrogen trough the solution. The optically clear solutions were studied by NMR techniques (NOESY, ROESY, DOSY, T_1 and T_2 relaxation). The

hydrophilic drugs do not penetrate the micelle, no interactions were found. The hydrophobic drugs penetrate the polyglycerol wall of the micelle as the NMR line shape and the relaxation measurements have been proven. NOESY experiments were proven that the drugs are solubilized in the PTHF phase. NMR signals of PTHF were also distorted indicating penetrating drug molecules in the core of micelle. Here we have to note, that the extremely hydrophobic drugs (like vinpocetine) penetrate the micelle, but the solubilization efficiency was given less effective. Cause of this weak solubilization is most probably the logP value of the core of micelle. A project was to start using more hydrophobic polymers as core in the block copolymers, but the synthetic work goes beyond the limit of recent research project. In my opinion, the most remarkable finding is that in a narrow logP range drug molecules show spatial distribution between water and micelle. Two series of signals were observed in several cases with different line shape and relaxation properties. Temperature dependent and NOESY (EXSY) measurements indicate active exchange between micellar and aqueous phase. The distribution of the drugs and the exchange were found dependent on logP.

This latter finding led us to preparing complex systems with multistep release characteristic. The aqueous micellar system itself is suitable for multistep release for example as nasal spray but we tried to prepare a solid form of the system applicable as mucoadhesive system. Solution of polyvinyl alcohol micellar solution were mixed together and dried out to a film. The mixture and the film were investigated with small angle X-ray scattering and NMR methods. In both cases the micellar structures could be identified but in the film a higher ordering was also found. Most probably the relative slow drying process led to these structure formations. To hinder this process electrospun nanofibers were prepared. The nanofibers were analyzed by scanning electron microscopy (SEM) and maximal micelle content was determined. High micelle content resulted instable fibers. Nanofibers were also prepared with micellar solution containing indole derivatives with different logP. The indole derivatives have amorphous structure in all cases independently on the spatial distribution of the drugs. Both a PTHF and the PVA hindered the crystallization of the indoles. The phase separated structure (independent HbPG-PTHF-HbPG and PVA phases) was proven by solid-state ^{13}C cross polarization magic angle NMR measurements. The build-up curves of the components were shown no difference between electrospun fibers and pure components.

Experiments under buccal administration condition (temperature, pH) were started to investigate the release properties of nanofibers containing filled micelle and compared with the pure drugs (polymeric components cannot penetrate the membrane). The test molecules were chosen to covering the logP range of 1-2.5. The first results show controlled release for

all the investigated drug molecules (tryptophan, tryptamine, gramine, 5-nitroindole). The hydrophilic tryptophan does not penetrate the micelle while 5-nitroindole occurs exclusively in the micelle. The other indole derivatives show distribution between inside and outside the micelle. These release experiments are still in progress, but the results show that solution rate of the hydrophobic 5-nitroindole was increased by two order.

Scientific dissemination of the project

Scientific papers: 11 (IF_{total}:39.124)

Conference papers: 15

PhD dissertation: 2

BSc and MSc thesis: 4

Scientific Students' Associations work: 3

Here I have to note, that all the risks mentioned in the research plan were handled, but the pandemic situation was not foreseen by anybody. This hindered the advance in the project. Some experiments are still in progress (reproducibility tests needed for scientific publication). One paper is under revision, two manuscripts are under revision by the coauthors and two manuscripts are waiting for finishing of the experiments.