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Stimuli-responsive Nanogels for Dermatology, Photothermal Therapy, and Detection of Circulating Tumor Cells

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This PhD thesis was carried out from July 2011 until September 2015 in the research group of Prof. Dr. Marcelo Calderón at the Institute for Chemistry and Biochemistry, Faculty of Chemistry, Biology, and Pharmacy of the Freie Universität Berlin.

I hereby declare that this PhD thesis was prepared autonomously and that no illegal help was used. Contributions of others, e.g., content, quotes, or figures are indicated by referring to the original work.

Berlin, September 2015 _____ (Mazdak Asadian Birjand)

1. Referee: Prof. Dr. Rainer Haag

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Date of Disputation: 9th of December 2015

بسم الله الرحمن الرحیم

تقدیم به فرزندم عیسی

که نور و شادی بخش زندگی من است

و همسرم آیلین

که همیشه تنها عشق من در دنیا خواهد بود

Dedicated to Isa & Aylin

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1 Introduction

1.1 Nanomedicine

In the last two decades, governments and business all over the world have been investing in nanoscience, because nanotechnology is widely seen as a platform with enormous potential to improve such diverse fields as water decontamination, drug development, communication technologies, and to develop lighter and stronger materials.[1] Many engineered nanomaterials are already being applied into our daily life in items such as sporting goods, electronics, cosmetics, tires, and sunscreens.[2] The medical application of nanotechnology that is referred as nanomedicine offers many opportunities in the improvement of healthcare, especially for cancer and for diagnosis, monitoring, control, and treatment of biological systems.[3, 4, 5] As cancer is the leading cause of death worldwide and the biggest health concern,[6] nanomedicine is exploring both the early detection of localized and disseminated tumor cells in patients and looking for specific cancer treatments with reduced systemic toxicity. Both methodologies are crucial for patients' survival rates and present great challenges for a future personalized oncology. Currently both natural and synthetic lipids and polymers as well as magnetic nanoparticles are commonly being used as drug delivery systems, imaging, and detecting agents in their clinically approved formulations (Table 1).[7, 8, 9] The vast variety of nanomedicine systems (nanosystems) that have been developed include polymer-protein and polymer-drug conjugates as well as lipid-based carriers such as liposomes and micelles, dendrimers, nanogels, carbon nanotubes, gold nanoparticles, including nanoshells and nanocages, silver nanoparticles, and iron oxide nanoparticles.[5, 7, 10, 11, 12, 13, 14, 15, 16]

These systems need to be biocompatible for rapid and effective clinical translation and well characterized for an easy functionalization of the used material. Additionally, the systems should have a higher uptake efficiency in the target tissue or cell than in healthy tissue, a greater solubility or colloidal stability in an aqueous environment for increased effectiveness, an extended plasma circulation, long shelf life, and low rate of aggregation. The main challenge for polymer-drug formulations, which is referred to as polymer therapeutics, a concept defined by Helmut Ringsdorf for drug delivery systems that dates back to the mid 70s of the past century,[17] is that they have to maintain certain advantages over pure drugs. This includes protection of a drug from premature degradation by preventing an interaction between drug and biological environment, an enhanced absorption into the targeted tissue, and hence achieve an improved control over the pharmacokinetics and pharmacodynamics, and an improvement of intracellular penetration.[10]

Table 1. Selected nanotechnology in cancer therapy.[7] Adapted from Ref. 7 with permission from The Wiley Company

Trade name	Technology	Indication	Status
Abraxane	Albumin-conjugated paclitaxel	Metastatic breast cancer	Approved
Doxil	Liposomal doxorubicin	HIV-related Kaposi sarcoma, metastatic breast and ovarian cancer	Approved
DaunoXome	Liposomal daunorubicin	HIV-related Kaposi sarcoma	Approved
Myocet	Liposomal doxorubicin	EGFR2-positive metastatic breast cancer	Approved
DepoCyt	Liposomal cytarabine	Intrathecal lymphomatous meningitis	Approved
Marqibo	Liposomal vincristine sulphate	Acute lymphoblastic leukemia	Approved
Oncaspar	Polymeric PEG-L-asparaginase	Acute lymphoblastic leukemia	Approved
Zinostatin stimalamer	Copolymer styrene maleic acid-conjugated neocarzinostatin	Unresectable hepatocellular carcinoma	Approved
Resovist	Carboxydextran-coated super paramagnetic iron oxide nanoparticle (SPIO)	MRI contrast agent for imaging hepatocellular carcinoma	Approved
Genexol-PM	Polymeric methoxy-PEG-poly(D,L-lactide) paclitaxel	Metastatic breast cancer	Approved
NanoTherm	Aminosilane-coated SPIO	Local ablation of glioblastoma multiform	Approved
Opaxio (Xyotax)	Poly-L-glutamic acid (poliglumex) conjugate with paclitaxel	Ovarian cancer and NSCLC	Phase III
NKTR-102	PEG micelle with irinotecan	Breast and colorectal cancer	Phase III
Mepact	Liposomal muramyl tripeptide phosphatidyl ethanolamine	Nonmetastatic resectable osteosarcoma	Phase III
ThermoDox	Liposomal nanoparticle with thermal release of doxorubicin	Hepatocellular carcinoma	Phase III

Up to date, several nano drug carriers, mainly liposomes, polymer-protein conjugates, and nano imaging agents have been approved for clinical use and have been reviewed elsewhere.[5, 10] Among the polymer-drug conjugates, of which 3 have been approved (Copaxone, Renagel, Welchol), currently one polymer-drug formulation has reached Phase III clinical trials, and at least 12 polymer-drug conjugates have entered Phase I and II clinical

trials that are mainly designed for targeting blood vessels in tumors.[3, 18] Despite advanced chemistries and the diversity of novel drug targets available, only a few drugs (doxorubicin, paclitaxel, camptothecin, and platinate) and polymers (poly(ethylene glycol) (PEG), dextran, poly-L glutamic acid, and N-(2-hydroxylpropyl)methacrylamide (HPMA) have been approved as a platform for the development of polymer-drug conjugates.

1.2 Passive and active targeting of polymer therapeutics

In the spite of the challenges for tumor targeting with nanomedicine and the need to develop a rational design for nanosystems for application in oncology, our understanding of tumor biology and tumor environment is fundamental. The characteristics of tumors include the absence of a working lymphatic system and leaky blood vessels in their environment. This results from an aggressive and rapid cancer tissue growth that affects angiogenesis and hypervasculature and leads to impaired lymphatic drainage and defective vascular architecture. Nanosystems that are applied into the bloodstream can therefore diffuse into the leaky malignant blood vessels and passively reach the tumor site, while the absence of a lymphatic system retains the accumulated nanosystems in the tissue and lets them interact with the tumor cells (Figure 1). Nanosystems, however, cannot pass through the epithelial walls of normal tissue. This characteristic is known as the enhanced permeability and retention (EPR) effect of tumor tissue and was first described by Maeda in the mid-1980s.[19] Based on the concept of passively targeting tumor tissue through the EPR effect, nanosystems made from liposomes and polymer-protein conjugates reached the market in the mid-1990s and were later approved for further use (Table 1).[10] Even though passive targeting enables a safer way to apply chemotherapeutics to the patient, because the systemic toxicity of the drug itself is reduced, passive targeting also has some drawbacks. A main point is the random nature of the process itself so that the applied drugs cannot efficiently diffuse into the tumor. The passive targeting approach shows that only less than 10% of the administrated nanoformulation reaches the target side, while the majority accumulates in the liver and kidneys.[20] Moreover, this lack of control may provoke and induce multi-drug resistance, a process in which overexpressed membrane proteins expel several chemotherapeutic drugs across the plasma membrane and out of the cell, while limiting the drug-target interaction.[21, 22, 23] Finally, some tumors are not suitable for passive targeting as the permeability of malignant blood vessels may differ throughout the tumor.[24]

One way to fight these limitations is the use of the so-called active targeting approach. In this approach the applied nanosystem is modified with a targeting moiety that actively binds to specific cell types. Nanosystems that are decorated with targeting moieties will recognize and bind to the target cell through interactions, such as ligand-receptor interaction, and

become internalized before the therapeutic agent is released into the cell (Figure 1). When designing an active targeting approach, two considerations are of main importance, for one, the targeting agent has to bind with high selectivity to molecules that are uniquely expressed on the target cell surface. Secondly, the target surface marker has to be overexpressed on the target cell surface in comparison to normal cells to improve the approach's specificity. Studies on the targeting efficiency of targeting agent-decorated liposomes, for instance, have shown that the density of receptors expressed on the cell surface has to lay in the range of 10^4-10^5 per cell when targeting the B-cell receptors. Cells that expressed receptors in a lower density were less effectively targeted by the liposomes.[25] In another study, it was observed that at least a receptor density of 10^5 of ErbB2 receptors per cell in a breast cancer model was necessary to show an improved therapeutic effect of a targeting moiety bearing a liposome-doxorubin formulation in comparison with its non-targeted counterpart.[26]

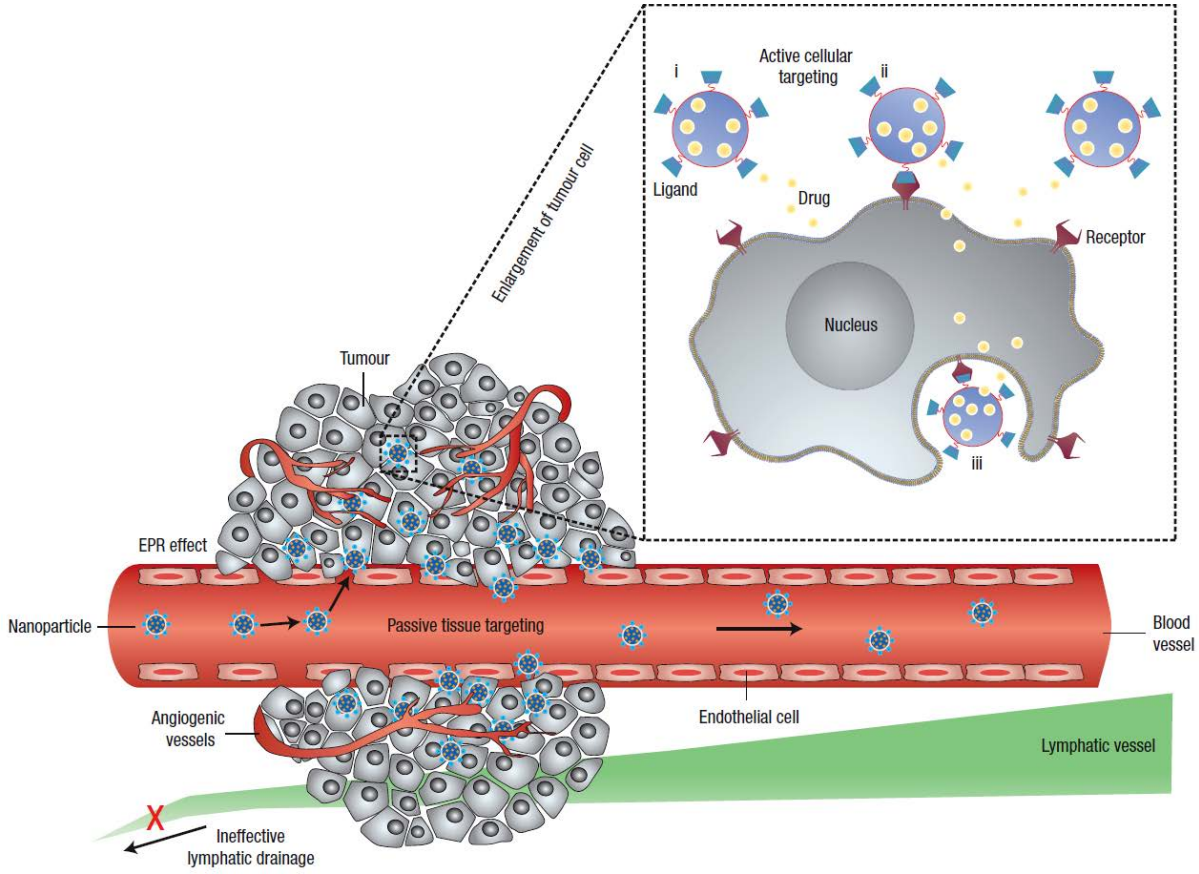

Figure 1. Illustration of different pathways by which nanosystems can deliver drugs to tumors. Nanosystems (nanoparticles) are shown as circles that can perform passive tissue targeting as a result of the EPR effect, and active cellular targeting if ligands are attached to the nanosystem that promote cell-specific recognition and binding.[10] Reproduced from Ref. 10 with permission from The Nature Publishing Group.

In addition, the ligand-receptor interaction between the nanosystem and the cell surface can result in a receptor mediated cell uptake, which is desirable in most of the cases when drug delivery into the cell is the objective.[27] For improving the targeting and enhancing the affinity to the cell surface, multivalent effects, also referred as avidity, play an important role. Multivalency is based on multiple interactions of ligands that are conjugated on a polymer which interact simultaneously with multiple receptor sites much stronger than a monovalent binding.[28, 29] For example, a folate decorated dendritic nanosystem showed, as a result of multivalency, an enhancement of binding avidity of up to ~170,000 fold to folate-binding proteins that were immobilized on a surface in comparison to free folate.[30] This example, among many others, demonstrates that the avidity along with receptor density on the target and the number of ligands per nanosystem have significant influence on the ligand-receptor interaction.[29, 31]

Up to date, a huge variety of targeting agents have been discovered and developed. Among targeting agents, such as proteins,[32, 33, 34] nucleic acids,[35] and other receptor ligands like vitamins,[36] peptides,[37] and carbohydrates,[38] monoclonal antibodies have achieved remarkable clinical success over the past two decades.[39] Since the mid-1990 at least 12 antibodies have been clinically approved by the FDA for treatment of a variety of solid tumors and hematological diseases. Moreover a large number of therapeutic antibodies are currently in early and late-stage clinical trials.[39] Non-antibody agents have the advantage that they are often readily accessible, easy to handle, and inexpensive to produce. On the other hand, they lack in high selective expression. Targeting agents such as transferrin and folate that target grow-factor receptors, and RGD, which targets cellular adhesion molecules, can also bind to non-target tissue. Through an advanced antibody engineering, however, a wide range of tissue affinities are being achieved today. Moreover, the possibility that is given through phage display libraries allows one to rapidly select antibodies or their fragments that obtain high specificity for the target tissue.[10, 40]

In some studies, however, active targeting has revealed some drawbacks. For solid tumors, for instance, it was shown that high binding affinities decreased the internalization of nanosystems as a result of the so-called “binding-site barrier,” a situation where the nanosystem binds so strongly to the target that penetration into the tissue is limited.[40, 41] In another study,[42] where antibody coated iron oxide nanoparticles were evaluated for imaging purposes, only 3.4% of injected particles were localized in the tumor after an *in vivo* biodistribution study, a value that was expected for non-actively targeted particles through the EPR effect.[20] Finally, evidence was found that antibodies may not increase the antibody-coated nanosystems’ tumor localization but may increase their internalization into the tumor significantly.[43]

These findings led to the conclusion that, for an advanced tumor targeting that allows the majority of applied dose to reach the disease site, a combination of passive tissue and active cellular targeting is required. Meanwhile moieties can also be attached to the nanosystems that enable targeting by external meanings, such as magnetic fields. Moreover, the smart design of nanosystems in the sense of shape, size, elasticity, and surface charge may have a significant impact on their uptake profile and their interaction with biological systems.

1.3 Considerations for the design of modern nanomedicines

The central question for the development of nanosystem in therapeutic applications is how to design and control material properties to achieve or to avoid biological responses. For example, nanosystems with special physico-chemical properties have been shown to influence many biological interactions such as circulation in the bloodstream,[44] phagocytosis,[45] adhesion, and targeting interactions, as well as toxicity profiles.[46] These material properties include their size, shape, elasticity, and their surface charge and chemistry.[31, 47, 48, 49] The motivation to use physico-chemical material properties to control biological functions derives from nature itself. When administrating systemic nanosystems, for instance, many biological barriers have to be overcome including organ-level clearance mechanisms, such as those operating in the kidneys, liver, and the spleen. Nanosystems with less than 5 nm in size get rapidly filtered from the circulation through renal clearance mechanism.[50, 51] Moreover systems that obtained sizes in the micrometer-scale get accumulated primarily in the liver, spleen, and bonemarrow.[52, 53, 54] On the other hand, red blood cells, which can be seen as natural nano and micron sized transporters, use their mechanical elasticity and their discoid shape to avoid clearance in the spleen. Moreover platelets, which can circulate up to 11 days,[55] are able to use their lens-like shape to assist their adhesion function and their movement along blood vessel walls.[56]

To be able to understand and control such behavior in biological systems, a closer look at the diverse nanosystems' physico-chemical properties with respect to the interdependency of the size, shape, and surface chemistry is necessary.

1.3.1 Size

The size of nanosystems applied *in vivo* has had a significant influence on biological relevant functions such as extravasation, targeting, circulation times, immunogenicity, internalization, degradation, intracellular trafficking, uptake mechanism, and clearance as shown in Figure 2.[57, 58, 59, 60]

Generally it is widely recognized that nanosystems that are designed for extravasation into tissue via systemic administration should be between 100 - 200 nm in size for a prolonged bloodstream circulation because they obtain sizes large enough to avoid uptake in the liver and are yet small enough to avoid filtration in the spleen.[48] A long circulation time is directly related with an increased tumor accumulation which is favorable if tumor retention, cell uptake, and drug release purposes are desired.[31] In addition, circulation of nanosystems can be increased through PEGylation, which is the attachment of polyethylene glycol (PEG) polymer chains to the system leading to increased water solubility and hydrodynamic diameter. As a result, renal clearance is reduced and a blood half-life of up to 55 h has been reported.[61, 62] Nanosystems that obtain sizes smaller than 5 nm are generally undesirable for intravascular injection as they get rapidly filtered through the renal clearance mechanism.[50, 51] Meanwhile nanosystems in the range of 1–10 μm in size have the disadvantage that they are taken up by macrophages, as this is the size range of bacteria, the most likely targets of macrophages, and accumulate in liver and spleen.[54, 63]

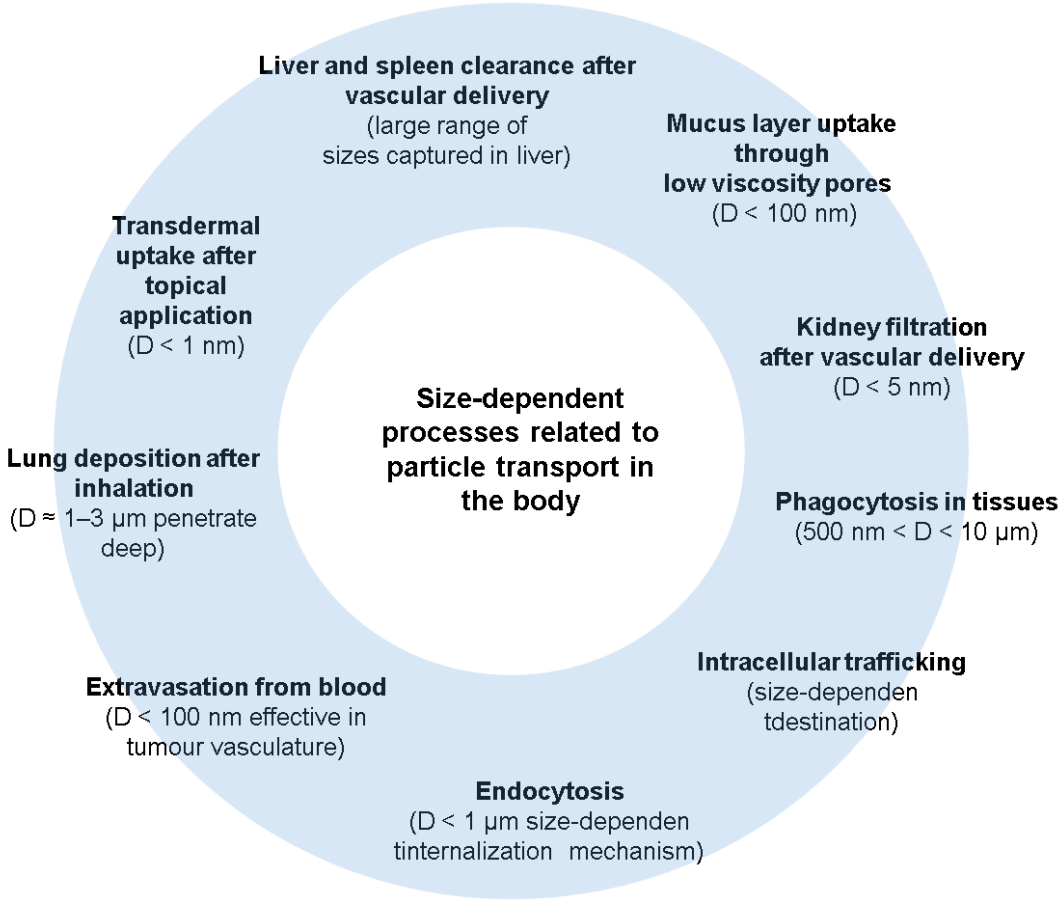

Figure 2. Size-related biological barriers in the human body for administrated nanosystems.[47] Reproduced from Ref. 47 with permission from The Nature Publishing Group.

Even though such general rules are very useful, the design of nanosystems has to consider important parameters, which will be explored in the following sections, and the literature has revealed well-documented exceptions to the general guidelines. For example, nanosystems larger than 300 nm escape from uptake by the liver and the spleen because they were designed to have such a mechanical deformability.[48] On the other hand, 1 – 3 µm sized nanosystems that may not be suitable for systemic administration could be considered for other applications, for instance pulmonary delivery, as they can diffuse deep into the lungs.[47] In addition, nanoparticles bigger than 1 nm are not supposed to be able to penetrate viable skin layers in dermatological applications. But recent studies have indicated that soft nanosystems, which were able to perform a phase transition, could be detected in the viable human skin layers after topical administration.[64, 65] Moreover, until recently, nanosystems bigger than 100 nm were thought to be unable to efficiently diffuse through mucus layers, as mucus has a fast flow rate and is quickly renewed, which results in rapid clearance from administration site. Studies, however, have demonstrated that nanosystems up to the size of 500 nm were able to penetrate mucus, if they were coated with a mucus-inert polymer.[66, 67]

1.3.2 Shape

In many instances, studies have shown that, along with particle size, shape plays a key role in a nanosystem's transport through the body, its internalization pattern, and its targeting efficacy.[45, 48, 68, 69] In this sense, some studies have even demonstrated the advantages of nanosystems engineered with unique shapes over their spherical counterparts, which is interesting as most of the approved nanosystems for clinical use have spherical shapes (liposomes, micelles, iron oxide nanoparticles, etc.) For example, recent studies have shown that oblate-shaped nanosystems demonstrated a prolonged circulation in the blood stream as a result of the lower uptake rates of macrophages,[70, 71, 72] which confirms the findings that phagocytosis by macrophages depends on the shape of the applied nanosystem.[45] In other studies investigating the binding efficiency of antibody-coated nanosystems, it was shown that spheres were internalized and degraded much faster in endothelial cells than their disc-shaped counterparts.[73] On the other hand, when protein-coated nanorods and nanospheres were compared, the rods showed twice as many specific binding interactions than their spherical analog.[74] In addition, a striking *in vivo* study recently compared the ability of nanochains (three covalently connected iron oxide nanoparticles) with respect to their spherical variant to perform vascular targeting in a tumor bearing mice model. Hereby, 45 min after systemic administration, vascular targeting of the nanochains was obtained twice as high as their spherical counterparts. In addition, vascular targeting resulted in more than 40% of the administered dose of nanochains that were localized in the primary tumor.[75]

When comparing antibody-coated nanorods with their antibody-coated spherical derivate *in vitro* a five time higher cellular growth inhibition for breast cancer cells was observed for the nanorods as a result of their 66% increase in binding and internalization by the breast cancer cells in comparison with the spheres.[76] In a further *in vivo* study, nanochains, composed of three connected nanoparticles with a doxorubicin bearing liposome, were compared with the drug bearing liposome alone. Here the nanochains revealed a twice as high internalization into the tumor in a mammary adenocarcinoma model. A radiofrequency triggered release study of the same nanochains demonstrated moreover a threefold higher therapeutic efficacy than the drug bearing liposome.[77, 78] An interesting *in vitro* study of nanogel with different shapes, sizes, and surface charges was performed using the top-down fabrication approach called Particle Replication in Non-wetting Templates (PRINT). Here three cationic and negatively charged cubically shaped particles ranging in side length by 1, 2, and 3 µm and a bunch of cylindrically shaped nanogels with diameter sizes from 100 – 500 nm were compared as shown in Figure 3. In terms of internalization, 84% of all cationic nanosystems were internalized after 1 h of incubation, whereas less than 5% of the negatively charged ones obtained uptake, suggesting that surface charge plays a significant role in internalization processes. Moreover, a clear correlation between the size and the shape regarding the rate of internalization was obtained, and it was demonstrated that particles with different shapes but similar volumes were internalized at diverse rates.[49] Finally, filamentous like nanosystems that obtained a one-dimensional length of 18 µm were compared in terms of biodistribution *in vivo* with their spherical counterpart with a similar chemistry. Interestingly, a circulation time of up to one week was obtained for the filamentous nanosystems[72] which defeated the circulation time of approved “stealth” liposomes.[79] Here shape effects in terms of flexibility had an important impact on the observed results.

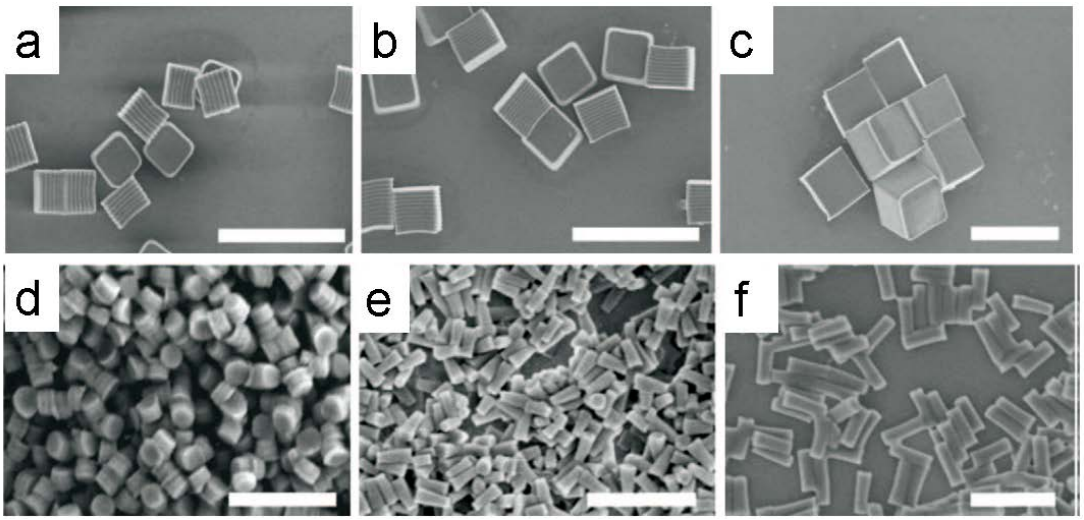

Figure 3. Scanning electron microscopy images of nanogels derived from the PRINT technique. (a–c) Series of the cubic nanogels where diameters equal to 2 µm (a), 3 µm (b), and 5 µm (c). (d-f) Series of cylindrical shaped nanogels. Diameter 200 nm, height 200 nm (d); diameter 100 nm, height 300 nm (e); diameter 150 nm, height 450 nm (f)]. Scale bars: (a-c), 20 µm; (d-e), 1 µm.)[49] Reprinted with permission from Ref. 49. Copyright (2008) Proceedings of the National Academy of Sciences of the United States of America.

1.3.3 Mechanical properties

The flexibility or elasticity, along with the size and shape of nanosystems, play an important role in their interactions with tissues of the human body. Human body tissues obtain selective mechanical properties that vary from very stiff, as obtained for calcified bone (> 30 kPa Young's modulus), to moderately soft, like skin and muscles (~10 kPa Young's modulus), and to very soft tissue as shown for the brain (0.5 kPa Young's modulus).[80] In addition, many cellular processes, like cell spreading and adhesion, depend on mechanical processes that originate from the cellular microenvironment.[81] An *in vitro* study has used these principles to investigate the phagocytosis of polyacrylamide beads with respect to their mechanical properties. Interestingly, macrophages showed a strong preference to trap rigid objects in comparison to their softer counterparts.[82] Moreover, a recent study compared PEG diacrylate nanogels of different softness (10 kPa and 3000 kPa) but similar size and shape (200 nm, spherical) *in vivo* with regard to their influence in blood circulation, phagocytosis, endocytosis, and antibody-mediated targeting. Soft nanogels offered a better circulation and improved targeting than their harder counterparts.[83]

The impact of a nanosystem's flexibility on their biological function has been explored to a far lesser extent than the impact of their size and shape. Even though the potential advantages of tuning their elasticity are not clear, a general tendency shows that softer nanosystems obtain longer circulation times and less immune system uptake.

1.3.4 Protein corona

When nanosystems come in contact with biological fluids such as blood, plasma, or interstitial fluids, they get immediately coated with proteins that form a so-called "protein corona" around the nanosystem.[84] This behavior is mainly driven by physico-chemical properties of the nanosystem and can cause unwanted effects.[85] The protein corona can hinder the binding ability of the nanosystem to the target and may induce an immune response that can cause the nanosystem to be removed from the bloodstream before

reaching its target.[86] Moreover, proteins that are adsorbed on the nanosystems surface can undergo conformational changes exposing binding sites to initially non-targeted tissue and therefore generating toxicity.[87] In addition, a rapid corona formation can lead to endothelial cell death, hemolysis, and thrombocyte activation.[84] For addressing these unwanted attributes, research has focused on modifying nanosystem's surface chemistry. A predominant strategy is to modify the nanosystems surface with PEG to render their surface more hydrophilic and to neutralize surface charges.[88, 89, 90, 91] Another approach is to dictate the protein coating on the nanosystems by conjugation of selected proteins to the nanosystem prior to administration. For example, albumin-coated liposomes were compared *in vivo* with PEGylated liposomes and displayed extended circulation times, higher therapeutic efficacy, and less toxicity. [92, 93, 94] This led to the conclusion that the shielding effect through PEGylation, however, had a transient character. The exploration of strategies to counteract the drawbacks of a protein corona still has to be addressed to be rationalized in a future design of nanosystems.

1.3.5 Safety

As mentioned above, the biological impact of nanosystems strongly depends on their physico-chemical properties like size, shape, chemical composition, solubility, and aggregation behavior. These parameters can influence their biodistribution, immunogenic responses, internalization, binding abilities, and toxicity.[95] Among such properties, studies have shown that size, hydrophobicity, and surface charge are the main parameters that have an impact on nanosystem's biocompatibility as mentioned in Figure 4. In general, hydrophobic particles show higher toxicity than hydrophilic ones while negatively charged nanosystems obtain better biocompatibility over cationic ones. Addressing concerns about long-term toxicities, it is noteworthy that nanosystems that are supposed to be designed in sizes bigger than 5 nm should obtain a degradable character, which is fragmented into smaller parts and removed by organ-level clearance mechanisms.

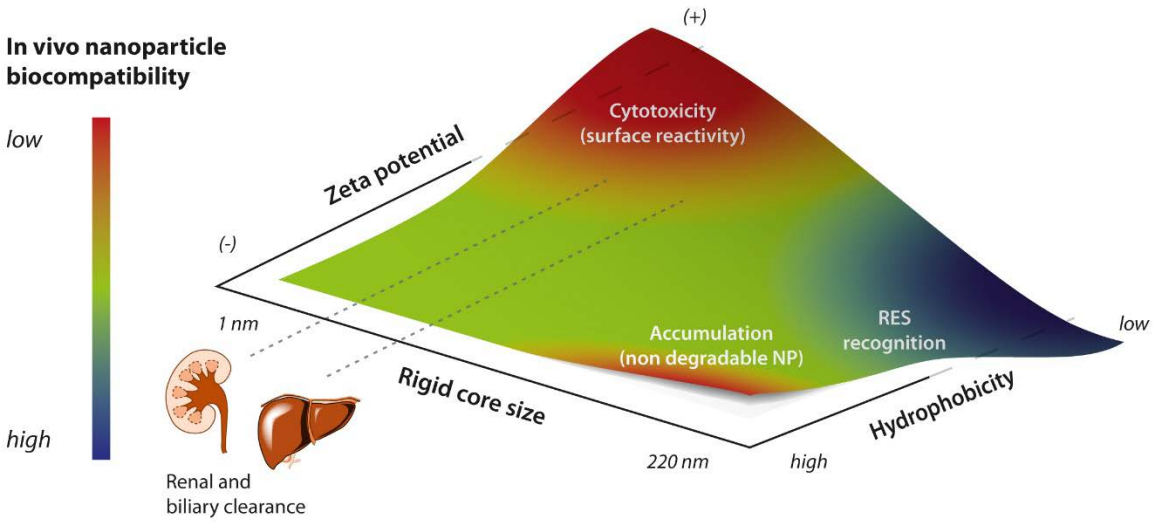

Figure 4. Three-dimensional phase diagram showing the biocompatible trends of 130 nanosystems after in vivo screening with respect to their physico-chemical properties. Color codes represent the degree of biocompatibility where, red is likely toxic, blue likely safety, and blue–green–yellow intermediate levels of safety.[95] Reproduced from Ref. 95 with permission from The Nature Publishing Group.

In conclusion, it was shown that many aspects in the design of nanosystems, including size, shape, elasticity, and charge, play significant roles when it comes to biodistribution profiles and therapeutic efficacies. Taking into account that the targeted biological environment is a complex system underlying complex biological, biochemical, and biophysical mechanisms, a single “one-design-fits-all” approach might not be the most effective option. Moreover, the nanosystems design should be shaped to the desired application.

1.4 Responsive polymer materials

One major aspect for the design of modern nanosystems is the introduction of responsive behaviors that can induce specific material properties in response to external stimuli. This kind of behavior derives from nature itself, when biological functions that require selectively tailored biomaterials respond to changes in their environment with chemical functions and structural changes.[96] The controlled responsive behaviors of designed materials that have arisen with the advances and developments in polymer chemistry are various. The most common abilities are the so-called coil-to-global transition, which is attributed to the behavior of linear responsive polymers that can undergo a phase transition in solution,[97] the swelling/de-swelling behavior of polymers in bigger constructs like hydrogels, the commitment of structural and functional changes both chemically and physically,[96] and the performance of degradation processes.[98] To trigger these responses various stimuli like temperature, pH, light, ionic strength, as well as magnetic and electric fields have been explored for use in diverse fields of application such as drug delivery, tissue engineering, and sensor material development.[99] A small portion of the huge variety of nanostructured responsive polymer materials that have been developed is illustrated in Figure 5. The family of thin films, for instance, includes immobilized polymer brushes, layer-by-layer films, thin films of cross-linked networks, and membranes that serve mainly purposes in reconstructable surfaces. These materials can change their permeability, adhesion properties, as well as mechanical and optical properties, in response to external stimuli, leading to applications such as self-healing coatings, switchable transparency film, biosensors, etc.[96] Out of all the responsive nanosystems, including miniemulsions, core-shell particles, nanocapsules, and micelles, which are highly relevant for purposes in nanomedicine, nanogels are perhaps the most attractive. These materials are formed by chemically or physically cross-linked polymer network chains resulting in nanosized hydrogel particles. Since nanogels are networks in the nanometer range, they reveal many interesting intrinsic properties such as high water content, soft and elastic nature, excellent water dispersibility, and cell and tissue compatibility. Moreover nanogels were initially developed as carriers for pharmaceutical agents and diagnostics due to their ability to reveal a high cargo payload. These properties, together with the possibility to tune their physico-chemical properties as size, shape, and elasticity (discussed in the following section), and their ability to respond to versatile triggers in diverse manner, as shown in Figure 7, make them ideal candidates for various applications in the biomedical field.[100] For example, in the spite of exploring novel diagnosis platforms for the early detection of cancer, a hydrophilic fluorescent nanogel thermometer was developed that was able to measure the intracellular

temperature with a resolution of 0.5 °C (Figure 6). From a clinical point of view, pathological cells usually have higher temperatures than healthy cells as a result of increased metabolic activity. Thus, the visualization of temperature changes in the cellular level through responsive nanogels is an interesting approach for the development of advanced diagnostic tools. In this case the nanogel was formed by poly(N-isopropylacrylamide) (PNIPAM), a thermoresponsive network chain that can undergo a phase transition from a hydrophilic to hydrophobic state upon a temperature change, and a water-sensitive fluorophore, and was furthermore decorated with negatively charged sulfate groups on the surface.

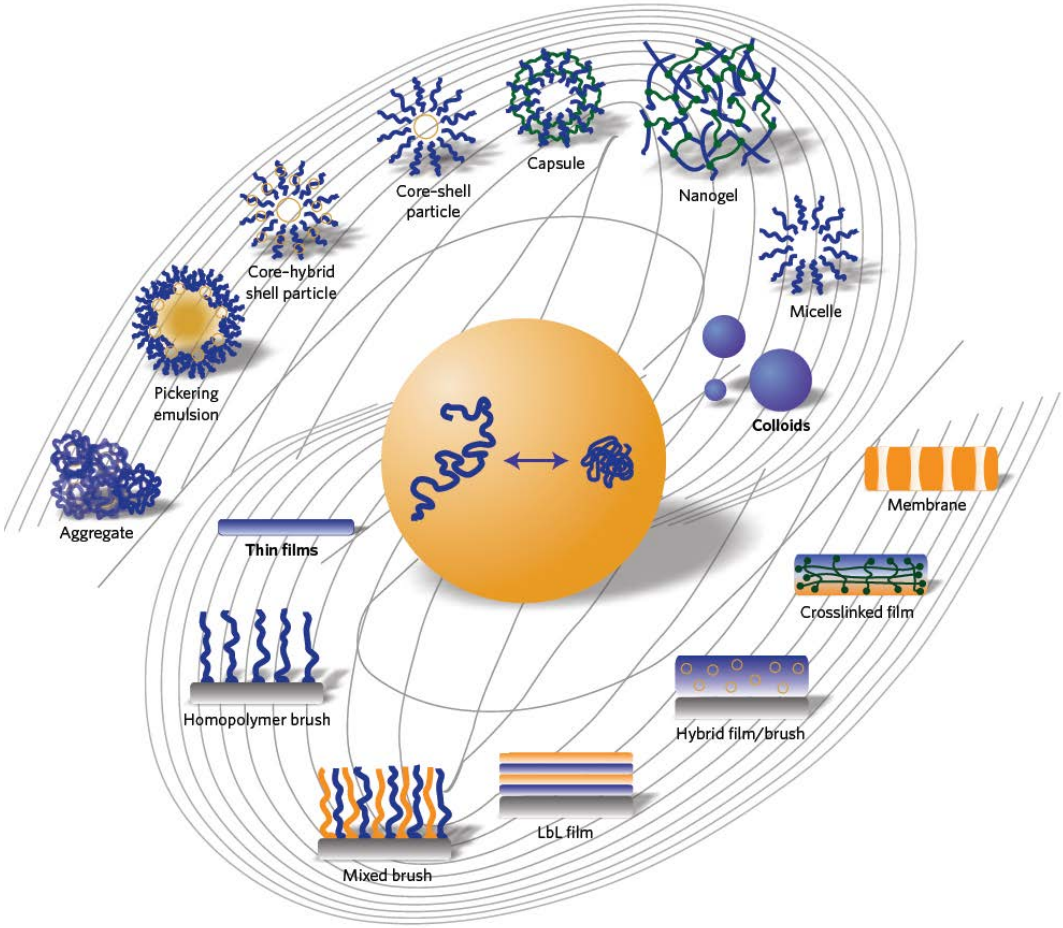

Figure 5. Stimuli-responsive polymers in diverse formulatios ranging from thin films, such as polymer bruches, and cross-linked film, to nanosystems like micelles, polymersomes, and nanogels.[96] Reproduced from Ref. 96 with permission from The Nature Publishing Group.

At low temperature when the nanogel was swollen with water molecules, fluorescence of the water-sensitive dye was quenched, but, with increasing temperature and as a result of water expelling by the thermoresponsive network chains, the nanogel changed its state to a shrunken state leading to high fluorescent intensities.[101] Another interesting approach of thermoresponsive nanogel for *in vivo* tissue targeting was recently reported. Here PNIPAM nanogels were engineered that had gold nanorods incorporated and were injected into mice.

Since gold nanorods are known near infrared (NIR) light-to-heat transducers,[102] and NIR light is known to penetrate viable tissue up to several centimeters ,[103] in this study, only one of the kidneys of the mice was externally exposed to a NIR laser immediately after intravenous administration. After biopsy, significantly increased accumulation was only observed in the kidneys that had been irradiated with the laser, while the non-thermoresponsive control showed no accumulation in the kidneys.[104] As a result of NIR light exposure, gold nanorods were heated up causing a collapse of the nanogel from a hydrophilic to a hydrophobic state that led to increased accumulation only on the irradiated side. This study represents a powerful demonstration of tissue targeting with the aid of responsive materials and most importantly with the absence of any targeting ligand.

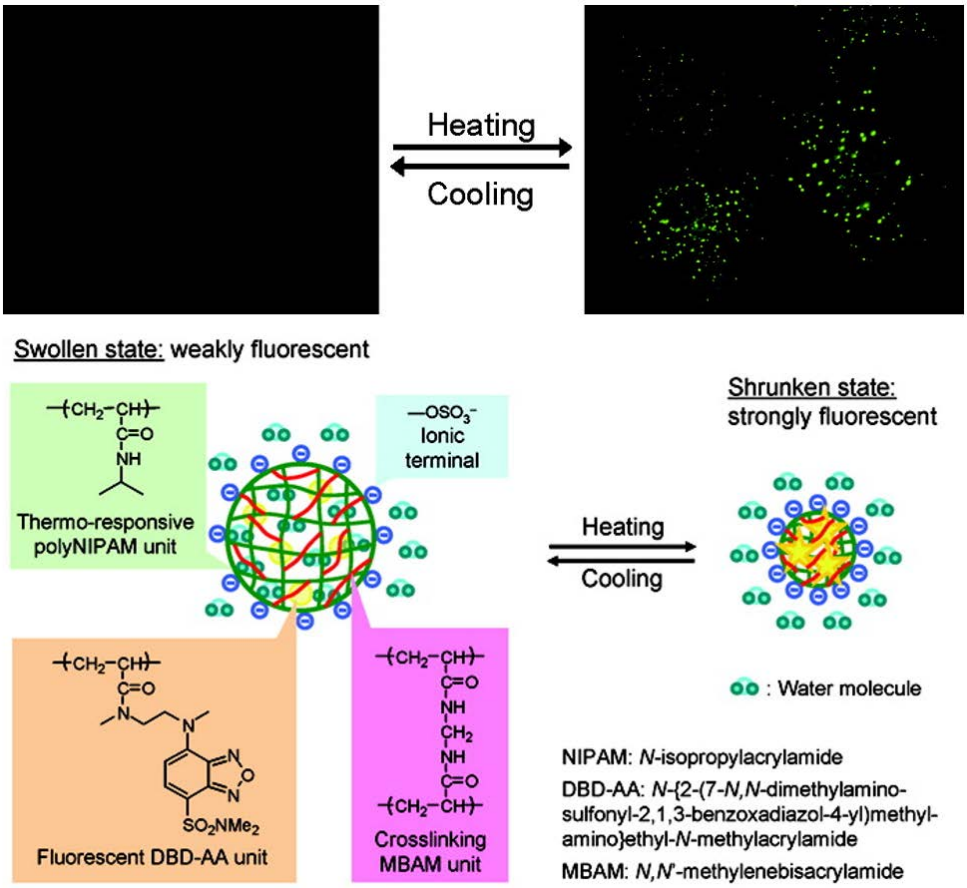

Figure 6. The thermoresponsive fluorescent nanogel thermometer. The nanogels consists of thermoresponsive polymer network chains (polyNIPAM) and has a water-sensitive dye (DB-AA) incorporated. When temperature is applied, nanogel changes its state from hydrophilic to hydrophobic and allows the dye to perform fluorescence. Reprinted with permission from Ref. 101. Copyright (2009) American Chemical Society.

In addition, a charge conversional “chameleon-like” nanogel for drug delivery purposes was designed that was negatively charged on the surface, but when coming in contact with the acidic environment of tumor tissue, the negatively charged moiety was cleaved and the

nanogel revealed positive charged amines on the surface. As a result, increased cell internalization and improved specific toxicity were achieved.[105] Finally, a dual responsive nanogel approach was developed for drug delivery purposes. The nanogels which were crosslinked by disulfides and had covalently bound doxorubicin through a hydrazone bond demonstrated both redox and pH responsiveness. Due to intracellular reductive conditions, cell internalization nanogels degraded to small fragments, which were below the clearance limitation of organ-level clearance mechanisms, while doxorubicin was efficiently cleaved in the acidic organelles of the cytoplasma and led to specific toxicity.[106]

The area of responsive polymer materials has recently made significant advances in the design of responsive nanosystems. Even though this emerging field of “smart” nanosystems, especially responsive nanogels, and nanocomposites, show outstanding features and potential for application in the biomedical field, the vast majority of generated systems are not able to perform the translation from pre-clinical studies to clinical trials. The main reason lies in the complexity and diversity of the systems along with non-trivial synthesis approaches that lack in efficiency and reproducibility. Hence, there is a need to address these drawbacks with development of strategies that allow an easy synthesis and reproduction of responsive nanosystems, while maintaining an intrinsic complexity that would be responsible for their “smart” properties.

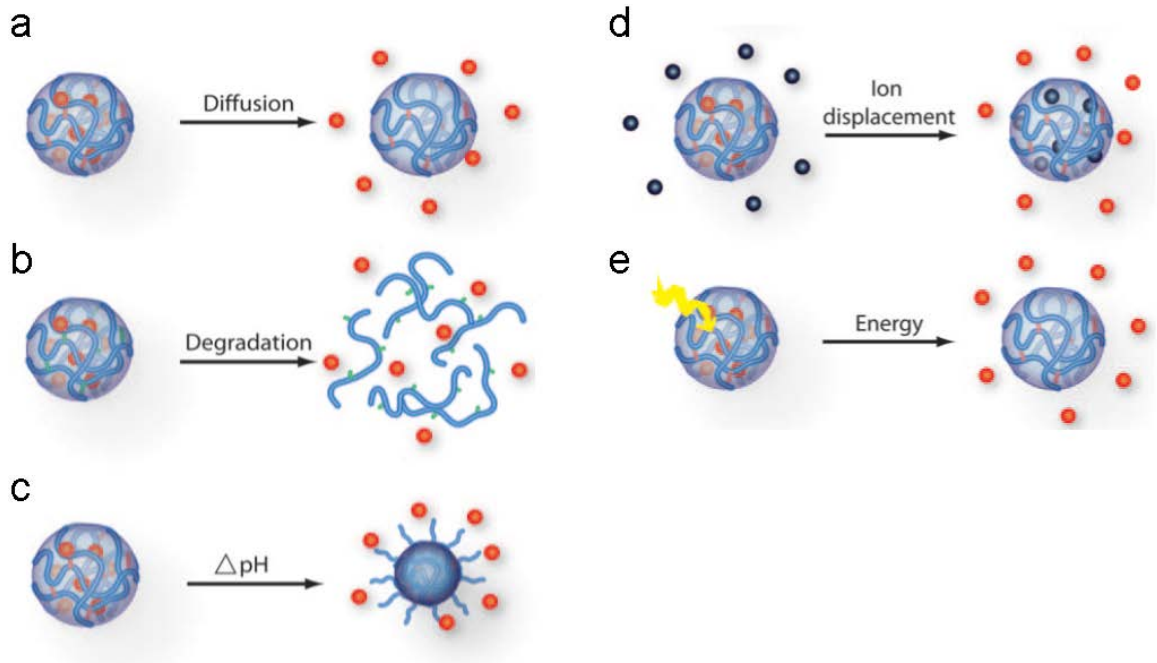

Figure 7. Diverse drug release mechanisms from nanogels. (a) Drug release by diffusion. (b) Release through degradation. (c) Release through deswelling as a result of pH change. (d) Ion displacement as a result of electrostatic interactions. e) Drug release induced by the

application of external energy such as temperature, electric field, ultrasound, etc.[100] Reproduced from Ref. 100 with permission from The Wiley Company.

1.5 Nanogel engineering approaches

For the generation of nanogels, two main synthetic approaches are currently being explored, namely, top-down or bottom-up. An example of a top-down method is the lithographic fabrication method called Particle Replication In Non-wetting Templates (PRINT) that performs a template-assisted nanogel synthesis. On the other hand, there is the huge arsenal of bottom-up techniques including the polymerization of monomers and crosslinkers in a nanoscale heterogeneous environment or in a homogenous phase, the physical self-assembly of functional polymers, and the cross-linking of pre-synthesized polymers. All these approaches have their specific advantages and disadvantages as well as important properties and setting options for nanomedical relevant parameters like size, shape, and yields, which will be discussed in detail below. The elasticity and surface charge of the generated nanogels play equally important roles in nanomedical applications. These parameters, however, are more determined by the chemistry involved, in terms of crosslinker density or applied functional chemical groups, than controlled by the fabrication technique itself.[107, 108]

1.5.1 Top-down approach

The great advantage of micromolding techniques such as PRINT is a precise control over the shape and size of nanogels that no other technique has given so far. Sizes between 10 nm and 200 µm with narrow size distributions and shapes ranging from spherical to cubic, cylindrical, and more can be easily obtained as mentioned in Figure 3. Moreover, different nanogel compositions can be generated, as this methodology does not require high shear stress, organic solvent, or high temperature.[109] Usually the initial precursor monomer mixture is applied into the non-wetting substrate, followed by mechanical stamping into the form through the mold with its specific pattern. After crosslinking initiation with external triggers like temperature or light, the nanogels can be harvested. Large-scale nanogel fabrications, along with low material limitations, are the highlights of this technique. The harvesting process, however, is limited, because surface modification of the molds may be required to facilitate particle removal and since special equipment and a clean room facility are necessary to install and run such devices.[110]

1.5.2 Bottom-up approach

For the synthesis of covalently cross-linked nanogels, a broad range of organic chemistry is available, which includes cyclo-addition reactions as well as controlled and free radical chemistry.[111] Among the vast variety of approaches available, free radical crosslinking of monovinyl and acrylic monomers remains the most common and simplest strategy since it requires a minimum of specific equipment. The main focus in this strategy relies on the prevention of long-range network formation that could end up in macroscopic gelation. Either performing the cross-linking reaction in highly diluted solvents that could be inconvenient when scale-up purposes are desired or the use of heterogeneous polymerization strategies can prevent this effect. The mechanism of such strategies, which includes techniques like miniemulsion, microemulsion, dispersion and precipitation polymerization, or nanoprecipitation, is based on the templation of the cross-linking reaction to process only within the formed nanodroplet. While the formation of such nanodroplets depends on various conditions that strongly differ in each technique, their dimensions during polymerization mainly influence the final size and shape distributions of produced nanogels.[111]

In miniemulsion processes such templates are formed through administration of high shear stress, like ultrasonification, on a mixture of two or more liquid phases that are not miscible with each other and result in a dispersed and a continuous phase, e.g., oil and water. The dispersed phase should contain the monomers even though the choice of which phase is supposed to act as the dispersed phase depends upon the nature of the reacting agents. The use of surfactants and co-stabilizer is crucial to stabilize the nanodroplets and prevent them from diffusional degradation. The controllable sizes for nanogels derived from miniemulsion strategies lay between 50 – 500 nm. Here, the parameters that control the size are mainly the amount of used surfactant and the power and duration of administrated shear stress.[112] Microemulsions, however, differ from miniemulsions only through the absence of high shear stress and the use of precisely aligned surfactant amounts that result in thermodynamically stable emulsion. Usually nanogel sizes between 10 and 150 nm can be achieved.[113] In contrast to emulsion strategies, dispersion and precipitation polymerization approaches are initially homogenous. Only in the progress of polymerization when the growing polymer chains reach a critical length, precipitation occurs from which later the nanogels derive. The difference between dispersion and precipitation polymerization is the use of surfactants in the first case that act as colloidal stabilizers and commonly lead to bigger nanogel sizes but narrower size distributions. Depending on the polymerization conditions, sizes between 100 and 600 nm can be obtained through precipitation polymerization[114] while dispersion polymerization usually yields nanogel sizes within the

range of 100 nm to 15 µm.[115] The synthesis of covalently cross-linked nanogels through the nanoprecipitation technique is a quite young methodology and not explored to such an extent as the above.described methods.[116, 117, 118] Its mechanism is based on the injection of homogenously dissolved functional macromonomers into a non-solvent that immediately forms nanodroplets in that the cross-linking reaction can occur (Figure 7). While the obtained nanogel sizes vary so far between 80 and 300 nm, bioactives can be easily incorporated into the forming nanogels.

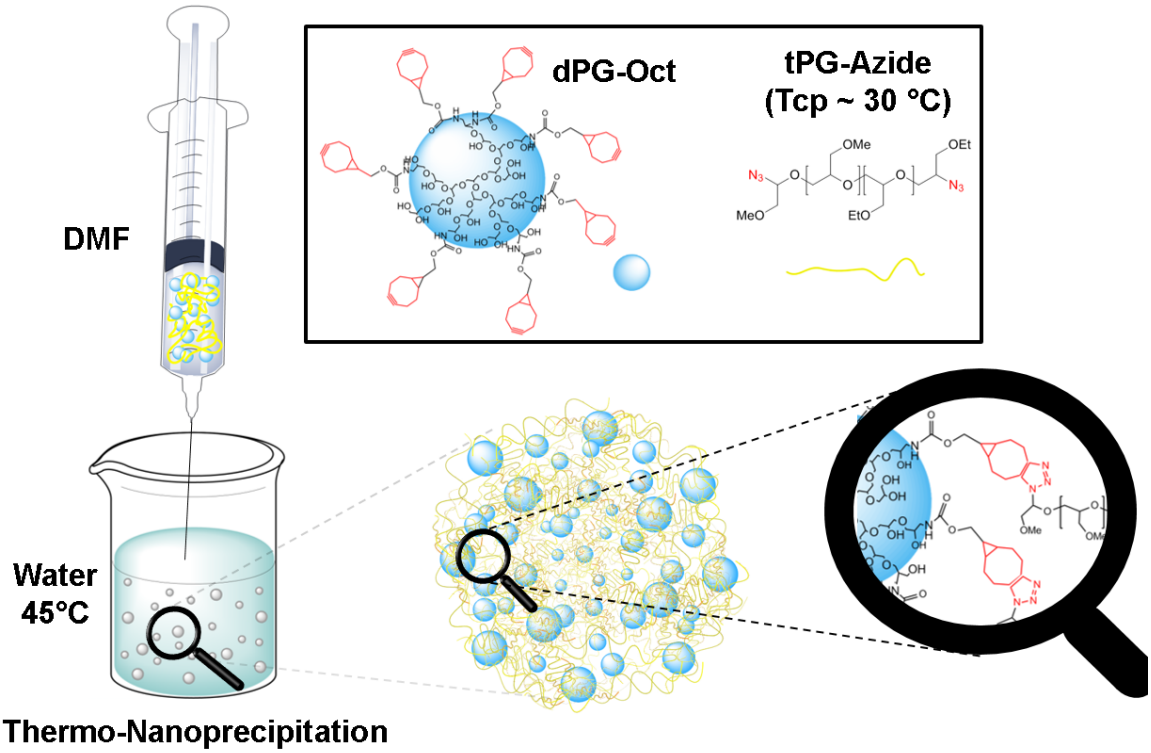

Figure 7. Example for the synthesis of chemically cross-linked nanogels through a modified nanoprecipitation approach. Bicyclononyne decorated dendtritic polyglycerol (dPG-Oct) served as the macrocross-linker while azide functionalized thermoresponsive linear polyglycerol (tPG-azide) represented the polymer netwotk chain. The cross-linking reaction occurred in nanodroplets through strain-promoted click chemistry.[118] Reproduced from Ref. 118 with permission from The Royal Society of Chemistry.

The synthesis of physically cross-linked nanogels is performed mainly by the self-assembly of functional polymers in aqueous environment[100] and by the nanoprecipitation technique.[119] These methodologies depend mainly on non-covalent interactions between the functional polymers, such as hydrogen bonds, electrostatic and hydrophobic interactions, as well as Van der Waals forces [120] and allow an *in situ* encapsulation of sensitive bioactives. While

self-assembly interactions between polymers are generally considered to be weak since they rely on equilibrium conditions, which, in fact, makes it difficult to synthetically control their size, the nanoprecipitation approach, by contrast, relies on solvent / non-solvent interaction which results in water soluble, physically crosslinked nanogels that are kinetically stable enough for *in vivo* evaluation.[121] Moreover the interplay of solvent ratio and polymer concentration can lead to reproducible results in terms of size, functionality, and stability. Along with the optimization of starting material fabrication, the nanoprecipitation method is easy to scale up and may be the only nanogel formulation approach that allows nanogels to enter into clinical trials.[121, 122] Table 2 summarizes limitations and advantages of commonly used methodologies for nanogel engineering.

Bottom-up and top-down approaches have demonstrated versatile possibilities to control physico-chemical characteristics of nanogels along with proper yields and the ability to load them with bioactive cargos through both efficient encapsulation and covalent conjugation. Even though the control over a narrow size distribution and compositional homogeneity are challenges that have to be addressed, the given developments allow their evaluation as targeted drug delivery systems and diagnostic tools. Moreover the choice of diverse monomers and conjugates for nanogel preparation together with the possibility of various templating techniques enable the development of nanogels that can combine the inherent properties of inorganic nanoparticles such as magnetization, transduction, etc., with their soft, responsive, and biocompatible nature.

Table 2. Pros and cons of common synthetic methodologies for nanogel engineering.[a)]

Methodology (type of cross-linking)	Key step	Advantage	Limitation	Ref.
Micromolding techniques (covalent)	Non-wetting stamps	Precise shapes, sizes, and various monomer compositions possible. Easy scaling up. Allows *in situ* encapsulation.	Requires clean room facilities and lithographic devices. Surface modification of molds necessary to improve harvesting.	109, 123, 124
Miniemulsion (covalent)	Nanodroplet formation through high shear stress	Broad range of cross-linking chemistry. Narrow size distributions for diameters in the 50 - 500 nm range. Allows *in situ* encapsulation.	Non-commercial surfactant often required. Special equipment necessary (ultra-sonic device). Difficulties with scaling up of synthesis.	112, 125
Microemulsion (covalent)	Nanodroplet formation through phase and surfactand composition	Very small 10 - 150 nm tuneable systems No shear stress necessary.	High surfactant concentration needed. Co-surfactant necessary.	113
Dispersion polymerization (covalent / physical)	Choice of stabilizer determines reaction process	Easiness: single batch process. Tuneable broad size range tuneable as a function of monomer and dispersant concentration: 0.1- 15 µm. Suitable for synthesis of core/shell systems.	Preferred for vinylic and acrylic functionalized monomers.	114, 126
Precipitation polymerization (covalent / physical)	Critical chain length of propagating polymer determines nanogel properies	Easiness: single batch process, no surfactant required. Tuneable sizes as a function of monomer concentration in the 100 – 600 nm range.	Only suitable for chemical compositions which are able to undergo phase separation	127
Nanoprecipitation (covalent / physical)	Solvent / non-solvent interaction determines reaction progeress	Huge usable variety of composition. Easy *in situ* encapsulation.	Solvent / non-solvent interaction has to fit to reagent's physico-chemical properties	117, 118, 121, 122
Physical self-assembly (physical)	Repelling / attracting forces and their equilibrium with the solvent determine nanogel formation	Mild reaction conditions. Suitable for encapsulation of highly sensitive bioactives.	Only suitable for specially designed polymer constructs with specific functionalities	128, 129

[a)] The highlighted sections have been published in the following review: M. Asadian-Birjand, A. Souza, J. Cuggino, D. Steinhilber, M. Calderón, Functional nanogels for biomedical applications., *Current Medicinal Chemistry* **2012**, 19, 5029-5043.[111]

1.6 External triggers in nanomedicine

For the stimulated response of smart nanosystems in biomedical applications there are generally two types of triggers available. The one are so-called internal triggers, such as pH and enzymes that are associated with tumor biology. These triggers allow the nanosystem to interact with their target biological environment and in response commit conformational changes, degradation, and cargo release. On the other hand, there are triggers from outside the biological environment, so-called external triggers such as temperature, light, magnetic field, and ultra sound that can provoke responsive nanosystems, along with their cargo release, to further generate heat or toxic radical species if specific responsive moieties are attached. Moreover external triggers reveal more control over the internal triggers for purposes like “remote-switches” and “on-demand” delivery. External triggers, however, also show some drawbacks since their implement is limited to the target localized conditions.[130, 131]

1.6.1 Temperature

Temperature has been widely used to demonstrate the capability on thermoresponsive systems to achieve remote controlled drug delivery. The explored nanosystems include thermoresponsive micelles, liposomes, and nanogels that expel their cargo in response to temperature by undergoing a reversible phase transition or by falling apart as demonstrated in Figure 8.[132, 133, 134, 135] Some approaches have combined thermoresponsiveness with other forms of responsiveness to achieve a more efficient release and internalization profiles as well as to generate imaging signals or local hyperthermia.[136] So far only one thermoresponsive liposomal formulation, namely Thermodox, has reached clinical trials. Thermodox is a thermoresponsive doxorubicin- loaded liposome and has shown rapid doxorubicin release under slight hyperthermia conditions in comparison with the non-thermoresponsive doxorubicin bearing commercially available liposome Doxil. Thermodox is currently being evaluated in phase III human clinical trials for colorectal and breast cancer. Moreover, it has completed phase III trials for primarily liver cancers.[131, 137, 138] Another interesting approach was recently shown for topical application in dermatology. Thermoresponsive nanogels sized around 100 nm showed skin penetration to the deepest layers after performing a temperature-triggered phase transition from hydrophilic to hydrophobic.[65]

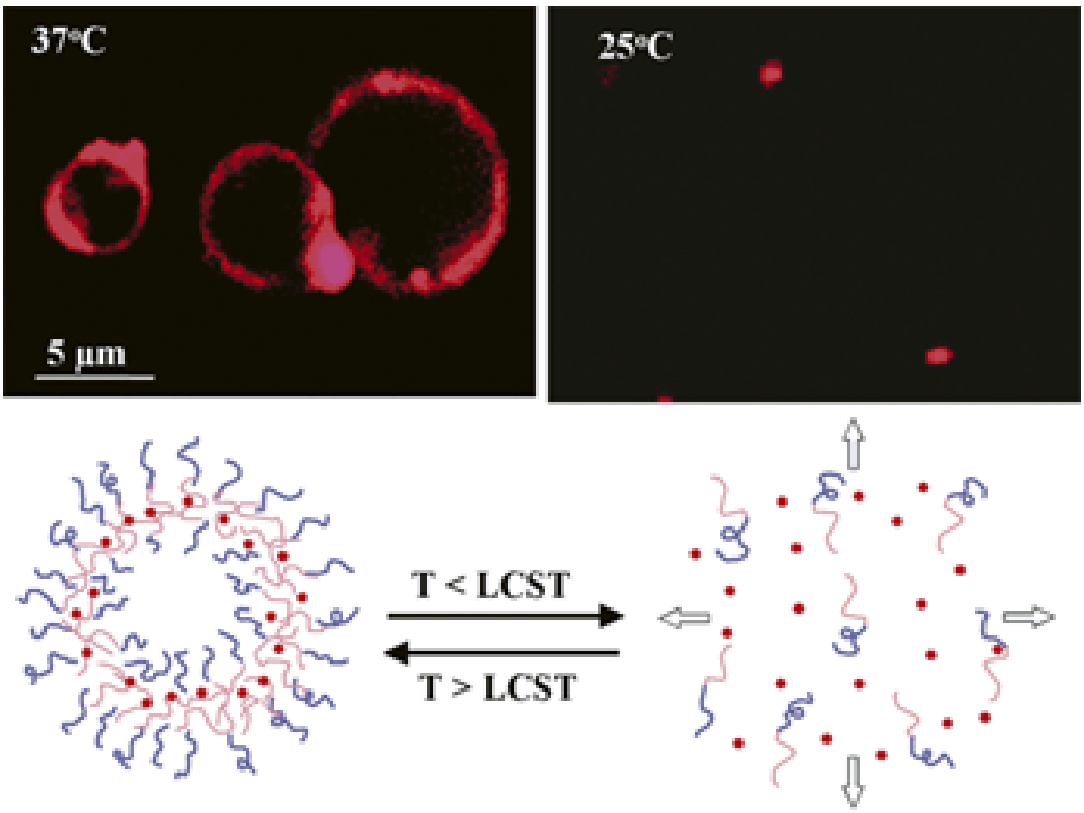

Figure 8. A thermoresponsive block co-polymer micelle that is able to release its cargo temperature controlled. [135] Reproduced from Ref. 135 with permission from The Wiley Company.

1.6.2. Light

Light responsive nanosystems can be generally divided into two classes, ones that undergo light-induced photochemical reactions, such as photoisomerization, photocleavage, and photooxidation, and others that are able to transduce the light into heat or generate reactive species. Especially the later class of nanosystems is of interest because they are widely recognized as potential agents for photothermal and photodynamic therapy. Materials such as gold nanostructures shown in Figure 9, carbon nanotubes, and cyanine dyes are able to perform light-to-heat transduction[139, 140] and are particularly relevant as their activation wavelengths lie within the so-called biological windows.[141] These windows can be described as a spectral range between 700 – 1400 nm where tissues become partially transparent due to a reduction in both absorption and scattering, leading to beneficial use of these transducers also in deeper tissue regions rather than only for superficial administration.[142] In addition, UV light that is commonly used for photothermal therapy has shown phototoxicity to healthy tissue and cannot penetrate deeply into tissue. Up to date, only one gold based nanoformulation has reached clinical trial phase I, which is a gold nanoshell system named

Ausoshell that is supposed to act for NIR light-triggered, localized thermal ablation of head and neck cancers.[143]

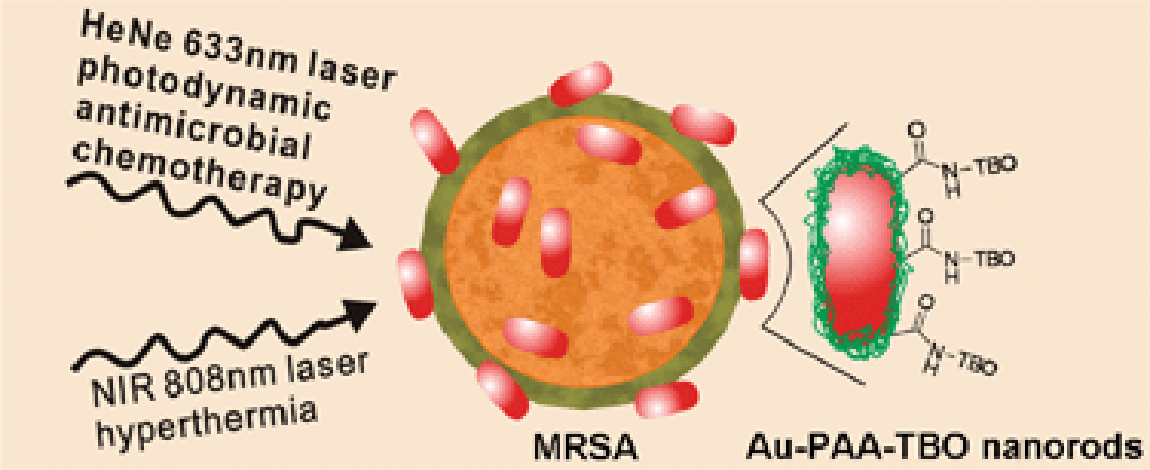

Figure 9. Example of a light responsive nanosystem. Gold nanorods (Au) were coated with an organic photosensitizer (TBO) for both photodynamic and photothermal antimicrobial purposes against methicillin-resistant Staphylococcus aureus (MRSA).[140] Reproduced from Ref. 139 with permission from The Royal Society of Chemistry.

1.6.3 Magnetic field

Nanosystems that respond to magnetic fields usually contain super paramagnetic nanoparticles such as iron oxide. Such responsiveness is mainly used for applications in hyperthermia since magnetic nanoparticles tend to generate heat when exposed to an alternating magnetic field. The combination of thermoresponsiveness and magnetic nanoparticles has generated many examples such as core-shell particles, nanocomposites, magnetic nanoparticle incorporated nanogels, and magnetic liposomes, that have shown simultaneous hyperthermia- and temperature-triggered drug delivery.[130] Moreover magnetic properties can be applied to nanosystems for approaches in tissue imaging like magnetic resonance imaging, targeting, and cell sorting abilities.[144] Along approved magnetic nanoformulations for imaging,[145] the only therapeutic approach that is currently under clinical trials is an aminosilane-coated iron oxide nanoparticle formulation, called Nanotherm, for the local ablation of glioblastoma multiforms.[146]

1.6.4 Ultrasound

Ultrasound-assisted drug delivery of nanosystems is an effective method to achieve drug release at the desired site without the use of targeting ligands. A variety of nanocarriers with diverse compositions can be used for this approach which include liposomes, nanocapsules, and microbubbles, since mechanical and thermal effects that are caused by cavitation phenomena and radiation forces trigger the cargo release instead of the responsive material properties used for the nanosystems.[130, 147] The ultrasound approach, however, is easy to apply and can increase internalization of nanosystems and their penetration into deeper tumor tissue.

The careful design and the optimization of responsive properties is crucial to achieve the development of smart nanosystems that are able to reach their target autonomous and to reveal their therapeutic potential only on the target site. The combination of different types of responsiveness such as thermo- and magneto-responsiveness has shown synergetic effects when hyperthermia and drug delivery purposes were targeted. Moreover, the combination of light- and thermoresponsiveness can lead to smart nanosystems that unite the possibilities given for thermosensitivity such as remote drug delivery, and remote targeting, and the abilities of phototransducers to improve efficacies of photothermal and photodynamic therapies. Thus, multi-responsiveness along with physico-chemical benefits of nanogels have the potential to combat the challenges of modern nanomedicine.

2 Motivation and summary

2.1 Motivation

Nanogels are increasingly recognized as potential platform for applications in nanomedicine due to their many interesting physico-chemical and intrinsic properties. In particular, responsive “smart” nanogel that are able to respond to external stimuli and therefore exhibit potential “on-demand” properties have gained great attraction among scientists across several disciplines. For an early clinical translation of responsive nanogels many consideration have to be made. Their production, for instance, should be easy feasible while their complex internal structure that contains their responsive nature has to be maintained. Moreover their design in terms of size, shape, elasticity, charge, etc. and their responsive ability should be developed with respect to the desired biological application along with the use of biocompatible building blocks. Therefore the main objective of this thesis is to evaluate strategies for the development of responsive nanogels that reveal ideal intrinsic properties such as soft and elastic nature, excellent water solubility, and narrow size distributions, along with sharp responsive behaviors. Moreover this thesis focuses on the biological evaluation of responsive nanogels in terms of biocompatibility and investigates their mode of action in diverse biomedical scenarios such as topical application in dermatology, photosensitizer in photothermal therapy, and cell sorting in early cancer detection. The main objectives are schematically highlighted in Figure 10.

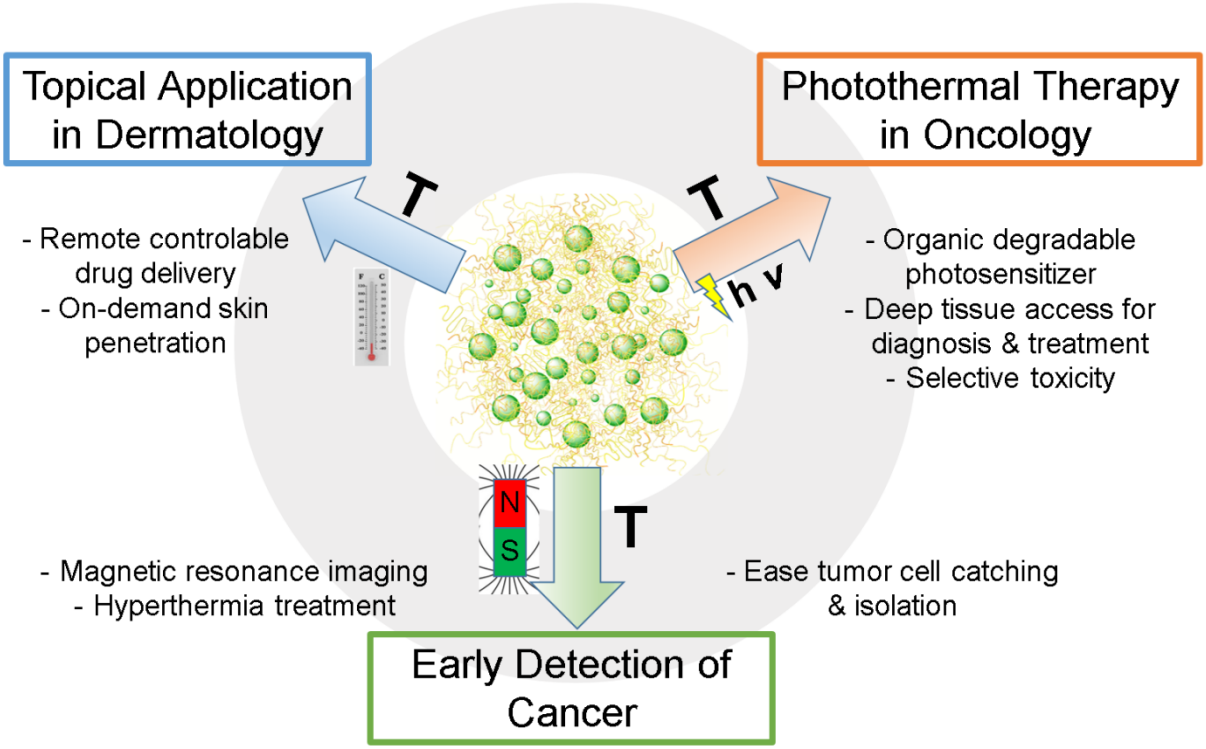

Figure 10. Illustration of the main objectives of this thesis. Stimuli-responsive nanogels for diverse biomedical application that can promote on-demand skin penetration in dermatology as a result of thermoresponsiveness; generate heat and selective toxicity as a result of light- and thermo-responsiveness; and that are able to perform magnetic cell sorting due to magneto-responsiveness.

2.1.1 Topical application in dermatology.

Transdermal drug delivery obtains many advantages over intravenous or oral administration that can lead, for instance, to reduced side-effects. Nevertheless efficient transdermal administration is quite challenging due to the strong barrier function performed by the stratum corneum (SC) in the skin. Nanoparticles bigger than 1 nm are generally not considered to cross this barrier while only hydrophobic molecules have been observed to actively cross through the SC to viable skin layers. Thus, we are interested in investigating the interaction of thermoresponsive nanogels with the stratum corneum of human skin and, based on our findings, to develop a nanogel system for on-demand skin penetration and remote controlled topical drug delivery. Thermoresponsive nanogels are known to undergo a phase transition above a certain temperature, where they change their state from hydrophilic to hydrophobic. Therefore the first goal is to design a strategy to develop thermoresponsive nanogels with precise control over their size and transition temperature while revealing excellent biocompatibility. Moreover, to evaluate these precisely engineered nanogels on human skin explants with respect to their thermoresponsive behavior, their localization in the skin layers, and their interaction with human skin cells.

2.1.2 Photothermal therapy in oncology

Photothermal therapy is currently being explored as an alternative therapy in oncology. It is based on the light induced thermal ablation of tumor cells through the administration of a photothermal agent that is able to transform light into heat. The clinical translation of the currently investigated photothermal therapy approaches is generally limited due to inherent properties of the photothermal agents. In one hand, long term cytotoxity is related to the use of inorganic photothermal agents. On the other hand the poor water solubility, high sensitivity, and low efficiency of organic photothermal agents represent big issues. Therefore the incorporation of such organic agents into a thermoresponsive nanogel could improve its sensitiveness and solubility, while generating a dual responsive system. The developed nanogel system has to be tested for its ability to perform light-to-heat transduction and to be

evaluated regarding its cell internalization / adhesion properties and its photothermal efficiency against cancer cell lines.

2.1.3 Early detection of cancer

Circulating tumor cells (CTCs) are known to play a significant role in metastases formation that is the main cause of high mortality in cancer. Their early detection and isolation is crucial for patients' survival rates. A strategy for an efficient detection and isolation of CTCs relays in the use of antibody decorated magnetic nanoparticles. Since size, shape, and elasticity of nanoparticles can improve their interaction with biological systems, magnetic nanoparticles should be incorporated into the soft nanogel matrix and be evaluated for cell capturing purposes. The introduction of a targeting ligand that binds to cancer cell overexpressing receptors could improve the capturing efficiency of such magnetic nanogel. Moreover magnetism offers many other potential application for magnetic nanogel such as for magnetic resonance imaging and hyperthermia purposes.

2.2 Conclusion and outlook

This thesis discusses in detail three projects involving the engineering of biocompatible and thermoresponsive nanogels for topical application in dermatology, the development of a dual light- and thermoresponsive nanogel for photothermal therapy, and the fabrication of magnetic nanoparticle incorporated thermoresponsive nanogels for cell sorting and magnetic resonance imaging purposes.

In the first part, thermoresponsive nanogels based on dendritic polyglycerol (dPG) as the cross-linker, and poly(oligoethylene glycol) (POEG) as the network chain were synthesized through a dispersion/precipitation polymerization methodology. This methodology offered the possibility to modify nanogel sizes and phase transition temperatures by changing the feed monomer ratio, the cross-linker feed and functionalization degree, and the initial dispersant concentration. Out of these studied parameters two rhodamine b labeled nanogels were synthesized that reveal comparable sizes and compositions but differed in their phase transition temperature. One nanogel obtained a transition temperature at 36 °C while the second one was non-thermoresponsive. When applied on human skin explants at temperature below and above the transition temperature of the nanogel, a temperature dependent interaction with the skin barrier and hair follicle channels was obtained. Other than for previously investigated nanoparticles, it was observed that thermoresponsive nanogels penetrated deep in to the stratum corneum and that significant amounts of thermoresponsive nanogels crossed the intact skin barrier reaching the first viable skin layer. High resolution cyro-SEM measurements confirmed their translocation across the stratum

corneum along with fluorescence intensity histograms of isolated epidermis cells that had nanogels internalized. The ability that nanogels crossed the skin barrier as whole moiety can be attributed to both their elasticity and their chemical stability. These properties can be helpful when the delivery of bioactive drugs to the skin cells in desired. Moreover thermoresponsive nanogels represent potential drug carries for various hair follicle and skin related diseases such as acne, hair loss disorder, and inflammatory skin disease like psoriasis. Since rhodamin b labeled thermoresponsive nanogels revealed such interesting abilities a future use as smart drug delivery system or diagnostic tool for both encapsulated and conjugated drugs and dyes has to be evaluated with respect to the influence of thermal triggers on the release profiles.

Based on the synthetic findings for the conjugation of fluorescent dyes to thermoresponsive nanogels, a novel methodology was developed that allowed the conjugation of the highly sensitive organic photothermal agent, namely IR806, a NIR light absorbing dye, to the thermoresponsive nanogel scaffold based on dPG and POEG. Thus a dual light- and thermoresponsive nanogel was designed that revealed narrow size distributions and spherical shapes. Upon NIR light exposure these nanogels were undergoing a phase transition since the conjugated photothermal agent caused a measurable temperature increase from room temperature to about 70 °C, depended on the concentration, respectively. Their evaluation against human ovarian carcinoma cells have shown a biocompatible profile and reduced toxicity that was an order of magnitude smaller in comparison to the free dye IR806 at the same concentration. Confocal fluorescence microscopy along with fluorescence intensity histograms confirmed that nanogels got internalized efficiently and showed a preferential accumulation in mitochondrial compartments of the cell. Upon NIR laser exposure nanogels could efficiently destroy carcinoma cells. Hence, these NIR light responsive nanogels represent a promising scaffold for their future use as photothermal agent. Since IR806 also generated toxic radicals upon NIR light irradiation, the nanogels profile as a potential photodynamic agent has still to be explored *in vitro*. The combination of photothermal and photodynamic therapy along with the possibility of thermally triggered drug release could reveal a synergetic effect and strike therapeutic efficiencies of known photodynamic agents. These possibilities have to be planed and evaluate in future studies.

To further evaluate the potential of responsive nanogels for the detection and isolation of circulation tumor cells (CTCs) through a magnet, a methodology was developed that reproducibly generated magnetic nanogels with sharp responsive behavior and narrow size distributions. This methodology was based on an ultrasound assisted and strain-promoted copper free cross-linking reaction between bicyclononyne decorated magnetic nanoparticles that served as both nanogel cross-linker and magnetic moiety, and azide functionalized

linear polyglycerol that served as the polymer network chain. Magnetic nanogels were further functionalized with the model targeting ligand transferrin (Tf) and evaluated for their cell capture ability against Tf receptor overexpressing cancer cells from an artificial CTC-like suspension. In this first study, capturing efficiencies of 30 % were obtained which were dependent on the polymer spacer length between targeting ligand and nanogel. Since super paramagnetic nanoparticles are commonly used as contrasting agents in magnetic resonance imaging (MRI), nanogels suitability as a contrasting agent was explored and found to equal to commercially available contrasting agents. Since the developed methodology is based on a building block concept, it allows the substitution and modification of polymer network chains and targeting ligands. Thus, in future studies the variation of these parameters have to be explored to investigate possible changes in nanogel size, swelling capacity, and elasticity over their cell capture efficiency. Moreover magnetic nanogels' ability is not limited to cell capturing purposes but can extended in the future for application as contrasting agent in MRI and as heat transducer for hyperthermia purposes.

2.3 Abstract

The carful design and the optimization of synthetic strategies are key steps for the development of responsive nanogels (NG) that achieve the desired "smart" interaction with biological systems. In this thesis responsive nanogels were engineered through different methodologies and evaluated for their ability in biomedical applications such as for dermatology, photothermal therapy, and as capturing system for circulating tumor cells.

It was found that thermoresponsive nanogels based on dendritic polyglycerol (dPG) and poly(oligoethylene glycol) (POEG) revealed an excellent biocompatible profile against human keratinocyte and fibroblast cell lines with a tolerable dose of 2 mg mL^{-1}. The developed synthetic methodology allowed, moreover, to set the size and transition temperature of generated NGs in the range of 50 – 250 nm and 30 – 40 °C respectively. When these NGs were fluorescently labeled and applied on human skin explants a temperature dependent translocation pattern through the skin barrier and hair follicles was observed. This behavior could be attributed to the elastic nature of NGs and their structural integrity. This study demonstrated that thermoresponsive NGs reveal a great potential as smart drug carries for various hair follicle and skin related diseases. Based on the findings for the conjugation of fluorescent dyes to thermoresponsive nanogels a methodology was developed that uses ultrasound assisted precipitation polymerization. Along with the covalent incorporation of IR806, a NIR absorbing dye, into the nanogel scaffold, this methology revealed spherical shape, nanometric size (90 nm), and narrow size distribution (DLS PDI 0.16) for the generated NGs. Since IR806 is a photothermal agent that transduces NIR light into heat,

NGs were evaluated for their photothermal ability against cancer cell lines. As a result selective toxicity was demonstrated when NG internalized cells were exposed to a NIR laser while excellent biocompatibility was demonstrated when cells were not exposed to a NIR laser. This study demonstrated the proof of principle that organic photothermal agents can be incorporated covalently into NGs to demonstrate reduced toxicity, improved water solubility, and excellent photothermal efficiencies. To investigate the ability of NGs for cell capturing purposes nanogels based on magnetic nanoparticles and linear polyglycerol were synthesized using strain-promoted click cross-linking chemistry. This methodology allowed moreover the surface decoration with targeting ligands. Magnetic nanogels showed cell capture efficiencies of 40 % that have to be improved in future work. Studies on the magnetic relaxations time, however, showed that the magnetic NGs obtain similar relaxivity values as commercial available contrasting agents. Hence, this study lead to the conlusion that the use of magnetic NGs is not limited to cell capturing purposes but can be extended for their use as a potential contrasting agent in MRI.

This thesis shows that responsive nanogels obtain a great potential for their use in nanomedicine. Mainly their physico-chemical properties such as their size, shape, and elasticity combined with their responsiveness play a key finction on their influence to applied biological systems. Controlling these porperties can enhance the translation of responsive nanogels from bench to bedsides.

2.4 Kurzzusammenfassung

Das sorgfältige Design und die Optimierung von Synthesestrategien sind Schlüsselschritte in der Entwicklung von responsiven Nanogelsystemen, mit denen eine „smarte“ Wechselwirkung mit biologischen Systemen angestrebt werden soll. In der vorliegenden Arbeit wird die Darstellung von responsiven Nanogelen und ihre biologische Evaluation für Anwendungen in der Dermatologie, in der photothermalen Therapie, sowie für die Isolation von zirkulierenden Tumorzellen beschrieben.

Mittels der freien radikalischen Fällungspolymerisation wurden die thermoresponsiven Nanogele, die auf dendritischem Polyglycerin und Poly(oligoethylenglycol) basieren, synthetisiert. Diese Synthesestrategie erlaubt das Maßschneidern der Größe der Nanogele in einem Bereich zwischen 50 – 250 nm, sowie die Kontrolle über die Phasenumwandlungstemperaturen (PUT) zwischen 30 und 40 °C. Alle synthetisierten Nanogele, unabhängig von deren Größe und PUT, weisen eine überaus hohe Biokompatibilität gegenüber menschlichen Keratinozyten und Fibroblasten *in vitro* auf. Farbstoff-Konjugate dieser Nanogele demonstrieren zudem eine temperaturabhängige Translokation durch die äußerste Hautschicht menschlicher Haut sowie in Haarfollikeln. Diese Eigenschaft ist hauptsächlich auf die hohe Elastizität und die strukturelle Integrität der

Nanogele zurückzuführen. Diese Studie zeigt das Potential thermoresponsiver Nanogele, die für den Einsatz als smarte Wirkstoffträger für Erkrankungen von Haut und Haarwurzel genutzt werden können. Basierend auf den Erkenntnissen für die Farbstoff-Konjugation an Nanogelen wurde eine weitere Synthesestrategie entwickelt, die es erlaubt den hochsensiblen Farbstoff, IR806, in das thermoresponsive Nanogelgerüst kovalent einzubinden. IR806 ist ein im nahen Infrarot (NIR) Bereich absorbierender organischer Farbstoff der die Fähigkeit besitzt absorbiertes NIR Licht in Wärme umzuwandeln. Er zeichnet sich allerdings durch schlechte Wasserlöslichkeit, hohe Toxizität und geringe photothermale Effizienz aus. Seine erfolgreiche Einbettung in das polymere Nanogelnetzwerk hat nicht nur seine Wasserlöslichkeit verbessert und seine Toxizität um das zehnfache reduziert, sondern gleichzeitig ein dual-responsives Nanogelsystem erschaffen. Diese Nanogele, die sich durch eine Größe von 90 nm mit schmalen Größenverteilungen (Polydispersitätsindex in dynamischer Lichtstreuung: 0.16), sowie hervorragenden licht- und thermoresponsiven Eigenschaften auszeichnen, sind in der Lage, nach erfolgter Internalisierung, Krebszellen *in vitro* durch Aussetzung von NIR Laserbestrahlung selektiv und thermisch zu zerstören. Diese Eigenschaften gepaart mit ihrer exzellenten wasserlöslich und ihre reduzierten Toxizität erweisen sich als überaus nützlich für zukünftige *in vivo* Anwendungen in der photothermalen Therapie.

Ferner wurde eine Synthesestrategie entwickelt, die es ermöglicht Nanogele für die Isolation zirkulierender Tumorzellen einzusetzen. Die Ultraschall-unterstützte Methode ermöglicht, in einer Kupfer-freien ‚Klick'-Reaktion, die Quervernetzung von Bycylononyn-funktionalisierten Nanopartikeln mit azid-modifiziertem linearen Polyglycerol, und die daraus resultierende Synthese von magnetischen Nanogelen. Darüber hinaus gelingt es diesem simplen Verfahren die Nanogeloberfläche mit Targeting-Liganden zu dekorieren die dann eine spezifische Bindung zu Zellrezeptoren ausüben können. Die magnetischen Nanogele zeigen eine 30 prozentige Zellerfassungseffizienz, die anhängig von der Länge des eingesetzten polymeren Abstandhalters zwischen Targeting-Ligand und Nanogel ist. Des Weiteren zeigen magnetische Relaxationsstudien, dass magnetische Nanogele eine ähnliche Relaxivität wie kommerziell erhältliche Kontrastmittel in der MRT aufweisen. Diese Erkenntnis führt zu dem Schluss, dass der Einsatz magnetischer Nanogele nicht auf Zellerfassungsuntersuchungen begrenzt ist, sondern das Potential besitzt Anwendung in der MRT zu finden.

Diese vorgelegte Arbeit verdeutlicht das große Potenzial von responsiven Nanogelen für biomedizinische Anwendungen. Es sind hauptsächlich die physikalisch-chemischen Eigenschaften wie ihre Größe, Form, und Elastizität die Schlüsselfunktionen in der Interaktion mit biologischen Systemen ausüben. Eine effiziente synthetische Kontrolle über diese Eigenschaften kombiniert mit ausführlichen biologischen Evaluationen kann ihre klinische Translation vom Labor zum Patienten ermöglichen.

3 Publications and manuscripts

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

3.1 Engineering thermoresponsive polyether-based nanogels for temperature dependent skin penetration

M. Asadian-Birjand, J. Bergueiro, F. Rancan, J. C. Cuggino, R.-C. Mutihac, K. Achazi, J. Dernedde, U. Blume-Peytayi, A. Vogt and M. Calderón*

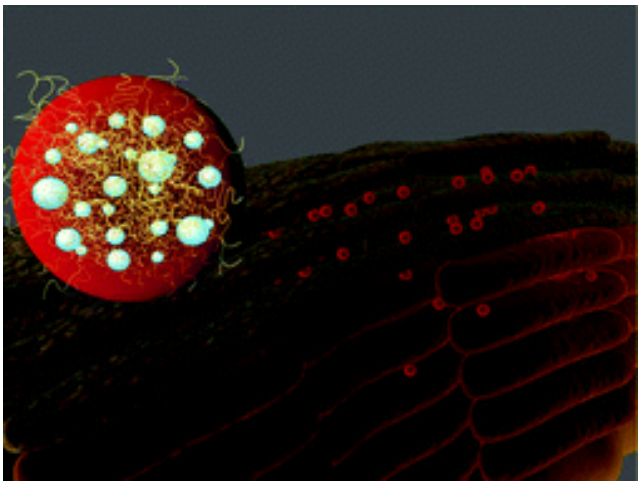

Figure 11. Reproduced from Ref. 139 with permission from The Royal Society of Chemistry.[65]

In this publication the author contributed with the synthetic evaluation of thermoresponsive nanogels and their labeling with a fluorescent dye. Moreover the author contributed with the physico-chemical characterization of both labeled and non-labeled nanogels, the data evaluation, and with parts of the report.

Asadian-Birjand, M.; Bergueiro, J.; Rancan, F.; Cuggino, J. C.; Mutihac, R. C.; Achazi, K.; Dernedde, J.; Blume-Peytayi, U.; Vogt, A.; Calderón, M., Engineering thermoresponsive polyether-based nanogels for temperature dependent skin penetration. *Polymer Chemistry* **2015,** *6* (32), 5827-5831.

DOI: 10.1039/c5py00924c

3.2 Effects of thermoresponsivity and softness on skin penetration and cellular uptake of polyglycerol-based nanogels

Fiorenza Rancan*, **Mazdak Asadian-Birjand**, Serap Dogan, Christina Graf, Luis Cuellar, Stefanie Lommatzsch, Ulrike Blume-Peytavi, Marcelo Calderón*, Annika Vogt

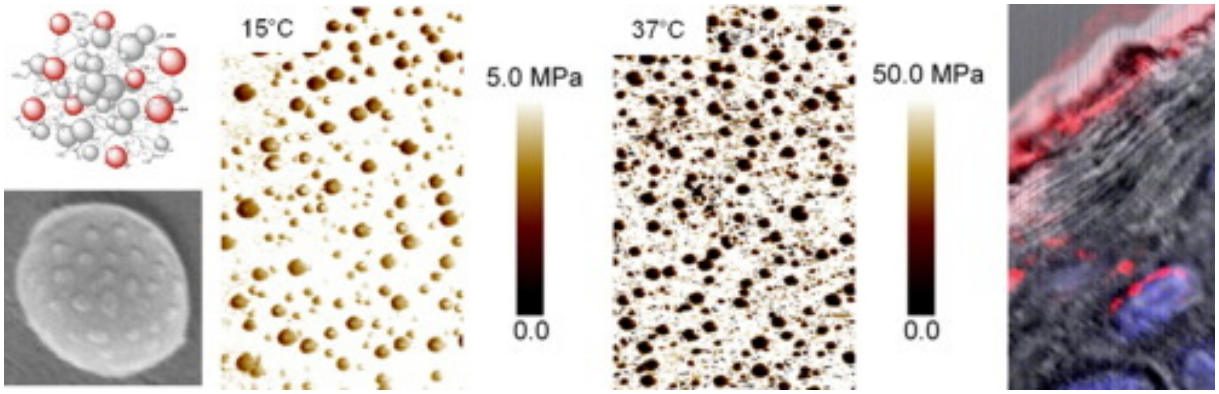

Figure 12. High resolution cryo-scanning electron microscopy images of spherical nanogels and their different elesticity modulus at 15 and 37°C recorded at a fluidic AFM chamber. Reproduced with permission from Elsevier B.V.

In this publication the author contributed with the synthesis of thermoresponsive nanogels and their labeling with a fluorescent dye. Moreover the author contributed with the physico-chemical characterization of the nanogels, the data evaluation, and with parts of the report.

Rancan, F*; **Asadian-Birjand, M**; Dogan, S; Graf, C.; Cuellar, L.; Lommatzsch, S.; Blume-Peytavi, U.; Calderón*, M.; Vogt, V. Effects of thermoresponsivity and softness on skin penetration and cellular uptake of polyglycerol-based nanogels**,** *Journal of Controlled Release* **2016**, *228*, 159–169.

http://dx.doi.org/10.1016/j.jconrel.2016.02.047

3.3 Near infrared dye conjugated nanogels for combined photodynamic and photothermal therapies

M. Asadian-Birjand, J. Bergueiro, S. Wedepohl, and M. Calderón*

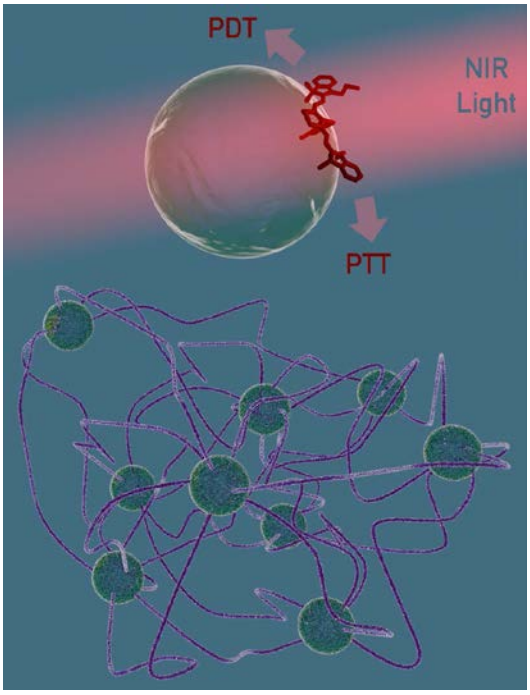

Figure 13. Schematic illustration of dually light- and thermoresponsive nanogels as a suitable agent for both photodynamic and photothermal therapy. Reproduced with permission from WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

In this publication the author contributed with parts of the concept and established the synthesis and characterization of dual responsive nanogels. Moreover the author established methods to quantify the photothermal and photodynamic efficiency of nanogels, and contributed with the data evaluation, and parts of the report.

Asadian-Birjand, M; Bergueiro, J.; Wedepohl, S.; Calderón, M.* Near infrared dye conjugated nanogels for combined photodynamic and photothermal therapies. *Macromolecular Bioscience* **2016**

DOI: 10.1002/mabi.201600117

3.4 Transferrin decorated thermoresponsive nanogels as magnetic trap devices for circulating tumor cells

Mazdak Asadian-Birjand,# Catalina Biglione,# Julian Bergueiro, Ariel Cappelletti, Chinmay Rahane, Govind Chate, Jayant Khandare, Bastian Klemke, Miriam C. Strumia, Marcelo Calderón.*

# Shared first authorship due to equal contribution.

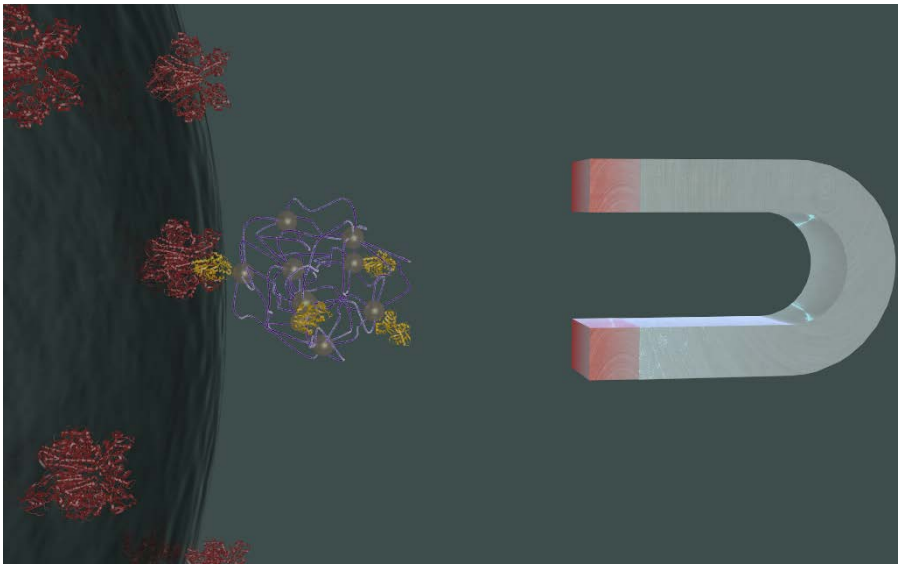

Figure 14. Schematic illustration of targeting ligand decorated magnetic nanogels. Reproduced with permission from WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

In this publication the author contributed with parts of the concept and established the synthesis and characterization the macromonomers such as linear polyglycerol and magnetic nanoparticles. Moreover the author contributed with parts of the physico-chemical characterization of magnetic nanogels, as well as the data evaluation, and parts of the report.

Asadian-Birjand, **M**.;# Biglione, C.; # Bergueiro, J.; Cappelletti, A.; Rahane, C.; Chate, G.; Khandare, J.; Klemke, B.; Strumia, M. C.; Calderón, M.* Transferrin decorated thermoresponsive nanogels as magnetic trap devices for crculating tumor cells, *Macromolecular Rapid Communications* **2016***, 37,* 439-445.

DOI: 10.1002/marc.201500590

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5 Appendix

5.1 Publications & Conference Contributions

Publications

Asadian-Birjand, **M**.;**#** Biglione, C.; **#** Bergueiro, J.; Cappelletti, A.; Rahane, C.; Chate, G.; Khandare, J.; Klemke, B.; Strumia, M. C.; Marcelo Calderon, M. Transferrin Decorated Thermoresponsive Nanogels as Magnetic Trap Devices for Circulating Tumor Cells *Macromolecular Rapid Communications* **2016***, 37,* 439−445. DOI: 10.1002/marc.201500590

Asadian-Birjand, M; Bergueiro, J.; Wedepohl, S.; Calderón, M. Near Infrared Dye Conjugated Nanogels for Combined Photodynamic and Photothermal Therapies. *Macromolecular Bioscience* **2016**, DOI: 10.1002/mabi.201600117

Rancan, F; **Asadian-Birjand, M**; Dogan, S; Graf, C.; Cuellar, L.; Lommatzsch, S.; Blume-Peytavi, U.; Calderón, M.; Vogt, V. Effects of Thermoresponsivity and Softness on Skin Penetration and Cellular Uptake of Polyglycerol-Based Nanogels. *Journal of Controlled Release* **2016**, *228*, 159-169. http://dx.doi.org/10.1016/j.jconrel.2016.02.047

M. Asadian-Birjand, J. Bergueiro, F. Rancan, J. C. Cuggino, R. C. Mutihac, K. Achazi, J. Dernedde, U. Blume-Petytavi, A. Vogt, M. Calderón. Engineering thermoresponsive polyether-based nanogels for temperature dependent skin penetration. *Polymer Chemistry* **2015**, *6*, 5827-5831. DOI: 10.1039/C5PY00924C

M. Molina, **M. Asadian-Birjand,** J. Balach, J. Bergueiro, E. Miceli, M. Calderón. Stimuli-responsive nanogel composites and their application in nanomedicine. *Chemical Society Reviews* **2015**, *44*, 6161-6186. DOI: 10.1039/C5CS00199D

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. DOI:10.1039/C4PY01186D

M. Asadian-Birjand, A. Sousa-Herves, D. Steinhilber, J.C. Cuggino and M. Calderón. Functional Nanogels for Biomedical Applications. *Current Medicinal Chemistry* **2012**, *19 (29)*, 5029-5043. DOI:10.2174/0929867311209025029

# equal contribution

Professional Experience

02.2013 - 03.2013 3 weeks research stay at CIPF – Centro de Investigation Principe Filipe in Valencia, Spain, under supervision of P.I. Dr. Maria J. Vicent in the frame of the European Commission - Seventh Framework Programme “Light-based functional in vivo monitoring of diseases related enzymes (LIVIMODE).”

11.2012 - 12.2012 4 weeks research stay at Universidad Nacional de Cordoba, Argentina, under supervision of Prof Dr. Miriam Strumia in the frame of the Federal Ministry of Education and Research (Germany) project “Design of novel dendritic nanocarrier architectures for biomedical applications.”

01.2011 – 06.2011 Student assistant at Department of Organic Chemistry, Freie Universaet Berlin (Berlin, Germany) in the research group of Prof. Dr. Rainer Haag. Focus: Intracellular organelles targeting mediated by dendritic polyglycerols

07.2010 - 09.2010 12 weeks internship at celares GmbH, Berlin, Germany under supervision of CEO Dr. Ralf Kraemer. Theme: Production of activated poly (ethylene glycol) derivatives.

06.2008 – 12.2010 Student assistant at Fraunhofer - Institut fuer Zuverlaessigkeit und Mikrointegration IZM (Berlin, Germany) under supervision of Andreas Ostmann and Lars Boettcher. Focus: Electroless nickel bumping and under bump metallization of semiconductor materials for flip-chip applications.

Grants and Awards

1. Price Poster Award during 8th Workshop Molecular Interactions - From molecules to product innovation, Berlin, Germany. 12-14.9. 2012. Poster Title: Novel Dendritic Nanocarriers for In Vivo Delivery of Small Interfering RNA.
M. Asadian Birjand,**#** F. Sheikhi Mehrabadi,**#** W. Fischer, M. Calderon, R. Haag.

# equal contribution

Contributions to Scientific Meetings

M. Asadian-Birjand, F. Rancan, J. C. Cuggino, K. Achazi, R. C. Mutihac, J. Dernedde, A. Vogt, and M. Calderon. Biocompatible Dendritic Nanogels for Thermally Triggered Skin Penetration. 41st Annual Meeting & Exposition of the Controlled Release Society. Chicago, Illinois, U.S.A. 13.07. - 16.07.2014

M. Asadian-Birjand, H. Krüger, M. Giulbudagian, M. Molina Soler, S. Wedepohl, J. Bergueiro, and M. Calderon. Thermonanogele: Neuartige thermoresponsive Nanogele für den zielgerichteten Wirkstofftransport und die kontrollierte Freisetzung von Zytostatika und/oder fluoreszierenden Substanzen. BMBF MediWING Wirkstoffinitiative für Industrie und Wirtschaft 2014. Nürnberg Germany, 02.07. – 03.07.2014

M. Asadian-Birjand, A. Sousa-Herves, and M. Calderon. Polym. Adv. Technol. 24 (Suppl. 1) 2013, 80–177. Responsive dendritic nanogels with tunable sizes and phase transition temperatures. 12th International Conference “Polymers for Advanced Technologies” 2013 Berlin, Germany, 29.09.2013 – 02.10.2013.

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5.2 Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.