Development and Adaptation of Thermoresponsive Nanogels for the Treatment of Inflammatory Skin Diseases

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1 Introduction

Topical administration of drugs is attractive and occasionally inevitable application due to the targeted and local therapy of inflammatory skin diseases. Yet, the function of skin as a natural and protective barrier does not allow the sufficient absorption of drugs to the site of action. Attempts to improve the per-cutaneous absorption of drugs vary among a broad range of approaches. Most approached are designed to overcome the barriers of the skin targeting the upper-most physical barrier, the *stratum corneum* (SC), as well as tight junctions, and the metabolic barrier (enzymes).[1-2] The main obstacle for reaching therapeutically relevant drug concentrations, and simultaneously avoiding undesired side effects, is the nature of commonly used drugs for autoimmune skin disease conditions like atopic dermatitis or psoriasis. Most small and medium molecular weight (Mw) drugs are of a hydrophobic nature, which challenges their solubilization for efficient transport. On the contrary, high Mw therapeutic molecules, i.e. biomacromolecules, suffer from extremely low bioavailability caused by the effective barrier function of the SC. The recently published protocol by the food and drug administration (FDA) specified that the vast majority (59%) of nanoparticle based formulation submissions are indicated for the intravenous use, followed by 21% for the oral administration, and only 4% for the topical delivery of drugs.[3] This emphasized the challenge in the cutaneous administration route, with Estrasorb, a micellar nanoparticle formulation for the delivery of estradiol, being one of the only efficacious examples.[4-5] Here, recent advances for dermal delivery of drugs with the aid of polymeric nanocarriers will be discussed. The focus will be given explicitly to polymeric nanocarriers while keeping in mind the essential research in the field of other particulate formulations such as solid lipid nanoparticles, nanocrystals or inorganic nanoparticles.[6-8] A critical discussion will be addressed to polymeric nanoparticle properties required for efficient dermal drug delivery.

1.1 Nanoparticles as Drug Delivery Systems for Topical Administration of Drugs

1.1.1 Skin Penetration Mechanisms of Nanoparticles

The penetration enhancement process of topically applied nanoparticle formulations is mainly governed by the skin barriers to overcome. Among them are physical barriers, tight junctions, and the metabolic barrier.[1] Barriers are to prevent external threats as physical damage, penetration of chemicals and pathogens. From the organism perspective, the barrier function is to prevent excessive desiccation. Under certain pathological conditions these barriers suffer from insufficient

functionality, especially on lesional skin, leading to a selective permeation through the barrier. Topically applied nanoparticles first come in contact with the SC, which is the first physical barrier of the skin that constitutes the rate determining step for penetration.[9-10] Colloidal nanoparticles were shown to be an effective tool in overcoming this barrier by underlying mechanisms, i.e. intercellular, intracellular, or the follicular (appendageal) pathways (Figure 1).[11-13] In particular, soft particles are able to penetrate through narrow channels due to their deformability.[14-16] In contrast to that, rigid nanoparticles preferably accumulate in the follicular openings and skin furrows.[17]

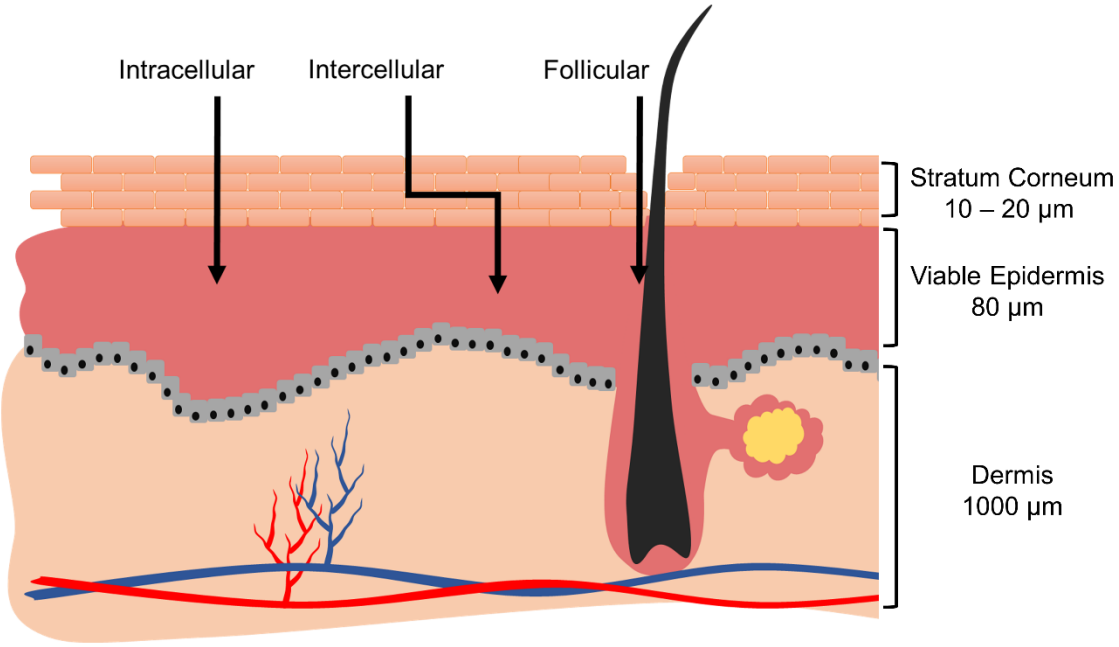


Figure 1. Schematic representation of major skin penetration pathways.

Though many studies report on a certain penetration mechanism, the analytical detection methods are often limited to fluorescence signal or the quantification of the penetrated moiety in the acceptor medium of the experimental setup. However, the structural integrity of the SC is based on the interaction and the close packing of lipids and proteins, which require a closer look on their deformation upon treatment with particulate formulations. The lipid-protein-partitioning theory postulates the following interactions as a foundation of penetration enhancement: interactions with intercellular lipids, interaction with the extracellular keratin, and the extraction of the skin components leading to increased solubility of the drug.[18-20] Emerging technologies and the development of the existing ones allow a more precise localization of active compounds in skin

sections. One example is the use of a combination of multiphoton tomography with fluorescence lifetime imaging microscopy (FLIM), which allowed to detect internalized nanoparticles in keratinocytes and the delivery of siRNA through intact human skin.[21] As high resolution methods, cross correlation - raster image correlation spectroscopy (CC-RICS) and stimulated emission depletion microscopy (STED) could be employed to localize lipid based vesicles in the SC.[22-23] However, no evidence of intact particles could be detected, suggesting their disassembly and integration with the lipid bilayer of the SC. For this reason, it is crucial for the understanding whether the nanoparticles act as carriers for the loaded compound or indirectly enhance the penetration by disrupting the skin barrier.

Many label-based spectroscopic, microscopic, as well as spectromicroscopic methods employed for the detection and characterization of nanoparticles in complex biological media and microstructures of the SC components suffer from low spatial resolution or the influence of the labeling moiety on the experimental readout. Label free methods allow the examination of skin sections and collection of structural information at a molecular level.[24] For the absorption in the infrared (IR) range, the confocal Raman spectromicroscopy enables the measurement of molecular concentration profiles and their spatial organization.[25] Identification of the spectral signatures for a certain component was already employed for the characterization of abdominal human skin and differentiation of tumor from a healthy brain, however, the potential of the technique could potentially reach broadened applications.[26-27]

1.1.2 Role of External and Internal Triggers

The utilization of responsive materials becomes extremely relevant for the topical delivery of drugs. External stimuli to which materials respond, such as temperature modulation or irradiation can be readily applied and regulated due to the accessibility of the organ. Internal stimuli, such as pH gradients, redox conditions or specific enzymatic activity are well studied at the cellular level or in tumor environments.[28-29] For the skin however, such conditions are much less understood, in order to efficiently use them as tools for modulating material properties for triggered delivery of drugs. The complexity rises not only from the specific pathogenesis of skin diseases but also due to the immobile environment driven by slow diffusion processes. Nevertheless, internal and external stimuli are used for degradation or structural modification of the carrier system causing to a release of the cargo.

Nanoparticle carrier systems based on polymeric building blocks, can either consist of individual polymer chains forming a colloidally stable suspension of the formed nanoparticles, or be crosslinked via stable, degradable, or physical bridges forming a large unimolecular architecture. Each system should be carefully examined to conclude whether the transport properties are mechanistically driven by a single carrier or by aggregation of many. These parameters are substantial not only for the synthetic methodology of the referred carrier, but also for the fate of the carrier and its building blocks upon dermal application. While the utilization of commercially available polymers approved for human use ease the manufacturing procedure and prohibit toxicity issues, the development of new materials designed by taking into consideration triggering modalities contributes to innovative delivery systems and a better understanding of their behavior in a complex biological environment.

Whether or not topically applied polymeric carriers shall consist of biodegradable units can be subjected to debate. On the one hand, the degradation of polymers into non-toxic building blocks is a straight forward approach for releasing the encapsulated cargo specifically at the desired target site. It also allows the clearance of the carrier system units once they reach the systemic circulation. On the other hand, most studies do show almost exclusive accumulation of nanoparticles in the uppermost SC, with no or very limited evidence of deeper penetration. In that scenario, the carriers deliver the drugs to the border of the stratum lucidum and only allow further diffusion of the drug to the viable epidermis. Therefore, the degradation of the polymers to low molecular weight building blocks could cause their penetration to the viable tissue and lead to potential immune response.

In order to obtain nanoparticles that are degradable, one can make use of intramolecular bonds that are susceptible to break upon exposure to certain conditions. One strategy is the introduction of acid labile bonds, which are stable under neutral pH and undergo hydrolysis at different acidic pH ranges depending on the substituents of the functional groups. Commonly used acid labile bonds are acetals, ortho-esters, imines, hydrazones, and cis-aconityls.[30] The pH of the skin however, is acidic on the top while gradually reaching a neutral value in the viable epidermis. This situation is true for healthy skin, while diseased skin suffers from elevated pH values.[31] Esters and carbamates provide a more robust alternative to labile bonds, preserving the unimolecular nature of the carrier,

and at the same time constituting a digestible network of polymers.[32] Depending on the accessibility and molecular structure, the metabolic hydrolysis of carbamate and ester based conjugates takes place at prolonged half lifetimes under physiological conditions.[33-34] The intact carriers accumulated in the SC are removed from the skin surface by a process known as exfoliation, the constant removal of corneocytes, being replaced by terminally differentiated cells from the basal layer.[35] This process is enhanced by friction forces applied on skin as a consequence of washing and contact with fabrics. Particles accumulated in the hair follicles reside for longer terms, being eventually removed by sebum flow and hair growth. Such clearance routes might be interrupted when polymeric nanoparticles prone to degradation, penetrating into the deepest tissue layers.

Trigger based structural modification of polymers is another strategy to release drugs from polymer networks. This can be done by exploiting the physiological conditions of the skin such as temperature and pH gradients (Figure 2).[36-39] Other targeting modalities can be utilized for the structural modification of lipid organization by SC hydration.[40-41] The swelling/deswelling of crosslinked polymeric networks as nanogels (NGs), is accompanied by diffusion of water into the carrier or out of the carrier leading to the release.[42-45] pH or thermoresponsive polymers, which are tuned to undergo a phase transition at a physiologically relevant conditions show this behavior. Yamazaki *et al.* described the application of dual responsive polymers in microenvironments of the skin.[46] In her work, the methacrylate based methoxy diethyleneglycol methacrylate could provide temperature sensitivity, taking advantage of the natural temperature gradient form 32 °C on the top of the skin to 37 °C in the epidermis. While the pH sensitivity was provided by methacrylic acid units, tuned to release the encapsulated content under acidic environment of the melanocyte endosome. Similarly, thermoresponsive polymers showed promising results as delivery systems being able to migrate across the SC of porcine ear skin.[47]

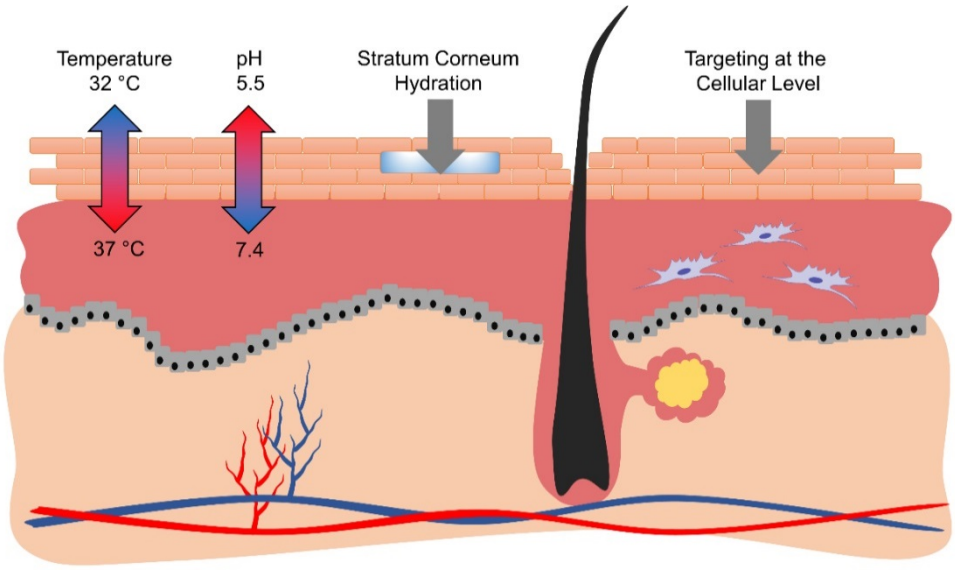


Figure 2. Approached for cutaneous triggered delivery of drugs.

Triggers may also play an important role for regulating delivery of drugs at the cellular level. The therapeutic activity of most drugs is related to a site specific cellular delivery. Hence, the utilization of external triggers for directing the payload to the site of action at a cellular level by inducing a specific uptake mechanism constitutes a fascinating, yet unexplored field. The uptake mechanism of polymeric nanoparticles is of main importance for their intracellular fate, the intracellular delivery of active compounds, and finally the secretion of the metabolized building blocks. Systematic studies have been performed to elucidate the influence of size, charge, and shape of nanoparticles on endocytosis mechanisms, while stimulated approaches to intervene, and therefore influence their cellular pathway represent an attractive strategy.[48-49] It has been shown that the nature of the polymeric particle, as well as the cell type, can have a significant impact on endocytosis. Endocytosis mechanisms substantially differ from each other regarding their molecular regulation basis, but all result in the formation of intracellular vesicles navigating in the plasma. Two main categories of endocytosis are distinguished by their dependency on a vesicle-coating structural protein called clathrin. The clathrin dependent uptake is considered to be the classical route of cellular internalization of some essential nutrients as well as vast majority of polymeric nanoparticles.[50-51] Apart from clathrin dependent mechanisms, there are also clathrin independent pathways, one of which is mediated by so-called caveolae. Originating in cholesterol rich plasma membrane regions, the hairpin structured protein caveolin-1 is able to surround particles of about 80 nm bound to the caveolae surface.[52-53] This mechanism is particularly

interesting because it gives rise to the opportunity of cargo to bypass lysosomal vesicles with high acidity, therefore being especially attractive for the delivery of proteins and genes.[54] Moreover, being the dominant trans-endothelial pathway, nanoparticles internalized via caveolae are attractive for trans-vascular delivery of drugs.[55]

1.1.3 Nature of the Carrier: Size, Shape, and Surface

Most studied polymeric nanoparticles are fabricated from well tolerated polymers like poly(N-isopropylacrylamide) (pNIPAM), poly(acrylic acid) (pAAc), poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), poly(ε-caprolactone) (PCL), chitosan or other carbohydrate derivatives.[47, 56-60] Apparently, delivery systems composed of such building blocks were able to increase the concentration of active molecules in the viable tissue in a sustained manner while avoiding severe irritation of the skin towards the applied formulation. An increasing amount of publications reporting successful delivery of challenging drug formulations has to be examined carefully for obtaining conclusive knowledge. This is due to different choices of the studied parameters and the non-standardized protocols having a significant impact on the experimental results. Such parameters vary from the used skin model, the therapeutic or model molecules used, the applied formulation, and the physicochemical characteristics of the polymeric carrier. Nevertheless, the importance of the various models should not be underestimated. While excised human skin provided a close approximation towards the development of drug delivery systems for human use, reconstructed skin models are powerful tolls for mimicking diseased conditions and systematic analysis.[61-62] Table 1 present recent studies reporting the utilization of polymeric nanoparticles for topical delivery of drugs, as well as their interaction with skin components.

Table 1. Topical delivery systems based on polymeric nanoparticles.

Polymer Architecture	Size and Surface Charge	Formulation	Skin Model	Drug	Nanoparticle Skin Penetration	Ref
PLGA						
Multi-layer nanogels	183 nm, 5.34 mV	Hydroxypro-pyl methyl-cellulose and Carbopol	*In-vivo* mice	Spantide II and Ketoprofen	Retained in the SC, epidermis and dermis	[63]
Polymeric nanoparticles	122 to 860 nm	Aqueous dispersion	Porcine ears	-	643 nm particles penetrate deep into the hair follicles	[64]

Polymer Architecture	Size and Surface Charge	Formulation	Skin Model	Drug	Nanoparticle Skin Penetration	Ref
Hydrogels	350 nm, -8 mV (with Azone® -33 to -51 mV	Carbomer 934	*Ex-vivo* skin human and male CD-1 mice	Pranoprofen	Formulation caused prolonged contact with the top of the skin. Azone® did not improve drug penetration	[65]
Cationic lipid-polymer hybrid nanoparticles	163 nm, 35.14 mV	Aqueous dispersion	*In vivo* mice, imiquimod - psoriatic plaque like model	Anti-TNFα siRNA	Delivery of siRNA to the dermis. Carrier penetration was not studied.	[66]
PLA						
Polymeric nanoparticles	164 to 365 nm, size and morphology modulated by incorporated substance	Aqueous dispersion	*Ex vivo* human skin	Hydrophilic and hydrophobic dye models	Penetration into hair follicles and release of the dye	[67-68]
Chitosan						
Cationic nanoparticles	229 nm, 39 mV	Cream	Albino Wistar rats	Hydrocortisone Hydroxytyrosol	Positively charged nanoparticles interacted with the negatively charged SC	[69]
Polymeric nanoparticles	191 to 393 nm, 12 to 47 mV, pH dependent, spherical to amorphous	Cream	NC/Nga mice	Hydrocortisone Hydroxytyrosol	Retention of the drugs in the skin. No evidence of nanoparticle penetration	[70]
Polymeric nanoparticles	190 to 287 nm, 17 to 41 mV, spherical	Aqueous dispersion	Baby and adult Sprague Dawley rats	Plasmid DNA	*β*-gal expression in the dermis. Possible follicular penetration	[71]
PCL						
Self-assembled poly(ε-caprolactone)-block-poly(ethylene glycol)	40 and 130 nm	Aqueous dispersion containing ethanol	Skin of both hairy and hairless guinea pigs	Minoxidil	Size dependent delivery only on hairy skin. Particles mainly found in the hair follicles	[57]
Nanocapsules and nanoparticles	210 to 560 nm	Gel	Guinea pigs porcine ear skin	Parsol MCX	Nanocapsule film formation on the skin surface	[56, 60]

Polymer Architecture	Size and Surface Charge	Formulation	Skin Model	Drug	Nanoparticle Skin Penetration	Ref
Other						
Nanogels poly(NIPAM-*co*-AAc) and pNIPAM (bis)	346 and 242 nm, spherical	Aqueous dispersion	Porcine ear skin	-	Particles found in the viable epidermis	[47]
Amorphous silica particles	42 – 292 nm, positive and negative surface coating	Aqueous dispersion	*Ex vivo* human skin	-	42 nm particles found in epidermis independent of surface charge	[72]
Dendritic, core-multi-shell	19 nm	Aqueous dispersion	*Ex vivo* human skin, human skin eq.	Dexamethasone, Nile red	Particles remain in the SC, enhanced topical delivery	[73-75]

Despite the increasing number of publications reporting on efficient topical delivery with the aid of polymeric nanoparticles, little attention was given to the interaction of the particulate formulation with the skin barriers and their influence on its function. One of the first studies reporting on the migration of NGs across the SC was performed using pNIPAM-*co*-pAAc block copolymer.[47] The authors took advantage of the thermoresponsive behavior of pNIPAM, which exhibits a volume phase transition temperature (VPTT) by collapsing into a hydrophobic state. This transition for pNIPAM occurs close to its lower critical solution temperature (LCST), which is at about 34 °C for a crosslinked polymer network.[76] The incorporation of AAc lead not only to pH sensitivity but also to increased size of 346 nm due to larger swelling capacity compared to pNIPAM NGs (242 nm). Incorporation of the negatively charged units had an unexpected effect on the transition temperature of the NGs, occurring at a lower temperature of 31.1 °C. Interestingly, the NGs applied on porcine skin and analyzed by transmission electron microscopy (TEM), could be found in and beyond the viable epidermis. While not explicitly addressed, this work raised fundamental questions about the mechanistic insight regarding a direct influence of temperature or pH on particle penetration.

Reports on enhanced dermal or transdermal delivery of drugs, often miss a comprehensive evaluation of the enhancement mechanism or the detection of the particulate delivery system within the used skin model. While the consensus on the fact that nanoscale delivery systems accumulate in the SC without reaching the viable tissue still exists, recent studies do speculate on the possibility

of polymeric carriers entering the epidermis (Table 1).[62] Therefore, future studies must give attention to possible toxicological effects and populations of cells coming in contact with the particles retained in the viable tissue. Nevertheless, common findings contribute to a general understanding of topical drug delivery with the aid of polymeric nanoparticles. For instance, the effectiveness of positively charged carriers has repetitively proven for its superior interaction with the SC, but at the same time their potential toxicity. Carriers with an amphiphilic surface, i.e. PEGylated, possess an outstanding ability to enhance percutaneous absorption of hydrophobic as well as hydrophilic biomacromolecules.[73, 77-79] Moreover, the utilization of potentially toxic penetration enhancers was not essential when drugs are formulated within effective polymeric carriers.[65]

1.2 Thermoresponsive Nanogels

1.2.1 Thermoresponsive Polymers Used for Biomedical Applications

Thermoresponsive polymers represent a class of stimuli responsive materials with attractive applications in the drug delivery field which are summarized in recently published reviews.[29, 76, 80-81] Thermoresponsive polymers found their application also in the bioanalysis and bioseparation such as thermoresponsive chromatography and thermally mediated cell separation.[82] The two main classes of thermoresponsive polymers are those possessing a lower critical solution temperature (LCST) and polymers with an upper critical solution temperature (UCST). LCST polymers are fully hydrated below their critical temperature and undergo a phase transition above this point. The UCST polymers however, adopt a globular conformation below the critical solution temperature. The vast majority of thermoresponsive polymers belong to the LCST group. The physical understanding behind the responsiveness of a polymer to temperature and the configuration of a polymer coil in a solution lies in a volume phase transition at a certain temperature which dominates the thermal free energy k_BT.[83-84] In contrast to the ideal chain model where polymers are represented by hard beads interconnected to each other, the real coil model takes into consideration the excluded volume interactions. According to that theory, large excluded volume interactions are a direct consequence of high free energy of a repeating unit which overpowers the attraction between them, and therefore leads to expanded coil conformation. In the swollen state of the coil, the enthalpy is the dominating contributor to the energy originated from intermolecular solvating hydrogen bonds between the water molecules and the polymer units. The

phase transition is accompanied by gain of entropy originating from dissociation of the water molecules as well as entropic contribution from the intermolecular interactions of the exposed hydrophobic units.

It is worth mentioning that any polymer in a solution responds to temperature changes by adaptive conformation of its coil. To differentiate materials which are defined stimuli responsive, or thermoresponsive in this case, from any other polymer solution, Hoffman *et al.* defined them as materials which respond to small physical or chemical changes with a large property change.[85-86] The large property change in the case of thermoresponsive polymers is the coil to globule transformation resulting in turbidity of the polymers solution. That is the reason why the temperature at which polymers undergo a phase separation is called also the cloud point temperature (Tcp). The Tcp of a polymer solution depends on parameters as molecular weight of the polymer, its concentration, and additives in the solvent. This dependence further classifies thermoresponsive polymers to three types. Type I polymers follow a classic Florry-Huggins behavior by decreasing LCST with increasing molecular weight.[87-88] Type II polymers are hardly affected by changes in the molecular weight or architecture.[89] Finally, type III polymers can be represented as bimodal phase diagram at different temperatures.[90]

Thermoresponsive polymers can be subdivided into categories of their chemical structure (Figure 3); substituted amide bonds contributing to the thermoresponsive behavior, poly(ether)s, and poly(vinyl ether)s. Among many thermoresponsive polymers, pNIPAM is one of the most studied ones in term of its precise mechanism of phase separation as well as its successful application in numerous fields.[84, 91] pNIPAM is typically synthesized by free radical polymerization which results in a polymer with an LCST of 32 °C.[92] As a type II polymer, the attractiveness rises from its robust behavior in an aqueous solution. The presence of amide bonds is the key contribution to the enthalpy through hydrogen bonds with water molecules, while in the hydrophobic state dominate intermolecular interactions between the isopropyl groups. Another interesting class of thermoresponsive polymers are those composed of a polyether backbone or alternatively bare oligo(ether)s as a side group. The oligo(ethylene methacrylates) are particularly interesting due to their biocompatibility and the ease of incorporation of different co-monomers, which allows a precise control over the LCST.[93-95]

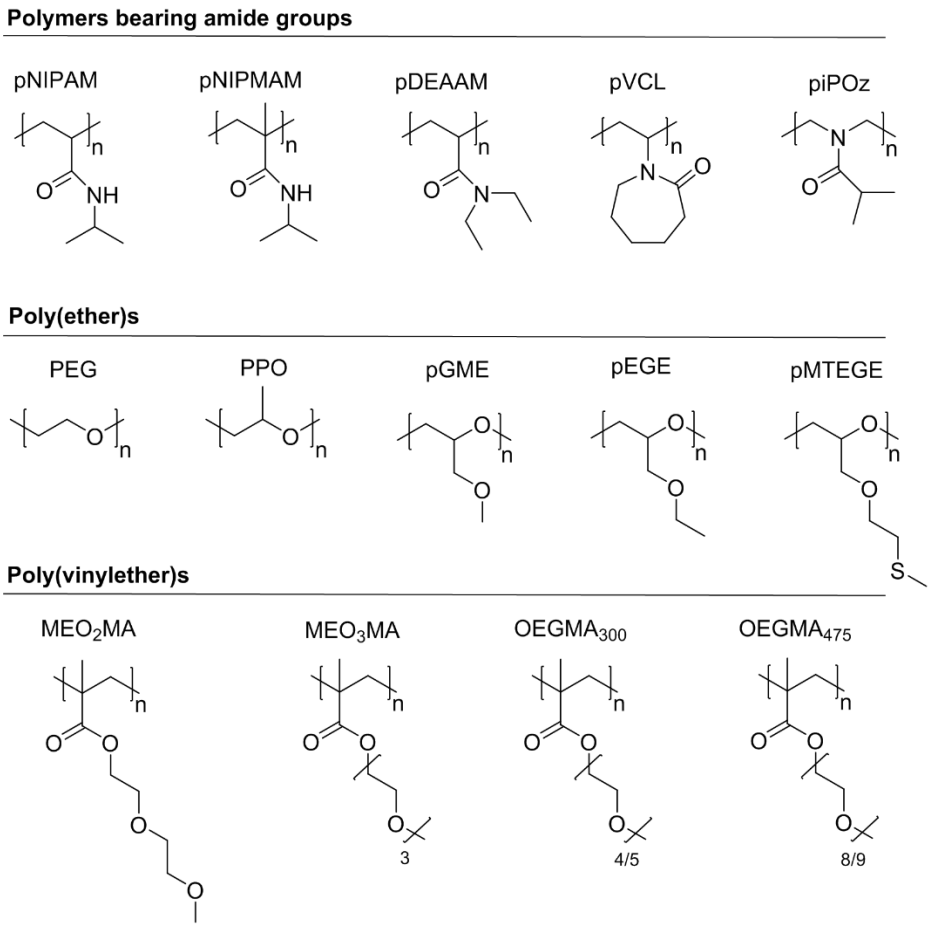
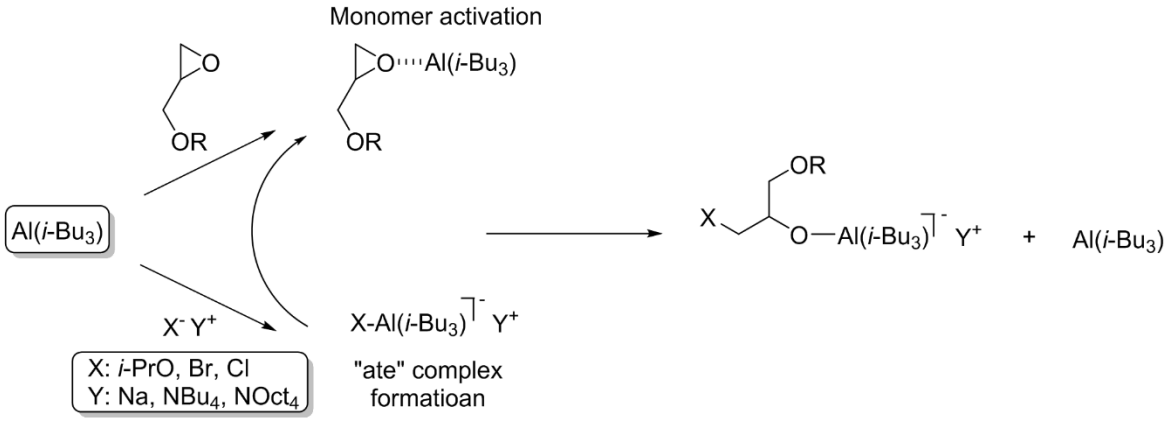


Figure 3. Selected thermoresponsive polymers used for biomedical applications. Polymers bearing amide groups: poly(N-isopropylacrylamide) (pNIPAM), poly(N-isopropylmethacrylamide) (pNIPMAM), poly(N,N‘-diethylacrylamide) (pDEAAM), poly(N-vinyl caprolactam) (pVCL), poly(2-isopropyl-2-oxazoline) (piPOz). Poly(ether)s: poly(ethylene glycol) (PEG), poly(propylene oxide) (PPO), poly(methyl glycidyl ether) (pGME), poly(ethyl glycidyl ether) (pEGE), poly(2-methylthioethyl glycidyl ether) pMTEGE). Poly(vinylether)s: poly(2-(2-methoxyethoxy) ethylmethacrylate)) (pMEO_2MA) and the corresponding oligo(ethylene glycol) methacrylates.

The polymerization of functional epoxides opens possibilities for a variety of water soluble polyether based materials with tunable properties. The living ring opening polymerization of epoxides allows the precise control over the molecular weight of polymers, and therefore their properties. While protected epoxides lead to the linear propagation of the polymer chain, AB_2 monomers such as glycidol lead to branched architectures.[96-97] The anionic polymerization does

allow the presence of functional groups such as allyls, methacrylates, epichlorohydrin, furfuryl, and methyl thioethers.[98] The introduction of hydroxyls or amines along the polyether backbone requires a suitable protecting group. The versatility of functional PEG-based materials expands even more since their copolymerization with carbon dioxide resulting in polycarbonate, a biodegradable material suitable for biomedical applications.[99] The traditional anionic ring opening polymerization occurs by an initiation step with an alkoxide, while the corresponding metal ion plays a crucial role in the stabilization of the propagating chain. However, since the coordinative polymerization method has been developed, the synthesis of homopolymers with molecular weights up to 100 kDa could be achieved by monomer activated anionic polymerization with the association of trialkylaluminum to alkali metal alkoxides or ammonium salts.[100-102] The combination of a Lewis acid with an alkali metal alkoxide or an onium salt leads to an “ate” complex, being able to open the ring of an activated epoxide (Scheme 1).[103-104]



Scheme 1. Mechanism of monomer activated anionic polymerization.

Particularly interesting monomers in the family of alkylglycidyl ethers are glycidyl methyl ether (GME) and ethyl glycidyl ether (EGE). Their corresponding polymers are thermoresponsive in a broad range of temperatures. On the contrary, PEG is a water soluble polymer while its thermoresponsive behavior is observed above 100 °C.[105] The introduction of hydrophobic units as methyls and ethyls along the backbone of the polymer tunes its polarity and induces thermoresponsiveness at a physiological temperature range. These phenomena are associated with a decrease in the number of water molecules bound to the linear polyglycerol, as well as their

configurational entropy. Above the cloud point temperature, water molecules are driven out of the hydration layer of the polymer resulting in a large entropy gain and collapse to a globular conformation.[98, 106] The LCST of these polymers is strongly affected by the composition of the monomers, molecular weight, concentration, and ionic strengths of the solution. The phase transition of the EGE polymer solution of 1 wt. % is observed at 14.6 °C while that of GME at 57.7 °C.[107] The LCST of the co-polymer can be fine-tuned by the ratio of the incorporated monomers with a linear dependence on the monomer composition.[108] It is well known that a gradient in the incorporation of the co-monomers can lead to broadening of the phase transition temperature, exhibiting rather block co-polymer behavior rather than of a random one. This is however not the case for the p(GME-co-EGE). The similar reactivity ratios of both monomers, $r_{GME} \approx r_{EGE} \approx 1$, lead to truly random co-polymers with no hysteresis upon heating and cooling cycles.[109] Interestingly, the similar reactivity ratios are unique for the monomer activated polymerization mechanism, and were not observed for conventional polymerization methods. Moreover, due to the structural similarity to the biocompatible PEG, and the branched analog, the dendritic polyglycerol (dPG), linear water soluble polyglycerols are particularly interesting building block as biomaterial due to their biocompatibility and modular properties.[97, 110-111]

1.2.2 Synthetic Strategies of Thermoresponsive Nanogels

Polymers can be crosslinked to form hydrogels, which, as the name suggests, are networks that hold large amounts of water. Hydrogels with a polymer network in the nanometer scale are termed NGs. NGs represent an attractive approach for drug carrier systems as they form colloidally stable particles benefiting from high surface area and swelling capacities which allow efficient encapsulation of therapeutic molecules. The physico-mechanical properties like the elasticity or softness of such carrier are determined by the three-dimensional structure of the polymer network. The characteristics of different polymer architectures can be quantified by a repulsive pair potential model. A polymer coil is represented as the softest colloidal system while a hard sphere composed of precipitated polymers or an inorganic composite, is the most rigid one - quantified by the repulsive pair potential (Figure 4).[112] The variability in the repulsive pair potential for grafted molecular architectures, hydrogels, or micelles is demonstrated by the dashed lines in between the two extremes of finite potential for a polymer coil and the infinite repulsion for a defined radius of hard spheres. NGs, placed in the center of the softness scale, are elastically deformable spheres being able to adjust their shape and volume while passing through narrow membranes. Moreover,

their properties are tunable when composed of responsive materials, and thermoresponsive polymers in particular.

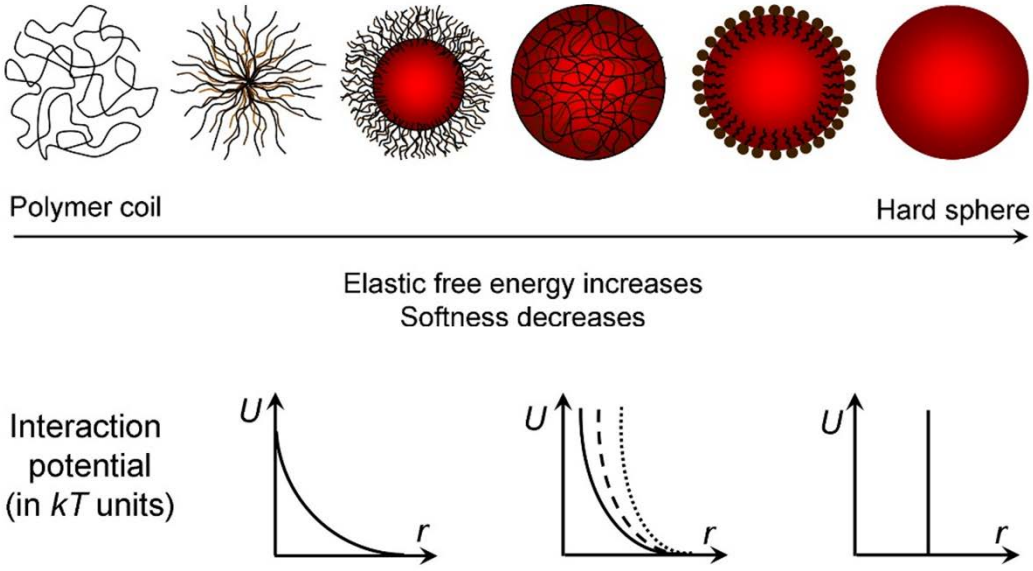


Figure 4. Representation of polymeric particles with different architectures with different softness and representative interaction potentials as a function of distance. Adapted with permission from Vlassopoulos *et al.* Copyright 2014 Elsevier.[112]

The synthetic route towards crosslinked polymeric networks in the submicron scale depends first and foremost on the properties of the utilized polymer such as its solubility and reactivity. NGs fabricated from the corresponding monomers can be templated via the micro-emulsion or the precipitation polymerization methods, typically by free radical polymerization of vinylic monomers in a presence of a multifunctional monomer.[113] The nanoparticulation of pre-synthesized polymers, is achieved by the precipitation of the polymers by solvent evaporation, salting out, or the nanoprecipitation techniques while the unique properties of stabilization and swelling associated with NGs are given by orthogonal crosslinking reactions.

While a method of choice can be found for any given polymer, or a polymer composite, the main challenge in the engineering of NGs is to establish a facile procedure without the input of high energy or potentially toxic surfactants and solvents.[114-116] The nanoprecipitation technique involves the precipitation of a concentrated polymer solution into a non-solvent for the polymer,

while both the solvent and the non-solvent are miscible in each other. Typically, this technique does not require the stabilization of the precursor particles by surfactants as the concentration of the dispersed polymers is tuned so that the particles reach colloidal stability. The nature of the solvent and the non-solvent, as well as the volume ratio between them, are the decisive factors towards the formation of polymeric particles. This is due to the fact the particles are formed as a consequence of rapid desolvation of the polymer during the diffusion process of one solvent into the other. The nanoprecipitation requires preferably solvents with similar dielectric constants, which therefore have a high affinity towards each other.[117-118] The affinity of two solvents towards each other is given by the interaction parameter χ, where the Hildebrand solubility parameter δ has a strong dependence on temperature (V_{NS} - molar volume of the non-solvent, ΔH_v – heat of evaporation).

$$(1)\ \chi = \frac{V_{NS}}{RT}(\delta_S - \delta_{NS})^2$$

$$(2)\ \delta = \sqrt{\frac{\Delta H_v - RT}{V_m}}$$

The most influential parameters are best described in a phase diagram of the three components, polymer, solvent, and non-solvent, which were obtained from the nanoprecipitation of PCL, dissolved in acetone (solvent) and precipitated into water (non-solvent) (Figure 5).[119-120] Zone A represents the concentration of PCL which is beyond its solubility in the solvent alone. Zone B is subdivided into three areas while the formation of nanoparticles in observed only in zone BI, having the correct polymer concentration and ratio of the solvent and the non-solvent, the so called “ouzo region”. In area BII, high polymer concentration and insufficient dissolution leads to flocculation, growth of polymeric aggregates due to processes such as Ostwald ripening.[121] Area BIII represents a regime where the volume of the solvent is too high to cause precipitation of the polymer.

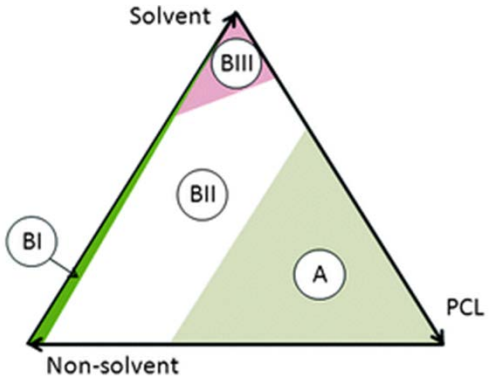


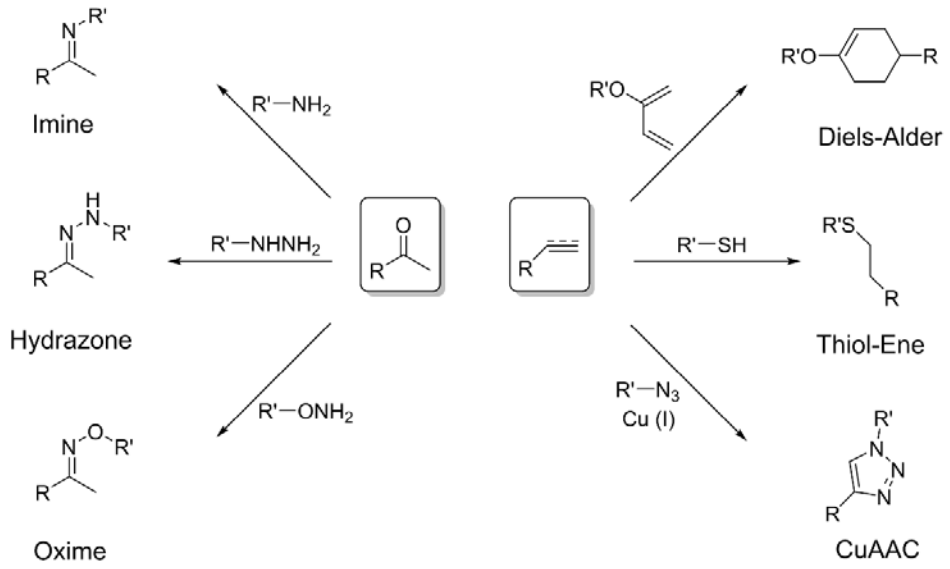
Figure 5. Phase diagram of PCL acetone solution precipitated into water. Adapted with permission from Schubert *et al.* Copyright 2010, Royal Society of Chemistry.[119]

While over decades the nanoprecipitation method was limited to the precipitation of water immiscible polymers only, such as PLGA, PLA or PCL, it was recently extended by inverse-nanoprecipitation to water soluble polymers.[122] Furthermore, high-throughput production of nanoparticles was achieved by implementing continuous flow apparatuses such as microfluidic flow channels that precisely control the mixing process of the two solvents, leading to reproducibility and low particle size dispersity.[123-124]

Despite its attractiveness, the nanoprecipitation technique was not applied for the fabrication of thermoresponsive NGs. The method of choice for the fabrication of thermoresponsive NGs is the precipitation polymerization of vinylic monomers.[125] In the presence of a surfactant below its critical solution temperature, free radical polymerization can be initiated via redox initiation by ammonium persulfate (APS) and N,N,N′,N′-tetramethylethylenediamine (TEMED), alternatively by heat or UV irradiation.[126] The initiation step is crucial for controlling the final size of the NG. The number of particles is determined by the initiation step and remains constant, while growing in size until full consumption of the monomers.[127] In addition to the controllable size, the co-polymerization of multi-functional crosslinking reagents allows controlling the architecture of the formed network. Calderón *et al.* developed a strategy for fabrication of tNG from pNIPAM and oligo(ethylene glycol) methacrylates.[95, 128] Rather than crosslinking the thermoresponsive polymers with a bifunctional monomer, they took advantage of the multifunctional surface of dendritic polyglycerol (dPG), which allows to grow multiple polymer chains from a single core.

1.2.3 Orthogonal Ligation in the Synthesis of Polymeric Nanoparticles

Orthogonality in chemistry has a broad meaning, spreading from the orthogonal removal of protecting groups, first reported 1977 by Merrifield, to the fine and selective chemistry of macromolecules of interest.[129] In contrast to chemo-selectivity where the reactivity of a certain compound can be directed by the appropriate selection of the reaction conditions, orthogonality is the exclusive reactivity of two functional groups towards each other.[130] The necessity for selective ligation in the field of polymeric nanoparticles arises from the fact that multiple functional groups have to be considered when crosslinking, surface decoration, or labeling of nanoparticles is performed. Click chemistry can be defined as a subdivision of orthogonal ligation because it is highly selective and high yielding under mild conditions with little or no byproducts (Scheme 2).[131-132] The development of the Cu(I) catalyzed azide-alkyne cycloaddition (CuAAC) established the foundation for a number of highly efficient conjugation reactions, which was followed by the development of many other related techniques. Among the most popular ones are reactions based on the carbonyl reactivity towards nucleophiles, which results in imines, hydrazones, or oxime ligation. Click chemistry reactions using alkenes or alkynes are based on Diels-Alder, thiol-ene (as well as thiol-yne or Michael addition), and the azide-alkyne cycloaddition.

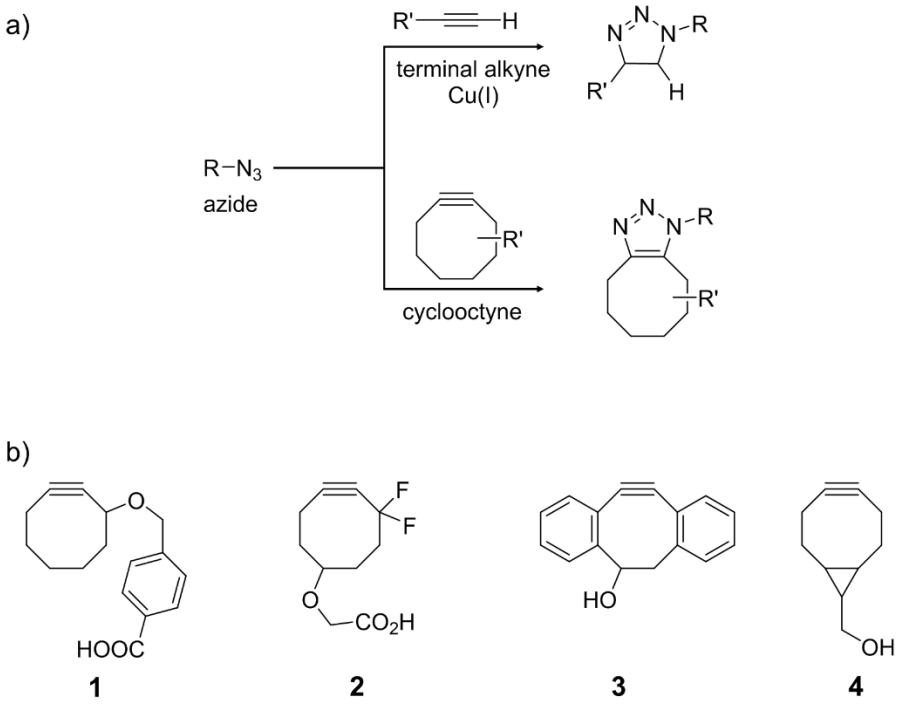


Scheme 2. Summary of commonly used click reactions resulting from aldehydes, ketones, alkenes, and alkynes.

The nearly quantitative yield of the Cu(I) catalyzed cycloaddition reaction and its reactivity in various solvents made it an attractive tool for crosslinking chemistry. Compared to the uncatalyzed reaction, Cu(I) allows a reduction of the reaction temperature. The association of the metal to terminal alkynes via σ and π interactions and the simultaneous activation of an organic azide leads to the exclusive formation of 1,2,3-triazoles.[133] The oxidation of Cu(I) to Cu(II) is typically prevented by addition of sodium ascorbate or other ligands that act as heterocyclic donors and tertiary amines for the acceleration of the reaction rate.[134-135] However, the purification of the copper salt from highly dense polymeric networks appears challenging using conventional purification methods such as dialysis, ultra-filtration or size exclusion chromatography. Remaining copper in the compound may negatively influence the applicability in the intended downstream application, especially in the biomedical field. While copper is an essential trace element in many organisms, elevated copper levels cause cytotoxicity by protein denaturation and intercalation into the DNA.[136-138]

Chemical reactions that shall be performed in the presence of biomacromolecules have to be compatible with the respective molecule to ensure its integrity, stability, and function during and after the reaction. Therefore material scientists have to develop techniques to allow the desired reaction at the respective conditions, usually close to the conditions in living organisms, with chemical approaches. This is to be considered when encapsulating therapeutic proteins during nanoparticles formation or creating hydrogel matrices for the immobilization of living cells.[139] The field of bioorthogonality, which lately evolved due to the findings of C. Bertozzi and her team, addresses this issue.[140] The primary condition that enables chemical reactions which do not interfere with biological processes is the utilization of unnatural functional groups which are integrated into polymeric building blocks. Bioorthogonality has evolved far above the selective targeting of cysteines and lysines, towards chemical recognition of specific amino acid sequences.[141] While the reactivity of ketones with amines is bioorthogonal, despite their abundance in living organisms, other nucleophiles can compete with this reaction under physiological conditions. The Staudinger ligation of organic azides with an ester substituted phosphine aryl has been proven to be highly efficient and selective, however its slow reaction kinetics and possible side reactions (Staudinger reduction) limit their exploitation.[142-143]

The ultimate solution for such limitations was presented by the copper free cycloaddition of alkynes and azides while the catalytic activity of Cu(I) is replaced by strain (Scheme 3a). The strain is introduced by a cyclooctyne which readily reacts with an organic azide under mild conditions.[144] The presence of an alkyne in a eight-membered ring induces a strain of 18 kcal mol^{-1}, which is almost completely released upon the [3+2] cycloaddition.[144] The first reported strained cyclooctynes (Scheme 3b **1**) had still limiting reaction kinetics and water solubility, which led to the development of next generation cyclooctynes. The dual substitution of electron withdrawing groups (fluorination) on the propargylic position drastically increased the reaction rate by lowering the energy of the alkyne's lowest unoccupied molecular orbital (LUMO) and favoring the cycloaddition with azides (**2**).[145] Additional strain could be induced to the ring by two benzoic groups of 4-dibenzocyclooctynol (**3**) increasing the reaction rate 3 times compared to the unsubstituted cyclooctyne.[146] Others contributed to the synthetic accessibility of strained water soluble cyclooctynes by the bicyclo[6.1.0]nonyne with modular functionalities for macromolecular conjugation chemistry (**4**).[147]



Scheme 3. a) Copper catalyzed and strained azide-alkyne cycloaddition. b) Generation of strained cyclooctynes used for copper free click reactions.

1.3 Encapsulation Strategies in Polymeric Nanoparticles

1.3.1 Topical Delivery of Hydrophobic Drugs

Nanoparticles are able to protect encapsulated compounds from oxidation or any other undesired interaction with living organisms before reaching the site of action. Efficient encapsulation of highly hydrophobic drugs in polymeric nanoparticles is the decisive milestone for increasing bioavailability in any application form. The strategies of encapsulation sum up in increasing the partition of the drug in the carrier by introducing complementary chemistry of specific interactions, or alternatively entrapment of the drug molecules (or homogenized crystals) in the polymer scaffold.[148] The diversity of drugs requires specific consideration of functional groups to be introduced to the polymeric building blocks, as well as the choice of organic solvents for the fabrication procedure. These factors have to be carefully analyzed in order to avoid detrimental toxic effects or changes in the physico-chemical properties of the carrier. More importantly, the fabrication method of the carrier dictates whether the encapsulation procedure is compatible with the synthetic conditions or if the drug has to be loaded into the readily synthesized particles. The selected route might have an unneglectable effect on the encapsulation efficiency and the release profile of the drug.

The synthesis of polymeric nanoparticles by variations of micro-emulsion methods provides a suitable platform for the encapsulation of many drugs by smart choice of solvents and surfactants. The utilization of volatile organic solvents such as methylene chloride or hexane, in which both the polymer and the drug are soluble, allows the *in situ* encapsulation during the micro-emulsion formation and facile removal of the solvent.[149-151] The exposure of drugs to high shear forces, remaining traces of organic solvents and surfactants make this method however disadvantageous in some cases. The nanoprecipitation technique can solve some of these issues allowing very mild reaction conditions with no need for surfactants. This approach is tolerated for a broader variety of solvents such as acetone, dimethyl formamide (DMF), dimethyl sulfoxide (DMSO) or N-methyl-2-pyrrolidone (NMP), which dictate the nature of drugs which can be co-precipitated with the pre-synthesized polymers.[117, 152-154]

A powerful post loading and efficient delivery of hydrophobic drugs could be achieved by using core-multishell (CMS) unimolecular carriers consisting of a dPG core, an inner alkyl shell, and an outer poly(ethylene glycol) methyl ether (mPEG) shell.[75, 155] Hydrophobic molecules, such as

dexamethasone and Nile red, could be successfully entrapped in the inner shell by the so called film method. Creation of a thin film of the drug by evaporation of the organic solvent followed by the addition of the CMS aqueous solution could increase the partitioning of the drug in the carrier. Interestingly, studies performed by electron paramagnetic resonance (EPR) spectroscopy of 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (PCA) labeled DXM, could locate the drug in the hydrophobic inner shell of the carrier.[156] A more specific encapsulation approach could be achieved by dPG based NGs decorated with a short peptide sequence with high affinity to Temoporfin, a lipophilic drug used for photodynamic therapy of skin cancer.[157] Decoration with the peptide of 9 amino acids selected by combinatorial means, could enhance the encapsulation of Temoporfin by 16 times and deliver the drug into the viable epidermis tested on intact and tape stripped human skin.

When referring to specificity, cyclodextrins (CD) represent a well-established and broadly employed approach for complexation of hydrophobic drugs in the inner cavity of this cyclic oligosaccharide. The inclusion complexes formed with many drugs that are for example used for the treatment of autoimmune skin diseases could drastically increase their solubility.[158-161] More importantly, cyclodextrins have been shown to act as topical penetration enhancers.[162] However, in contrast to conventional penetration enhancers (alcohols, fatty acids, sulfoxides), they are not able to penetrate the skin. Apart from the enhanced solubilization of drugs, improved drug delivery can be mediated by acting as channeling agent and promoting diffusivity through various matrices.[163] Zhang *et al.* reported on the skin penetration ability of hydroxypropyl-β-CD decorated with polyethyleneimine (PEI).[164] The carriers could enhance the delivery of vitamin B_{12} by changing the secondary structure of keratin in the SC. In a different approach, hydroxypropyl-β-CD was used as an additive to PEGylated PCL nanoparticles.[165] The encapsulated drug could be delivered and retained in the skin in a higher amount than the control groups of the nanoparticles alone. Confocal Raman spectromicroscopy analysis of the treated skin sections revealed that the penetration enhancer altered the hydration degree of the SC, promoting nanoparticle transport.

1.3.2 Topical Delivery of Therapeutic Proteins

The dermal delivery of therapeutic proteins is an attractive route of administration not only for the treatment of local inflammatory skin diseases but also as a way to reach the systemic circulation by bypassing the hepatic first-pass metabolism, which is highly relevant for biomacromolecules. It

has been reported that after reaching the systemic circulation, topically applied proteins showed longer half lifetimes and more steady concentrations compared to intravenous administration, which leads to potentially lower costs of existing therapies by a substantial reduction of the administered dose.[166-167] Furthermore, topical administration of proteins provides a distinctive vaccination route, for skin being an exceptionally immunoactive organ capable of prompting both the innate and the adaptive immune responses.[168-171] Proteins are almost completely restricted from penetrating the skin due to their size and the effective barrier function of the SC. The available platforms that allow the penetration of high Mw, hydrophilic molecules across the SC involve physical approaches such as microneedle technology, electroporation, or thermal ablation.[172-176] All these techniques however, may cause skin irritation or activity loss of the delivered substance. Maybe the most obvious advantage of using polymeric nanoparticles as delivery systems for topical applications is their unique ability to deliver therapeutic proteins into the viable epidermis via noninvasive strategies.

The emerging potential in cutaneous delivery systems for proteins was recognized in the past years and lead to great progress in the treatment of severe diseases. Chemical conjugation of proteins to vehicles or their PEGylation was extensively studied as means to enhance the structural stability of the bioactive, yet covalent linkages may cause to loss in the biological activity of the protein and provoke immunogenicity.[177-179] Encapsulation of biomacromolecules into nanocarriers is advantageous in that sense by providing a protective environment to the protein from enzymatic degradation or its denaturation. Particulate delivery systems such as microemulsions, liposomes, niosomes and ethosomes have been developed and successfully employed for delivering proteins to the skin.[180-182] Consisting of phospholipids or other surfactants, these vehicles confine a hydrophilic core where proteins can be encapsulated in quite high yields. The disadvantage of surfactant based protein delivery is the potential skin irritation and the challenging control over the release kinetics.

The encapsulation procedure of proteins into polymeric nanoparticles has to take into account the resistance of the protein to the reaction conditions or to exposure to organic solvents. The nanoprecipitation technique is widely favored for protein encapsulation due to the absence of extensive shear forces, sonication or heating. Organic solvents such as DMSO, acetonitrile, and acetone have been used as solvent components for protein encapsulation during nanoprecipitation.

Morales-Cruz *et al.* reported on the development of a two-step nanoprecipitation where lysozyme and α-chymotrypsin were precipitated in acetonitrile.[183] The formation of precipitated protein particles served as a template for the second precipitation of PLGA in water. Bilati *et al.* studied the precipitation of tetanus toxoid, lysozyme, and insulin from a DMSO solution and reported on improved drug loading efficiencies compared to emulsion based techniques.[184] However, the lack in comprehensive characterization of the protein structure and activity upon release from the polymeric carriers, question the suitability of the fabrication procedure.

A category of nanocarriers which are especially suited for the encapsulation of proteins are NGs. The attractiveness of NGs as carriers for biomacromolecules rises from their loose framework of polymers, allowing to embed proteins in a protective environment and trigger their release when responsive material are used for their fabrication. Successful encapsulation of asparaginase, IgG, lysozyme, and BSA was achieved by inverse nanoprecipitation to prepare NGs from orthogonally functionalized monomers precipitated into acetone.[122] The NGs could be degraded under acidic environment and released the intact proteins. A remarkable achievement in protein delivery could be realized by mucoadhesive physically crosslinked NGs based on cholesteryl-group-bearing pullulan in intranasal application.[185] An isolated vaccine could be released from the NGs and trigger an immune response without the administration of mucosal adjuvants. Calderón *et al.* has shown the benefit of utilizing thermoresponsive NGs for the topically triggered release of proteins.[186] Transglutaminase-1 was encapsulated in the NGs while the release was triggered by the natural temperature gradient of the skin. Interestingly, the NGs stabilized the protein in making it resistant to freeze-thaw cycles and causing a prolonged shelf life. When a transglutaminase-1 deficient skin model was treated with the NGs, the barrier function could be restored upon the delivery of the protein.

2 Scientific Goals

The topical treatment of auto-immune skin diseases requires efficient delivery systems for drugs in order to reach the site of action at the therapeutic concentration. The main obstacles to reach the target are numerous chemical as well as physical barriers preventing an efficient absorption of therapeutic molecules. The following thesis addresses the aspects of dermal drug delivery with the aid of thermoresponsive nanogels as adaptive carriers as well as tools for elucidating the impact of the carriers on the topical penetration mechanisms. As illustrated in Figure 6, the development and application of thermoresponsive nanogels will be presented in three chapters: *Synthetic Strategies*, *Skin Penetration Mechanisms*, and *Dermal Drug Delivery*.

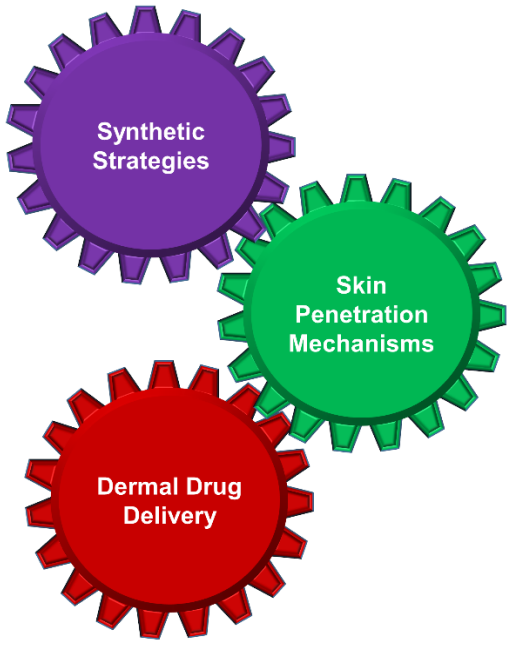


Figure 6. Aspects of dermal drug delivery with the aid of thermoresponsive nanogels

***Synthetic Strategies (Chapters 3.1 – 3.3)*:** The goal of this work is first and foremost focused on the development of synthetic procedures for the fabrication of thermoresponsive nanogels as versatile drug delivery systems. Facile and universal approaches shall be studied to enable the reproducible synthesis of thermoresponsive nanogels under mild conditions to enable the integration of sensitive therapeutic molecules. The nanoprecipitation approach shall be extended to yet unexplored choices of solvents which are suitable for certain classes of polymers. Working

in close collaboration with toxicologists that provide regular feedback regarding the biocompatibility of the compounds, the synthetic strategies and the purification procedures shall be optimized. A closer look on the interaction of the nanogels at a cellular and sub cellular levels shall broaden the understanding on the cellular uptake mechanisms and explore the possibility of controlling their intracellular fate.

***Skin Penetration Mechanisms (Chapters 3.4 – 3.6)*:** Utilizing various up-to-date detection methods, nanogels shall be labeled with fluorescent dyes, and loaded with model molecules for the investigation of the mechanisms by which nanogels enhance the percutaneous absorption of drugs. Label based as well as labeled free techniques will be applied for obtaining structural information regarding the spatial organization of the stratum corneum components upon the application of nanogels. The effect of temperature shall be evaluated as a stimulus of penetration enhancement. The follicular route of penetration will be evaluated for thermoresponsive nanogels of different sizes and their ability to act as reservoirs for prolonged release of the encapsulated moieties.

***Dermal Drug Delivery (Chapters 3.7 – 3.8)*:** As many of the therapies for the treatment of inflammatory skin diseases are based on low molecular weight hydrophobic molecules, the adaptation of the nanogels for the encapsulation of dexamethasone as a model drug for this class of molecules, shall be investigated. As the crosslinked polymeric network of the nanogels consists of highly hydrophilic cavities at its swollen state, the polymers will be decorated with functional units which have beneficial properties as topical penetration enhancers. Moreover, the fabrication of the nanogels will be optimized for allowing the *in situ* encapsulation of biomacromolecules, while preserving the structure and activity of the biomolecule upon the triggered release. The anti-inflammatory biopharmaceutical Etanercept will serve as the drug for the development of a non-invasive topical therapy.

3 Publications and Manuscripts

In the following section the published articles and submitted manuscripts are listed and the contributions of the author are specified.

3.1 Fabrication of Thermoresponsive Nanogels by Thermo-nanoprecipitation and *in situ* Encapsulation of Bioactives

M. Giulbudagian †, M. Asadian-Birjand †, D. Steinhilber, K. Achazi, M. Molina and M. Calderón, *Polymer Chemistry*, **2014**, *5*, 6909-6913.

† These authors contributed equally to this work

http://dx.doi.org/10.1039/C4PY01186D

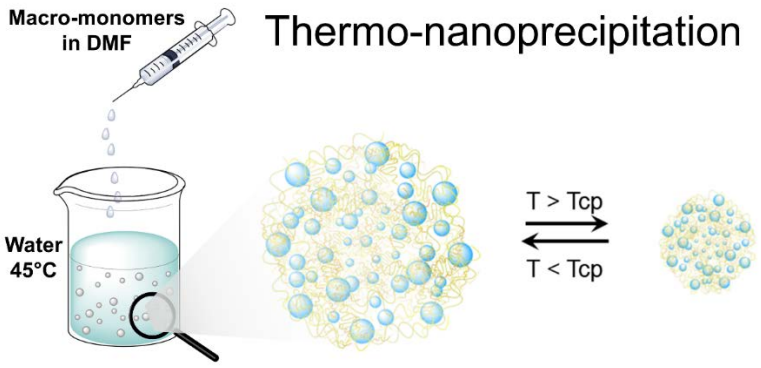


Figure 7. Adapted from Giulbudagian *et al.*[187] with the permission of The Royal Society of Chemistry.

Author contribution: In this work the author contributed with concept, the development of the synthetic methodology of the nanogels as well as their characterization, labeling, drug loading experiments, and part of the written report.

Abstract: A synthetic method for thermoresponsive, glycerol based nanogels has been developed. The nanogels were synthesized by nanoprecipitation of the orthogonally functionalized macromonomers and their gelation in water. The crosslinking points were generated by strain promoted azide–alkyne cycloaddition which enabled the in situ encapsulation of Doxorubicin HCl. The mild and surfactant free reaction conditions make these nanogels ideal candidates for biomedical applications.

3.2 Specific Uptake Mechanisms of Well-tolerated Thermoresponsive Polyglycerol-based Nanogels in Antigen-presenting Cells of the Skin

A. Edlich, C. Gerecke, **M. Giulbudagian**, F. Neumann, M. Schäfer-Korting, N. Ma, M. Calderón, B. Kleuser, *European Journal of Pharmaceutics and Biopharmaceutics*, **2017**, *116*, 155-163. http://dx.doi.org/10.1016/j.ejpb.2016.12.016

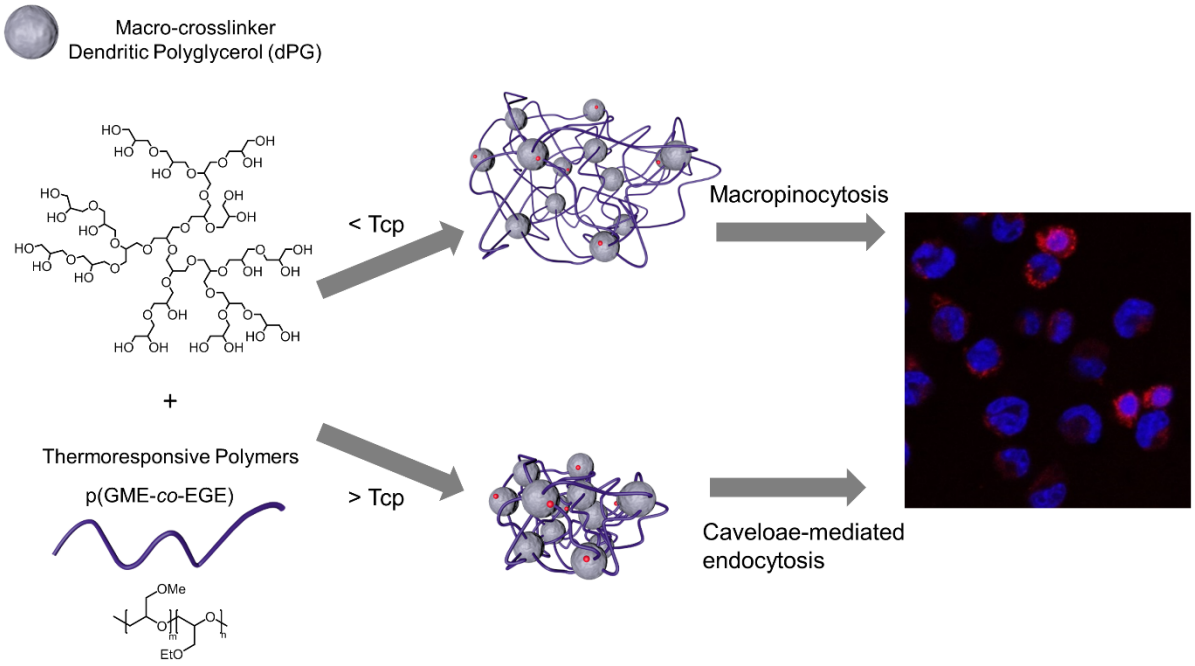


Figure 8. Adapted from Edlich *et al.*[188] with permission of Elsevier.

Author contribution: In this work the author contributed with the synthesis and characterization of thermoresponsive nanogels, tuning their transition temperatures, their labeling with a fluorescent dye, evaluation of the results with the collaboration partners, and part of the written report.

Abstract: As it has been indicated that nanogels possess a high ability to penetrate the stratum corneum, it cannot be excluded that nanogels interact with dermal dendritic cells, especially in diseased skin. In this study the potential crosstalk of the thermoresponsive nanogels (tNGs) with the dendritic cells of the skin was investigated. Although the tNGs were taken up, they displayed neither cytotoxic and genotoxic effects nor any induction of reactive oxygen species in the tested cells. Interestingly, specific uptake mechanisms of the tNGs by the dendritic cells were depending on the nanogels cloud point temperature, which determines the phase transition of the nanoparticle.

3.3 Biocompatibility and Characterization of Polyglycerol-based Thermoresponsive Nanogels Designed as Novel Drug Delivery Systems and their Intracellular Fate in Keratinocytes

C. Gerecke, A. Edlich, **M. Giulbudagian**, F. Schumacher, N. Zhang, A. Said, G. Yealland, S. B. Lohan, F. Neumann, M. C. Meinke, N. Ma, M. Calderón, S. Hedtrich, M. Schäfer-Korting, B. Kleuser, *Nanotoxicology* **2017**, *11*, 267-277.
http://dx.doi.org/10.1080/17435390.2017.1292371

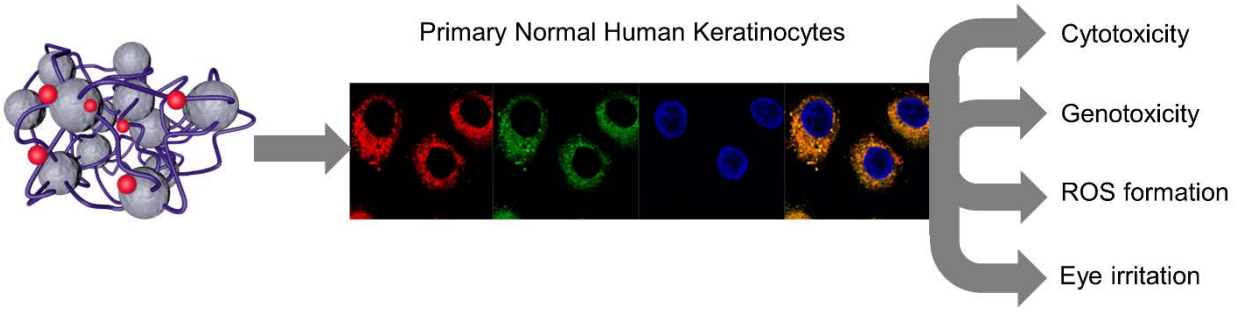


Figure 9. Adapted from Gerecke *et al.*[189] with the permission of Taylor & Francis Online.

Author contribution: In this work the author contributed with the synthesis and characterization of thermoresponsive nanogels, optimization of the synthetic procedure with regards to toxicological concerns, labeling of NGs with a fluorescent dye, development of encapsulation protocols for dexamethasone and tacrolimus, as well as part of the written report.

Abstract: Thermoresponsive nanogels (tNGs) are capable of enhancing penetration through biological barriers such as the *stratum corneum* and are taken up by keratinocytes of human skin. In this study, tNGs were synthesized from dendritic polyglycerol (dPG) and two thermoresponsive polymers: poly(glycidyl methyl ether-co-ethyl glycidyl ether) (p(GME-co-EGE)) and poly(N-isopropylacrylamide) (pNIPAM). Both tNGs were able to incorporate high amounts of dexamethasone and tacrolimus, drugs used in the treatment of severe skin diseases. MTT assay, comet assay and carboxy-H2DCFDA assay, demonstrated neither cytotoxic or genotoxic effects, nor any induction of reactive oxygen species of the tNGs in primary normal human keratinocytes (NHK). In addition, both tNGs were devoid of eye irritation potential as shown by bovine corneal opacity and permeability (BCOP) test and red blood cell (RBC) hemolysis assay.

3.4 Correlation between the Chemical Composition of Thermoresponsive Nanogels and their Interaction with the Skin Barrier

M. Giulbudagian †, F. Rancan †, A. Klossek, K. Yamamoto, J. Jurisch, V. C. Neto, P. Schrade, S. Bachmann, E. Rühl, U. Blume-Peytavi, A. Vogt, M. Calderón, *J. Controlled Release* **2016**, *243*, 323-332.

† These authors contributed equally to this work

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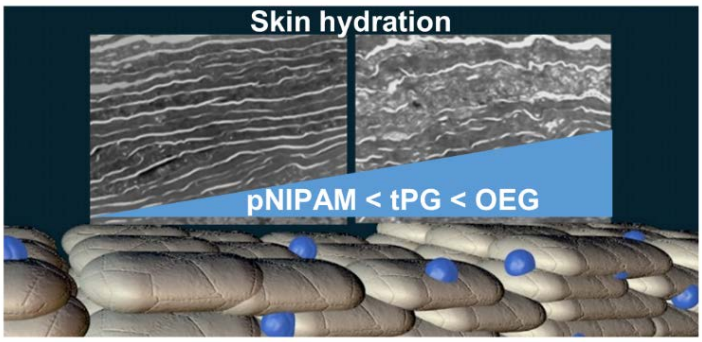

Figure 10. Adapted from Giulbudagian *et al.*[190] with the permission of Elsevier.

Author contribution: In this work the author contributed with the synthesis and characterization of thermoresponsive nanogels, their labeling with a fluorescent dye, encapsulation of model molecules, analysis of the experimental results, as well as part of the written report.

Abstract: Given the unique properties of thermoresponsive nanogels (tNGs) with regard to their thermoresponsive properties, a particular mode of skin penetration enhancement mechanism was proposed, i.e. hydration of the *stratum corneum*. A set of tNGs, differing in their chemical composition, were fabricated. Excised human skin was investigated by means of fluorescence microscopy, which enabled the detection of significant increment in the penetration of tNG as well as the encapsulated dye. The morphology of the treated skin samples was thoroughly investigated by transmission electron microscopy and stimulated Raman spectromicroscopy. It was found that tNG can perturbate the organization of both proteins and lipids in the skin barrier, which was attributed to tNG hydration effects and correlated well with tNG chemical composition.

3.5 Drug Delivery across Intact and Disrupted Skin Barrier: Identification of Cell Populations Interacting with Penetrated Thermoresponsive Nanogels

F. Rancan, **M. Giulbudagian**, J. Jurisch, U. Blume-Peytavi, M. Calderón, A. Vogt, *European Journal of Pharmaceutics and Biopharmaceutics*, **2017**, *116*, 4-11.
https://doi.org/10.1016/j.ejpb.2016.11.017

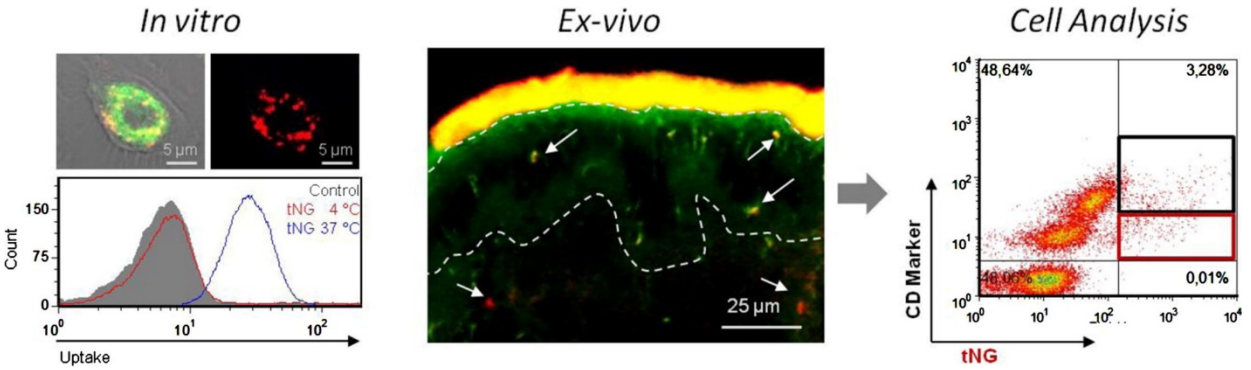

Figure 11. Adapted from Rancan *et al.*[191] with the permission of Elsevier.

Author contribution: In this work the author contributed with the synthesis and characterization of thermoresponsive nanogels, their labeling with a fluorescent dye, the encapsulation of the model molecule fluorescein, as well as part of the written report.

Abstract: In this study polyglycerol-based thermoresponsive nanogels (tNG) with diameter of 156 nm were investigated for penetration and release properties upon topical application on *ex vivo* human skin with intact or disrupted barrier. Temperature-triggered effects and the internalization of tNG by skin cells upon translocation to the viable skin layers were analyzed. The investigated tNG were tagged with indodicarbocyanine and loaded with fluorescein, so that fluorescent microscopy and flow cytometry could be used to evaluate simultaneously particle penetration and release of the fluorochrome. Topically applied tNG penetrated into the *stratum corneum* (SC) of both intact and disrupted skin explants. Only in barrier-disrupted skin significant amounts of released fluorochrome and tNG penetrated in the epidermis and dermis 2 h after topical application. When a thermal trigger was applied by infrared radiation, a significantly higher penetration of tNG in the SC and release of the dye in the epidermis were detected with respect to non-triggered samples. Penetrated tNG particles were internalized by and identified with skin cell populations.

3.6 Dendritic Polyglycerol and N-isopropylacrylamide Based Thermoresponsive Nanogels as Smart Carriers for Controlled Delivery of Drugs through the Hair Follicle

F. F. Sahle, **M. Giulbudagian**, J. Bergueiro, J. Lademann, and M. Calderón, *Nanoscale* **2017**, *9*, 172-182.

http://dx.doi.org/10.1039/C6NR06435C

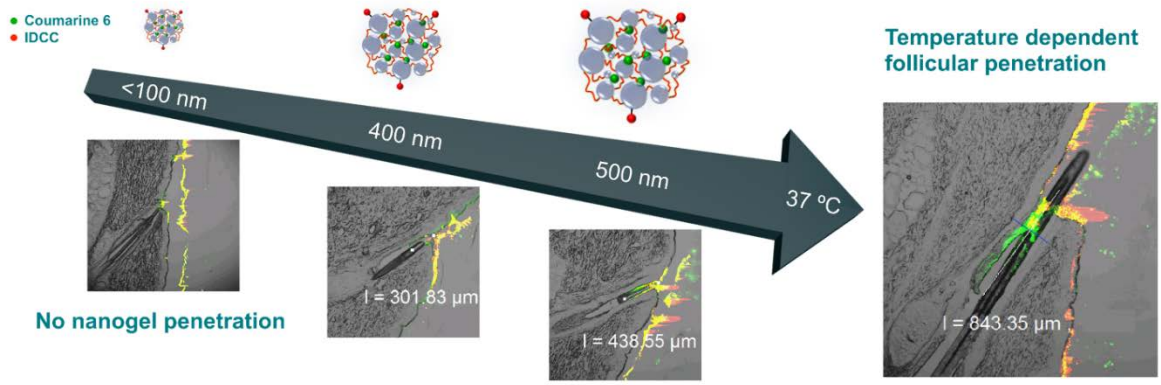

Figure 12. Adapted from Sahle *et al.*[192] with the permission of The Royal Society of Chemistry.

Author contribution: In this work the author contributed with concept, labeling of the polymers with fluorescent dyes, evaluation of the experimental analysis, as well as part of the written report.

Abstract: Thermoresponsive nanogels with a phase transition temperature of 32-37 °C were synthesized by the precipitation polymerization technique using N-isopropylacrylamide as a monomer, acrylated dendritic polyglycerol as a crosslinker, VA-044 as an initiator, and sodium dodecyl sulphate as a stabilizer. The follicular penetration of the indodicarbocyanine labeled nanogels into the hair follicles and the release of coumarin 6, which was loaded as a model drug, in the hair follicles were assessed *ex vivo* using porcine ear skin. Confocal laser scanning microscopy (CLSM) enabled independent tracking of the nanogels and the loaded dye. The results showed that, unlike smaller nanogels (<100 nm), medium and larger sized nanogels (300-500 nm) penetrated effectively into the hair follicles with penetration depths proportional to the nanogel size. The release of the loaded dye in the hair follicles increased significantly when the investigation on penetration was carried out above the cloud point temperature of the nanogels. The follicular penetration of the nanogels from the colloidal dispersion and a 2.5% hydroxyethyl cellulose gel was not significantly different.

3.7 Enhanced Topical Delivery of Dexamethasone by β-Cyclodextrin Decorated Thermoresponsive Nanogels

M. Giulbudagian, S. Hönzke, J. Bergueiro, D. Işık, F. Schumacher, S. Saeidpour, S. B. Lohan, M. C. Meinke, C. Teutloff, M. Schäfer-Korting, G. Yealland, B. Kleuser, S. Hedtrich, and M. Calderón, *Nanoscale* **2018**, Advance Article.
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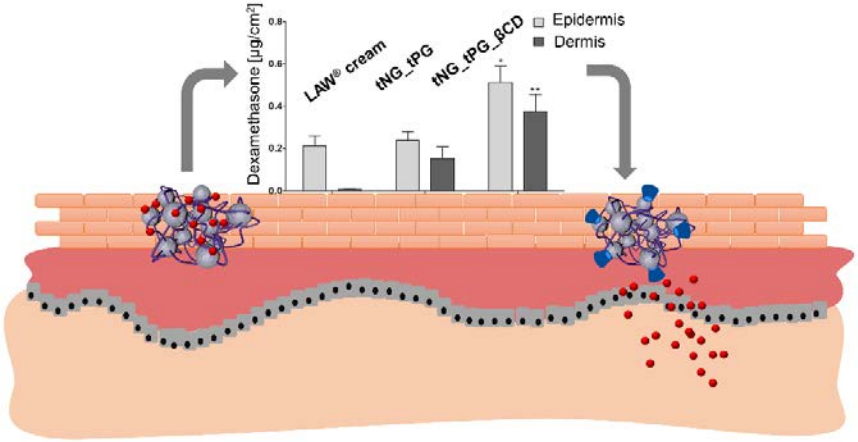

Figure 13. Adapted from Giulbudagian *et al.* (DOI: 10.1039/C7NR04480A) with the permission of The Royal Society of Chemistry.

Author contribution: In this work the author contributed with the concept development, the synthesis and characterization of the tNGs, encapsulation and release of DXM, and part of the written report.

Abstract: Highly hydrophilic, responsive nanogels are attractive as potential systems for the topical delivery of bioactives encapsulated in their three-dimensional polymeric scaffold. Yet, these drug carrier systems suffer from drawbacks for efficient delivery of hydrophobic drugs. Addressing this, β-cyclodextrin (βCD) could be successfully introduced into the drug carrier systems by exploiting its unique affinity toward dexamethasone (DXM) as well as its role as topical penetration enhancer. The fabricated carriers resulted in an efficient delivery of DXM to the epidermis and the dermis of human skin *ex vivo* (enhancement compared to commercial DXM cream: ~2.5 fold in epidermis, ~30 fold in dermis). Furthermore, DXM encapsulated in βCD decorated nanogels applied to skin equivalents down regulated the expression of proinflammatory thymic stromal lymphopoietin and outperformed a commercially available DXM cream.

3.8 Breaking the Barrier – Potent Anti-Inflammatory Activity following Efficient Topical Delivery of Etanercept using Thermoresponsive Nanogels

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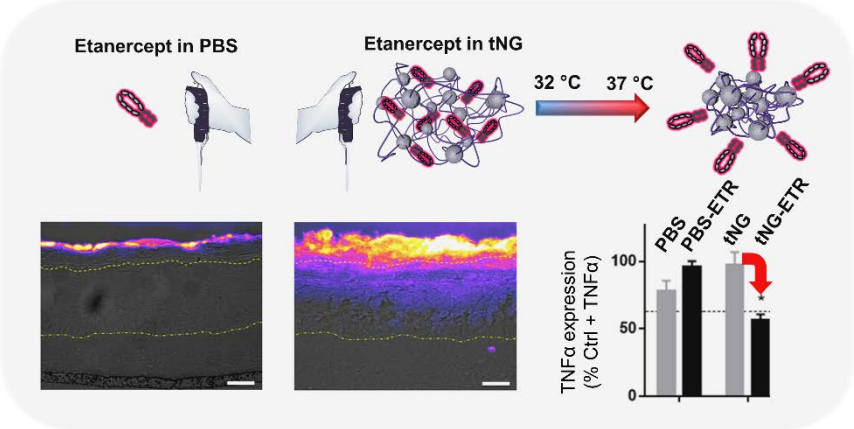

Figure 14. Adapted from Giulbudagian *et al.* (DOI: 10.7150/thno.21668) with the permission of Theranostics.

Author contribution: The author contributed to the concept of this work for the non-invasive delivery of Etanercept. The author developed the synthetic methodology of the tNGs, their labeling with fluorescent dyes, as well as the encapsulation and release of proteins, their characterization, and part of the written report.

Abstract: Novel nanoprecipitiation technique of precursor polymers, in which phosphate buffered saline was used as both solvent and non-solvent, formed linear thermoresponsive polyglycerol (tPG) based tNGs and permitted *in situ* protein encapsulation during their synthesis. Topical application of Etanercept (ETR) loaded tNGs to TNFα treated skin equivalents, and subsequent exposure to an increase in temperature over the tNG's Tcp resulted in ETR delivery throughout the *stratum corneum* and into the epidermis. This also correlated with apparent reductions in several inflammatory markers found up-regulated in the TNFα treated skin equivalents. Together these results indicate tNGs hold promise as a vehicle for stable protein encapsulation and delivery into inflamed skin.

4 Conclusions and Outlook

This work presents the potential of polymeric nanoscale transporter systems for the cutaneous delivery of drugs. Based on their unique physico-chemical properties, tNGs were investigated for their ability to deliver therapeutic moieties across the SC, while examining possible penetration pathways. This work is presented in three sections focusing on novel synthetic strategies for tNGs, a fundamental investigation of the interaction of tNGs with skin and skin cells, and finally, their realization for the treatment of inflammatory skin diseases.

Along with the implementation of the well-established tNGs based on dPG as a macro-crosslinker and thermoresponsive polymers as pNIPAM and OEGMA, novel platforms were established introducing adaptable chemistries and properties. Hence, a synthetic method for thermoresponsive, glycerol based NGs has been established. Such tNGs were synthesized by thermonanoprecipitation of the orthogonally functionalized macromonomers, i.e. dPG-bicyclooctyne (dPG-BCN) and tPG-N_3. Taking advantage of the thermoresponsive behavior of tPG allowed the utilization of water above the Tcp of the polymer as a non-solvent for precursor particle formation. Furthermore, the utilization of pre-synthesized polymers for tNG fabrication enabled not only to precisely control the phase transition temperature by tuning the co-monomer ratios (GME and EGE), but also to obtain determined distances between the crosslinking points. This approach served as a modular foundation for the synthesis of tNGs avoiding high energy input. Working hand in hand with toxicologists, we fine-tuned our synthetic protocols but also looked deeper on the cellular level interactions since it cannot be excluded that NGs interact with dermal dendritic cells (DCs), especially in diseased skin. Although the tNGs were taken up, they displayed neither cytotoxic nor genotoxic effects nor any induction of reactive oxygen species in the tested cells. Interestingly, we were able to identify specific uptake mechanisms of the tNGs by the DCs depending on their Tcp, which determines the phase transition of the nanoparticle.

Given the unique properties of such molecular architectures we were interested in the mechanism by which tNGs enhance dermal penetration. A particular mode of penetration enhancement mechanism, i.e. hydration of the SC was suggested. Different tNGs were fabricated using dPG as a multifunctional crosslinker and three different kinds of thermoresponsive polymers as linear counterpart: pNIPAM, OEGMA, and tPG. Excised human skin was investigated by means of

fluorescence microscopy, TEM and stimulated Raman spectromicroscopy. It was found that tNGs can perturbate the organization of both proteins and lipids in the skin barrier, which was attributed to tNG hydration effects. Most importantly, different drug delivery properties were detected and the ability of each investigated tNG to enhance skin penetration correlated well with the degree of induced SC hydration. Furthermore, it was shown that tPG based tNGs could create a drug depot in the SC, releasing it gradually into deeper skin layers in response to IR irradiation. Simulating diseased skin conditions by SC disturbed integrity, several cell populations were identified with internalized tNGs. To target follicular penetration, a method was developed to allow control over the size of dPG-pNIPAM based nanogels in the range of 100 - 500 nm. The follicular penetration of the indocarbocyanine labeled nanogels into the hair follicles and the release of coumarin 6, which was loaded as a model drug, were assessed *ex vivo* using porcine ear skin. The results showed that, unlike smaller nanogels (< 100 nm), medium and larger sized nanogels (300-500 nm) penetrated effectively into the hair follicles. The release of the loaded dye inside of the hair follicles increased significantly when the penetration experiment was carried out above the Tcp of the nanogels.

Finally, the delivery of therapeutic moieties and their effect on inflamed skin models was realized by the adaptation of tNGs for the transport of hydrophobic drugs as well as hydrophilic biomacromolecules. For that, a synthetic methodology for the introduction of βCD to the tNG surface was developed. This approach was proven to be advantageous for exploiting the unique complexation of βCD with DXM, along with its role as a cutaneous penetration enhancer. The βCD decorated tNGs were superior in their ability to deliver DXM into the epidermis and dermis of excised human skin, compared to a commercially available DXM formulation. Moreover, the expression of the proinflammatory cytokine thymic stromal lymphopoietin, a key player in atopic dermatitis pathogenesis, could be down regulated. The compatibility of tNG synthesis with *in situ* encapsulated proteins could be achieved by an advanced thermonanoprecipitation approach. Here, the tNGs were fabricated by replacing the conventionally used organic solvents by differently tempered aqueous buffer. Etanercept, the anti-TNF fusion protein, could be delivered into reconstructed skin equivalents providing the first evidence for its efficient non-invasive efficacy. Moreover, it could be shown that the delivery of the protein into the viable epidermis occurred explicitly upon its temperature triggered release.

As a future perspective, the established here thermonanoprecipitation methodology could be further optimized. Its up-scaling remains to be challenging for obtaining sufficient quantities of material, fabricated in highly reproducible manner. The fine tuning of the used polymeric building blocks and the mechanical properties of the obtained carriers constitute a fascinating field when correlated with a certain biological behavior. Therefore, the modification of the tNGs deformability could have a significant impact on their interaction with biological barriers. Further, the encapsulation and release of bioactives should be further investigated to achieve a more precise control over their kinetics. As the tNGs were proven to be efficient delivery systems when adapted for hydrophobic drugs as well as for therapeutic proteins, their implementation has to be established using *ex vivo* and *in vivo* disease models.

5 Zusammenfassung und Ausblick

Mit dieser Arbeit wurde das Potential polymerer Nano-Transportsysteme für den kutanen Transport von Wirkstoffen aufgezeigt. Basierend auf den einzigartigen physikochemikalischen Eigenschaften wurden thermoresponsive Nanogele (tNGs) auf ihre Fähigkeit hin getestet, Wirkstoffe über die *stratum corneum* (SC) zur Epidermis und Dermis zu transportieren. Zudem wurden mögliche Penetrationswege und -mechanismen der tNGs durch die Haut untersucht. Die Arbeit ist in drei Kapitel unterteilt: Entwicklung und Evaluierung neuer Methoden für die Nanogelsynthese, grundlegende Analyse von Wechselwirkung der tNGs mit Haut und Hautzellen, und abschließend Evaluierung der therapeutischen Wirkung in dermalen chronischen Entzündungskrankheiten.

Neben den bereits etablierten tNGs basierend auf dendritische Polyglycerol (dPG) als makromolekularer Vernetzer und den thermoresponsiven Polymeren poly(N-isopropylacrylamide) (pNIPAM) und poly(oligoethylene methacrylates) (OEGMA) wurden neue Systeme mit anpassungsfähigen Eigenschaften entwickelt. Diese tNGs wurden mittels Thermo-Nanopräzipitation mit orthogonal funktionalisierten Makromonomeren, wie beispielsweise dPG-bicyclooctyne (dPG-BCN) und lineares thermoresponsives Polyglycerol Azid (tPG-N_3), hergestellt. Durch die thermoresponsiven Eigenschaften von tPG kann die Bildung der Präkursorpartikel in Wasser oberhalb der Trübungspunkttemperatur (Tcp) erfolgen. Außerdem erlaubt die Nutzung von vorsynthetisierten Polymeren neben der exakten Kontrolle der Tcp über das Co-Monomerverhältnis von glycidyl methyl ether (GME) und ethyl glycidyl ether (EGE) einen definierten Abstand zwischen den Vernetzungspunkten im Nanogel. Außerdem erlaubt dieser modular basierte Ansatz eine Nanogelsynthese mit geringerem Energieinput. In enger Zusammenarbeit mit Toxikologen wurde das synthetische Protokoll optimiert. Zeitgleich wurden die Wechselwirkungen der tNGs mit dendritischen Hautzellen (DCs) mit besonderem Augenmerk auf Wechselwirkungen mit entzündeter Haut analysiert. Trotz einer Internalisierung der tNGs in Zellen konnten weder zytotoxische noch genotoxische Effekte oder die Induktion von reaktiven Sauerstoffspezien beobachtet werden. Vermutlich durch den Volumenphasenübergang der tNGs hervorgerufen, konnte außerdem eine Abhängigkeit des spezifischen Aufnahmemechanismus von der Übergangstemperatur der tNGs festgestellt werden.

Angeregt durch die einzigartigen Eigenschaften der Nanotransporter und ihrer Fähigkeit, eine erhöhte Wirkstoffpenetration in die Haut herbeizuführen, studierten wir den zugrundeliegenden Mechanismus. Dafür wurde zunächst eine Hydrierung der SC als Mechanismus angenommen und untersucht. Für die Experimente wurden unterschiedliche tNGs basierend auf dPG als makromolekularer Vernetzter und drei verschiedenen thermoresponsiven Polymeren (pNIPAM, OEGMA und tPG) hergestellt und ihre Penetration in exzidierte menschliche Haut anhand von Fluoreszenzmikroskopie, Transmissionselektronenmikroskopie (TEM) und Raman Spektroskopie verfolgt. Hierbei konnte gezeigt werden, dass die tNGs die Anordnung von Proteinen sowie die Anordnung von Lipiden in der Hautbarriere störten, was auf die Hydrierungseffekte der tNGs zurückgeführt wurde. Besonders bemerkenswert waren die unterschiedlichen Wirkstofftransportprofile in Abhängigkeit vom verwendeten thermoresponsiven Polymer. Hierbei konnte eine Korrelation zwischen einer erhöhten Penetration und dem Ausmaß der induzierten SC-Hydrierung herausgearbeitet werden. Des Weiteren wurde aufgezeigt, dass tPG basierte tNGs ein Wirkstoffdepot in der SC ausbildeten, aus dem der Wirkstoff unter Stimulierung mit Infrarotstrahlung in tiefere Hautschichten abgegeben wurde. Mit Modellen erkrankter Haut, die eine gestörte SC Integrität aufweist, konnten mehrere Zellpopulationen identifiziert wurden, die tNG internalisiert hatten. Um einen follikulare Penetration der tNGs zu ermöglichen, wurde außerdem eine Synthesestrategie entwickelt, die eine genaue Kontrolle der Partikelgröße von dPG-pNIPAM basierten Nanogelen im Bereich von 100-500 nm zulässt. Indocarbocyanin-markierte Nanogele wurden mit dem Modellfarbstoff Coumarin 6 beladen und die follikulare Penetration und Farbstofffreisetzung wurden *ex vivo* an Schweineohren untersucht. Die Ergebnisse zeigten, dass im Gegensatz zu kleinen Nanogelen (< 100 nm) mittelgroße und große Nanogele (300-500 nm) effektiv in die Haarfollikel penetrieren. Außerdem konnte eine signifikante Erhöhung der Farbstofffreisetzung bei Versuchsdurchführung oberhalb der Tcp aus den tNGs gezeigt werden.

Abschließend wurde die Effizienz des Wirkstofftransports sowohl mit hydrophoben Wirkstoffmolekülen als auch mit hydrophilen Biomakromolekülen und ihre therapeutische Wirksamkeit anhand von Hautmodellen im entzündeten Stadium untersucht. Hierfür wurde eine synthetische Route zur Einführung von β-Cyclodextrin (βCD) auf die Oberfläche des tNGs entwickelt. Dieser Ansatz stellte sich als besonders vorteilhaft heraus, da so die Komplexbildung zwischen βCD und Dexamethason (DXM) genutzt werden konnte und βCD als kutaner Penetrationsverstärker verwendet werden kann. Im Vergleich mit kommerziell Verfügbaren DXM-

Formulierungen zeigten die mit βCD-ausgestatteten tNGs eine erhöhte Effizient für den Wirkstofftransport in Epidermis und Dermis von exzidierter menschlicher Haut. Zudem konnte eine Herunterregulation der Expression des entzündungsfördernden Cytokins Thymic-Stromal-Lymphopoietin beobachtet werden, dem besonders bei der Entwicklung von atopischer Dermatitis eine wichtige Bedeutung zugeschrieben wird. Kompatibilität von tNG-Synthese und *in situ* Verkapselung von Proteinen ohne Veränderungen der Proteinstruktur und -aktivität hervorzurufen, konnte durch eine verbesserte Thermonanopräzipitationsmethode erreicht werden. Hierbei wurden die üblicherweise genutzten organischen Lösemittel durch wässrige Pufferlösungen unterschiedlicher Temperatur ersetzt. Etanercept, ein anti-TNF-Bindungsprotein, konnte erfolgreich in rekonstruierte Haut transportiert werden – ein erster Hinweis auf seine effiziente, nicht-invasive Wirksamkeit. Außerdem konnte eine ausschließlich temperaturstimulierte Freisetzung des Proteins demonstriert werden.

Perspektivisch sollte die entwickelte Methode der Thermonanopräzipitation weiter optimiert werden. Eine höhere Skalierung der Synthese der tNGs in ausreichender Reproduzierbarkeit bleibt weiterhin herausfordernd. Die Feinabstimmung der genutzten Bausteine sowie die mechanischen Eigenschaften der erhaltenen Transportsysteme in Korrelation mit spezifischen biologischen Verhaltensweisen stellt ein weiteres zu bearbeitendes Forschungsgebiet dar. Eine systematische Modifikation der Verformbarkeit der tNGs könnte einen signifikanten Einfluss auf die Wechselwirkung zwischen Nanotransporter und biologischen Barrieren aufweisen. Außerdem sollte die Verkapselung und Freisetzung biologisch aktiver Substrate weiter untersucht werden um eine präzisere Kontrolle über die Freisetzungskinetik zu erlangen. Es konnte bereits die Effizienz der tNG als Wirkstofftransportsystem für hydophobe Wirkstoffe sowie therapeutische Proteine nachgewiesen werden. Daher sollten *ex vivo*- und *in vivo*-Modelle entwickelt und implementiert werden.

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7 Appendix

7.1 Publications and Conference Contributions

List of Publications

1. **M. Giulbudagian** †, M. Asadian-Birjand †, D. Steinhilber, K. Achazi, M. Molina, M. Calderón, Fabrication of thermoresponsive nanogels by thermo-nanoprecipitation and in situ encapsulation of bioactives, *Polymer Chemistry* **2014**, *5*, 6909-6913.
2. M. Molina, **M. Giulbudagian**, M. Calderón, Positively Charged Thermoresponsive Nanogels for Anticancer Drug Delivery *Macromol. Chem. Phys.* **2014**, *215*, 2414-2419.
3. **M. Giulbudagian** †, F. Rancan †, A. Klossek, K. Yamamoto, J. Jurisch, V. C. Neto, P. Schrade, S. Bachmann, E. Rühl, U. Blume-Peytavi, A. Vogt, M. Calderón, Correlation between the chemical composition of thermoresponsive nanogels and their interaction with the skin barrier, *J. Controlled Release* **2016**, *243*, 323-332.
4. F. F. Sahle, **M. Giulbudagian**, J. Bergueiro, J. Lademann, M. Calderón, Dendritic polyglycerol and N-isopropylacrylamide based thermoresponsive nanogels as smart carriers for controlled delivery of drugs through the hair follicle, *Nanoscale* **2017**, *9*, 172-182.
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7.2 Curriculum Vitae

For reasons of data protection, the curriculum vitae is not included in the online version.