Targeted delivery of advanced functionality by nanomaterials: Focus on nucleic acids delivery by novel block copolymers

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Abstract

Smart drug delivery systems are versatile examples of successful nanomedicine with potential in diagnostics and medical therapy. The thesis presents selected approaches in current drug delivery systems in the (pre-)clinical trials, and deals with potential side effects, including complement activation and hypersensitivity reactions as well as the design requirements of the delivery systems. Furthermore, it presents approaches of cationic block copolymers, which are capable to condense negatively charged nucleic acid molecules such as plasmid deoxyribonucleic acid (pDNA) and small interfering ribonucleic acid (siRNA) with the aim of efficient cell gene delivery and specific gene suppression, respectively.

The first part addresses the transfection efficiency of circular versus linearized plasmid DNA using a green fluorescent protein expressing vector with Lipofectamine 2000 and linear 25 kDA polyethylenimine (PEI). These results show a considerably improved transfection efficiency with the circular compared to the linearized DNA for the two transfection reagents. The electron microscopy images with Lipofectamine or PEI demonstrate that the circular DNA gives rise to random coil appearance of compact, spherical shape, while linearized DNA appear as worm-like strands. Particle size and shape are important in the cell biology of endocytosis and phagocytosis. The findings indicate that the shape of the transfection particle is vital for successful gene transfer.

To develop a delivery system for gene therapy, two cationic diblock copolymers consisting of primary and tertiary amines were synthesized and analyzed with respect to DNA condensation properties, morphology of the condensed plasmid DNA and transfection efficiency using two cell lines. This study revealed proof-of-concept showing an order of magnitude lower transfection efficiency of primary amine diblock copolymers compared to PEI after 48 h with increasing plasmid DNA concentration. Furthermore, primary amines compared to tertiary ones show much stronger binding to DNA and improved transfection efficiency. Transmission electron and atomic force microscopy data revealed morphologies of primary and tertiary amines regarding the condensation of the plasmid DNA, in agreement with the transfection efficiency.

In a second part the design and characterization of pentablock-based polyplexes based on the combination of cationic pentablock copolymers with folic acid functionalized copolymers for targeted specific siRNA delivery is described. The achieved 31 % knockdown efficiency shows its potential regarding cancer gene therapy. The pentablock architecture allows the formation of highly stable micelleplexes of (21 ± 3) nm in 10 mM PBS buffer solution with a neutral surface charge, excellent siRNA condensation properties, outstanding colloidal stability in 10 % serum over 24 h and biocompatibility deduced from the absence of considerable cytotoxicity even after 48 h incubation. Furthermore, selective delivery of the siRNA could be proven by the introduction of a ligand-linked block copolymer, resulting in 31 % compared to 8 % gene suppression for targeted a non-targeted micelleplexes. This pentablock-based delivery system might yield impact to future delivery systems as well as being a potential platform to be applied *in vivo* for cancer gene therapy.

Zusammenfassung

Innerhalb des Bereichs der Nanomedizin weisen intelligente Wirkstoffabgabesysteme ein großes Potenzial auf, sowohl hinsichtlich der Diagnostik wie auch der medizinischen Therapie. Die vorliegende Arbeit stellt im Rahmen einer Literaturrecherche ausgewählte Wirkstoffabgabesysteme vor, welche sich in (vor-) klinischen Studien befinden, den Nebenwirkungen welche durch diese entstehen können, im speziellen der Komplementaktivierung und Überempfindlichkeits-reaktionen, sowie deren Konstruktionsanforderungen. Des weiteren werden in einem experimentellen Teil kationische Block-Kopolymere präsentiert, welche in der Lage sind, negativ geladene Nukleinsäuremoleküle zu binden - wie etwa Plasmid Desoxyribonukleinsäure (pDNA) und kleine interferierende Ribonukleinsäuren (siRNA) - mit dem Ziel der Transfektion von fremder DNA in die Wirtszellen und damit der spezifischen Unterdrückung der Genexpression.

Der erste Teil der experimentellen Arbeit untersucht die Transfektionseffizienz von zirkulärer gegenüber linearisierter Plasmid-DNA mittels eines Vektors, welcher ein grün fluoreszierendes Protein exprimiert. Transfiziert wurde einerseits mit Lipofectamine 2000 und andererseits mit linearem 25 kDa Polyethylenimin (PEI), zwei etablierten Transfektionsreagenzien. Die Ergebnisse zeigen eine wesentlich verbesserte Transfektionseffizienz der zirkulären, verglichen mit der linearisierten DNA für beide Transfektionsreagenzien. Die elektronenmikroskopischen Bilder von Lipofectamine sowie PEI komplexiert mit DNA zeigen, dass die zirkuläre DNA zufällige, kompakte Kugelformen bildet, während die linearisierte DNA wurmartige Stränge aufweist. Partikelgröße und -form spielen in der Zellbiologie eine wichtige Rolle bei der Endozytose und Phagozytose. Die Ergebnisse legen die Vermutung nahe dass die Form der zu transfizierenden DNA-Transfektions-Komplexen eine wichtige Rolle einnimmt für einen erfolgreichen Gentransfer.

Für die Entwicklung eines intelligenten Wirkstoffabgabe-Systems für die Gentherapie wurden zwei kationische Diblock-Kopolymere, die aus primären und tertiären Aminen bestehen synthetisiert und im Hinblick auf deren DNA-Kondensationseigenschaften, Morphologie der kondensierten Plasmid-DNA sowie Transfektionseffizienz unter Verwendung von zwei Zelllinien analysiert. Die Studie bestätigt trotz einer um den Faktor 10 schwächeren Transfektionseffizienz der primären Amin-Diblock-

Kopolymeren im Vergleich zu PEI nach 48 h mit zunehmender pDNA Konzentration eine Bestätigung des Konzepts. Außerdem weisen die primären Amin-Block-Kopolymere im Vergleich zu den tertiären eine viel stärkere Komplexbildung der DNA auf - wie transmissions-elektronen- und rasterkraft-mikroskopische Daten ergaben - als auch eine verbesserte Transfektionseffizienz. Diese physikalisch-morphologischem Erkenntnisse über die Kondensation der primären und tertiären Amine mit Plasmid-DNA konnten mittels der biologischen Transfektionseffizienzdaten validiert werden.

Der zweite Teil der experimentellen Arbeit befasst sich mit dem Design sowie der Charakterisierung von pentablock-basierten Polyplexen für einen gezielten siRNA Transport. Diese Polyplexe beruhen auf einer Kombination von kationischen Pentablock-Kopolymeren mit folsäure-funktionalisierten Kopolymeren. Die erreichten 31% Gen-Suppression in einem Krebszellkulturmodell, zeigen das Potenzial des Wirkstoffabgabesystems in Bezug auf eine Krebstherapie auf. Die Architektur ermöglicht die Bildung von sehr stabilen Mizellen mit einer Grösse von (21 ± 3) nm in 10 mM PBS Pufferlösung, eine neutrale Oberflächenladung, ausgezeichneten siRNA-Kondensationseigenschaften, hervorragender kolloidaler Stabilität in Zellkulturmedium supplementiert mit 10 % Serum über 24 h, sowie guter Biokompatibilität aufgrund fehlender erheblicher Zytotoxizität auch nach 48 h Inkubation in einem Zellkulturmodell. Ferner konnte durch die Einführung eines liganden-gebundenen Block-Kopolymers der selektive Transport der siRNA nachgewiesen werden, was zu einer Gen Suppression von 31% gegenüber 8% nicht funktionalisierter Polyplexen führte. Das in dieser Arbeit eingeführte und charakterisierte pentablock-basierte Wirkstoffabgabesystem könnte Auswirkungen auf das Design zukünftiger Wirkstoffabgabesystem haben als auch als eine potentielle Plattform für *in vivo*-Krebsgentherapien angewendet werden.

Chapter 1: General Introduction

Since several years, nanotechnology has a significant impact within the field of medicine and its development. A broad range of novel nanomaterials have emerged and have been tested for biomedical applications such as scaffolds for skin wound healing, bone tissue engineering and intelligent drug delivery (1-3). Intelligent drug delivery systems belong to the most successful and versatile examples of nanomedicine, with a large potential regarding imaging, sensing or therapy. Several nanomaterial based platforms have been reported in the last decade and been tested for biomedical application such as drug delivery and cancer therapy (4). The proper design of a delivery system is a key issue since the therapeutic efficacy of many payloads is often limited in the administration by their stability, safety and bioavailability (5,6). In addition, the large freedom in design allows the construction of intelligent delivery systems with complex functionality to expand possible diagnostic and therapeutic options in medicine. Functional materials turn delivery systems intelligent through stimuli-responsiveness, which then can take advantage of unique patterns or multimodal factors of the diseased area. Intelligent systems include locally/internally triggered (pH, redox potential, enzymatic activity, temperature), externally switchable (temperature-, light-, electromagnetically-, radiation - ultrasound sensitive) and actively targeted systems (7). Ligand decorated nanoparticles for actively targeted delivery, have been found to improve control of biodistribution. Prominent examples include the molecule GalNAc, which is been used as a ligand to target receptors on the surface of hepatocytes and folate, targeting delivery of rapidly dividing cancer cells (8,9).

Polymers have gained significant attraction as delivery systems, due to the large variety of properties that can be programmed into polymers by design, rendering them attractive for the development of new applications in the biomedical field (10). Different types of polymers, such as biodegradable, non-degradable, synthetic and natural are being investigated (11-14). They can either form small sized micelles (10-100 nm) with an inner hydrophobic core or they can self-assemble to polymeric vesicles (polymersomes) containing an inner aqueous compartment, allowing the control of the membrane thickness (15). Both variants can exhibit the ability to accumulate in tumor tissue trough Enhanced Permeability and Retention (EPR). In

addition, certain synthetically produced polymers offer the advantage of longer circulation times in blood, low cytotoxicity, improved ability of local drug delivery, active targeting and controlled cellular uptake (16). Various types of functional multiblock copolymers including biodegradability, stimuli-responsiveness and surface modification properties are opening new possibilities for efficient delivery (16-18). A topic of strong interest in nanoparticle based therapies is the development of cationic polymers, which are capable to condense negative charged nucleic acid molecules such as plasmid deoxyribonucleic acid (pDNA) and small interfering ribonucleic acid (siRNA) with the aim of efficient cell gene delivery and specific gene suppression, respectively. Such synthetic cationic vectors for nucleic acids may protect the nucleic acids from premature degradation and may enhance cellular uptake. While several platforms have been developed for this task, a major drawback of many designs is the overall positive surface charge. Positive charged polymers often show unspecific binding to plasma proteins and nonspecifically to cell surfaces, potentially resulting in premature elimination from the circulation by opsonization, lack of target specificity, and risk of increased toxicity (including immunotoxic phenomena like complement activation).

The development of cationic polymer based gene and siRNA delivery systems with characteristics compatible with in vivo application, minimizing toxicity but maximizing specificity within drug targeting, remains a very important task. Small interfering RNA (siRNA) has gained significant attraction since its discovery, and is claimed by some to be one of the most important developments in biology in the last decade (19-21). In cancer research, siRNA therapeutics are seen as an innovation with a huge potential for personalized cancer treatment. Specific siRNA can interfere with a specific gene and therefore suppress cancer growth by post-transcriptional silencing of the target gene (22).

Likewise, gene therapy has received significant attraction in medicine, because of its potential in treating acquired genetic diseases such as cancer, inflammation or cardiovascular diseases. Delivering naked DNA and using viral vectors for this task have significant limitations (23-25). For this reason, synthetic vectors based on polymeric nanosystems are attractive (26). DNA delivery is a difficult task and achieving high levels of transfection remains a challenge, associated with the

understanding of structure-activity correlations (27). However, due to their polyanionic characteristics, siRNA molecules and plasmid DNA are not able to spontaneously cross the cell membrane and thus require a delivery platform. Furthermore, several biological barriers must be overcome to achieve efficient delivery. These barriers include binding to the cell surface, traversing the plasma membrane, escaping lysosomal degradation, and overcoming the nuclear envelop. Therefore an optimal carrier should be versatile enough to target a cell of choice and be stable enough to avoid premature destruction. It should also show no unspecific adherence to proteins and cells, which could lead to its elimination from the circulation. In addition, it should be able to carry a load of molecules to its destination, where it would interact in a controlled way with the target receptor or target cell.

Thesis goals and contributions

Since the field of nanomedicine is highly interdisciplinary, this thesis required combined expertise from several fields, which were provided through several group members within our interdisciplinary research group as well as by interaction with scientists from other groups through the different projects. Following is a summary of my contribution to this manifold work.

Extensive literature research: An initial literature survey was conducted with the aim to give an unprecedented overview of current intelligent drug delivery systems in the preclinical, clinical trial, and approved stages within the field of nanomedicine and the critical subject of possible unwanted side effects, such as complement activation and hypersensitivity reactions, in particular against polyethylene glycol (PEG) which is often being considered in the design of a drug delivery system, illustrating their advantages and disadvantages for further clinical application.

Polymer design and synthesis: My emphasis was on the study of polymer-nucleic acid interaction and cellular impact of novel nanomaterials, in view of medical application, while the polymer synthesis has been carried out by our chemist, Kegang Liu. Iterative design trials of several polymers, based on an extensive literature survey, led to selection of the appropriate polymers for final nucleic acid delivery. The

selection criteria included biocompatibility, processability, specificity, reproducibility as well as colloidal and serum stability.

Preparation of nucleic acid/polymer complexes: The siRNA and pDNA/polymer complexes were prepared by aqueous self-assembly. The polymers, depending on their structural composition, were dissolved in ethanol or phosphate buffered saline (PBS) and mixed accordingly to the N/P ratio. Different salt concentrations were tested to better understand binding behavior of polymers with nucleic acids and a standard preparation protocol has been established.

Loading capacity determination: The loading capacity and condensing ability of the polymers play a crucial role for proper cellular uptake of the nanoparticles. The relative mobility of the nucleic acid/polyplexes at different charge ratios was studied by agarose gel electrophoresis. Agarose gel electrophoresis is a standard method used in molecular biology to separate nucleic acids or proteins in an agarose matrix accordingly to their charge and size. Complexation of nucleic acid with cationic polymers, prevent migration at a given N/P ratio, which indicates full neutralization of the negative charge of nucleic acids. In the case of siRNA, agarose gel electrophoresis revealed full neutralization at N/P ratio 5, considered to be appropriate for further experiments.

Particle size and surface charge: The particle size and the zeta potential of the nanoparticles were examined using a zeta sizer (Nano ZS, Malvern Instruments Ltd., Malvern, UK) by dynamic light scattering (DLS) and electrophoretic mobility respectively for characterization of the nanoparticles. Nanoparticles with stable properties regarding size and charge could be prepared. Zeta potential measurement is currently the simplest and most straightforward way to characterize the surface of charged nanoparticles based on the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed nanoparticles.

Transmission Electron Microscopy (TEM) measurements: Size and morphology determination of the nucleic acid/polymer complexes were conducted with great support from V. Oliveri (Biozentrum, Microscopy Center) confirming the data observed by DLS.

Atomic Force Microscopy (AFM) measurements: To better understand proper morphological behavior, plasmid DNA condensation properties regarding diblock copolymers and in comparison to TEM measurements, atomic force microscopy imaging using a Bruker MultiMode V microscope was conducted, with great support from R. Wagner. AFM images gave further agreement and deeper understanding of polymer DNA interactions.

Cell culture and transfection procedure: As a model system for gene knockdown studies, HeLa cells expressing green fluorescent protein (GFP) as well as folate receptor positive HeLa and negative HEK293 cells for targeted cellular uptake studies were used. Cells were passaged split and seeded in 24-well plates. Improvements of the standard manufacturers transfection protocols were achieved, regarding optimization of transfection time, wash procedure, cell medium composition and concentration of polyplexes applied to the cells.

Fluorescence microscopy: Visualization of the cellular uptake and localization of siRAN/polymer complexes was achieved by introduction of fluorescently labeled siRNA and studied with introductional help of M. Abanto with an Olympus BX61 Diana fluorescence microscope The observations showed clear correlation of the localization and the silencing efficiency but also left speculation regarding the proper cellular uptake mechanism involved, which still has to be identified. Furthermore, qualitative analysis of plasmid DNA transfection was conducted.

Knockdown analysis: As a method of choice for quantification, the well established Western Blot technique was chosen to quantify green fluorescent protein suppression and folate receptor expression. Data analysis showed clear differences of folate receptor expression of HeLa and HEK293 cells, enabling receptor mediated uptake studies as well as receptor-mediated knockdown of GFP upon application of the own designed polymers.

Stability studies: Colloidal stability and possible agglomeration of the polymer solely and siRNA complexed with the polymer were investigated by dynamic light scattering using a zeta sizer. Time dependent studies showed clearly that the micelleplexes do not aggregate in the presents of serum as compared with polyethylenimine.

Aggregation of cationic polymers is very important topic, since it determines the fate for successful in vivo application.

Cytotoxicity measurements: The cytotoxicity of the polymers used for siRNA delivery was tested using the Resazurin reduction assay. The results showed that cytotoxicity occurs at more then 10 x higher concentrations then the ones used

Cloning experiments: Plasmid DNA used in all experiments has been produced as following: Circular plasmid DNA, pEGFP-C1 (Clonetech) was transformed into competent bacterial cells (DH5α) and inoculated with the appropriated selective antibiotic. Purification and elution of plasmid DNA was done using a MaxiPrep Kit (Qiagen). The DNA quantitation was obtained spectrophotometrically and confirmed by the comparison with a high DNA mass ladder by gel electrophoresis.

Flow Cytometer studies: Transfection efficiency was determined by flow cytometry with BD Accuri C6 (Becton Dickinson, San Jose, CA) in close collaboration with X. Wang. The measurements gave qualitatively understanding regarding different N/P ratios and cell lines on transfection efficiency as well as dependency of primary versus tertiary amide based polymers for condensing of the plasmid DNA and showed proof of concept.

Structure of the thesis

In this thesis, required properties and differences of basic delivery platforms in regard to deliver smart functionality, on building block suited to enhance tissue-, cell- and receptor specific targeting are discussed. Next it discusses advantages and disadvantages of those platforms for future clinical application with regard to the subject of immune system responses. It also deals with the highly relevant issue of advancing from the current, mostly passive medical nanosystems towards active nanosystems with advanced functionality, by the development of stimuli responsiveness and of multi-functionality, which permit progressive optimization of diagnosis and therapy and can be adapted to the needs of individual patients. Furthermore, the synthesis, characterization and efficiency of new block copolymers for siRNA and gene delivery are described.

A general introduction is presented in **Chapter 1**, providing the aim and the general background of the work and the structure of the thesis. In **Chapter 2**, a state of the art overview of intelligent nanomaterials for medicine such as polymers, liposomes, dendrimers, quantum dots, carbon nanotubes, nanogels, metallic as well as chitosan and peptide based nanoparticles is given. It presents a summary of current drug delivery systems in the preclinical, clinical trial, and approved stages within the field of nanomedicine and deals with the crucial subject of possible unwanted side effects, such as complement activation and hypersensitivity reactions, in particular against polyethylene glycol (PEG), illustrating their advantages and disadvantages for further clinical application.

In **Chapter 3 and 4**, stimuli-responsive nanosystems and their potential for medical application are discussed. A variety of new nanomaterials have been introduced in the field of nanomedicine and found their way into clinical domain as drug delivery systems, for imaging, sensing and therapy. An important issue for the future design of nanomaterials is the development of stimuli responsive and multifunctional nanosystems for optimization of diagnosis and therapy. The wide variety of internal (pH, redox potential, enzyme responsive, temperature) and external stimuli (light, temperature, ultrasound, electromagnetically and radiation sensitive), as well as advantages and disadvantages of these stimuli and their challenges for possible medical application are described **(Chapter 3)**.

Among all stimuli, light represents a promising candidate due to its attractive features such as high sensitivity, ease of controllability and physical properties, which allow a wide variety to design sensitive nanomaterials. **Chapter 4** thus gives an overview on light and its applications within the field of nanomedicine, identifies opportunities and describes gaps supported by the state of the art.

Chapter 5 deals with the topic of the development of a suitable system for transfection of plasmid DNA (pDNA) into eukaryotic cells. The greatest obstacle in the field concerns the engineering of appropriate delivery systems able to deliver the plasmid DNA, inefficient long-term expression, low transfection rates and immunogenicity. In addition to the choice of the delivery system, the topology of the DNA seems to play a key factor for efficient transfection. To compare transfection efficiency in regard to the topology of the DNA, experiments of circular versus linear

plasmid DNA using a cationic lipid system (Lipofectamine) or a cationic polymer (polyethyleneimine) were conducted. Furthermore, cationic diblock copolymers were synthesized to test condensing of pDNA into compact nanostructures and been used for in vitro delivery studies. To understand condensing of the pDNA, the size and molecular morphology of the pDNA/polymer complexes was observed by atomic force microscopy and transmission electron microscopy at increasing charge ratios of the complexes. Transfection efficiency of the diblock copolymers was evaluated by flow cytometer analysis. In **Chapter 6**, a novel pentablock based block copolymer siRNA delivery system with targeting properties is presented. The concept of the study was to assess our novel siRNA drug delivery systems that expand the established PMOXA-PDMS-PMOXA triblock copolymer concept into a pentablock copolymer system that combines several desired characteristics. In combination with receptor-specific ligands, such polymeric constructs promize target specific interactions. The pentablock copolymer based siRNA delivery system was characterized concerning size, surface charge and serum stability by dynamic light scattering, zeta potential and transmission electron microscopy measurements. Gene silencing mediated by siRNA loaded pentablock based nanoparticles, was quantitatively determined by Western Blot analysis. The cellular uptake efficiency was investigated by fluorescence microscopy and cytotoxicity was determined by measuring cell viability using the resazurin reduction assay.

The dissertation closes with **Chapter 7** giving conclusions along with recommendations for future work.

The work described in this thesis has either been published or is currently being patented or to be submitted for publication:

Chapter 2: R Lehner, X Wang, S Marsch, P Hunziker: *Intelligent nanomaterials for medicine: Carrier platforms and targeting strategies in the context of clinical application.* Journal of Nanomedicine: Nanotechnology, Biology, and Medicine 9 (2013) 742-757

Chapter 3: R. Lehner, X. Wang, M. Wolf, P. Hunziker: *Designing switchable nanosystems for medical application.* Journal of Controlled Release 161 (2012) 307-316

Chapter 4: R. Lehner, P. Hunziker: *Why not just switch on the light?: light and its versatile applications in the field of nanomedicine.* European Journal of Nanomedicine 2012;4(2-4):73–80

Chapter 5: R. Lehner, X. Wang, P. Hunziker: *Plasmid linearization changes shape and efficiency of transfection complexes.* European Journal of Nanomedicine 2013; 5(4): 205–212

Chapter 6: R. Lehner, K. Liu, P. Hunziker: *Efficient receptor mediated siRNA delivery in vitro by folic acid targeted pentablock copolymer-based micelleplexes*. (Material currently being patented, manuscript ready for submission)

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Review

Intelligent nanomaterials for medicine: Carrier platforms and targeting strategies in the context of clinical application

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Abstract

Nanomedical approaches are a major transforming factor in medical diagnosis and therapies. Based on important earlier work in the field of liposomal drug delivery and metallic nanomaterials, the last decade has brought a broad array of new and improved intelligent nanoscale platforms which are not only suited to deliver drugs and imaging agents but also to carry advanced functionality including internal and external stimuli-responsiveness in a highly targeted fashion to a diseased area. This review focuses on required properties and differences of basic delivery platforms in regard to deliver smart functionality, on building blocks suited to enhance tissue-, cell- and receptor-specific targeting and on nano-bio interaction. Further it discusses advantages and disadvantages of those platforms for future clinical application with regard to the subject of complement activation and hypersensitivity reactions in particular against polyethylene glycol (PEG) and possible functionalization with nanosize switches.

From the Clinical Editor: This review focuses on the properties of platforms designed to deliver smart functionality, using appropriate building blocks to enhance tissue-, cell-, and receptor-specific targeting. The authors also discuss potential complications such as complement activation and hypersensitivity reactions, and possible functionalization with nanosize switches.
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Key words: Nanoplatforms; Targeted delivery; Stimuli-responsive nanocarriers; Active targeting; Passive targeting

During the last decade, nanotechnology has had a steadily increasing impact on preclinical development in medicine, shaping the emerging scientific field of nanomedicine. Today, many of these developments are entering the clinical domain. An important topic is the development of composite nanosystems for diagnosis and therapy within the body. Such systems often consist of i) a carrier platform, ii) a payload for imaging, sensing, or therapy and iii) optional targeting ligands. Beyond such basic systems, the large freedom in design allows to compose *nanosystems with complex functionality* that pave the way to intelligent and responsive behavior, potentially applicable in medicine. Different basic nanomaterial platforms have emerged and have been tested for biomedical applications such as drug delivery and cancer therapies. Nanoparticles (NPs) are nanosized materials (diameter 1–200 nm) that can carry different payloads such as small molecular drugs, imaging agents, proteins, nucleic acids or other content.[1–4] Nanocarriers are designed to improve efficacy and safety for drug delivery in general and for target specific non-viral drug delivery in particular.[5,6] The design of such nanomaterials requires the ability to control particle size, to assure biocompatibility and stealth properties, to optimize specificity, and to achieve controlled release and functionality.[7,8] As basic materials, liposomes, dendrimers, polymers, carbon nanotubes, metallic NPs, organic NPs, quantum dots, nanogels and peptidic nanoparticles have been applied as possible nanotechnological carrier platforms (Figure 1).

With an emphasis on medical therapy, this review aims to shed light on design principles suited to create complex nanosystems by combining carrier platforms, engineering nanomaterial–cell interactions and enabling such systems to show stimuli responsiveness by equipping them with elements from the toolbox of switches on the nanoscale, considering the critical factors of such systems for success in clinical application with regard to complement activation and hypersensitivity reactions in particular against polyethylene glycol (PEG).

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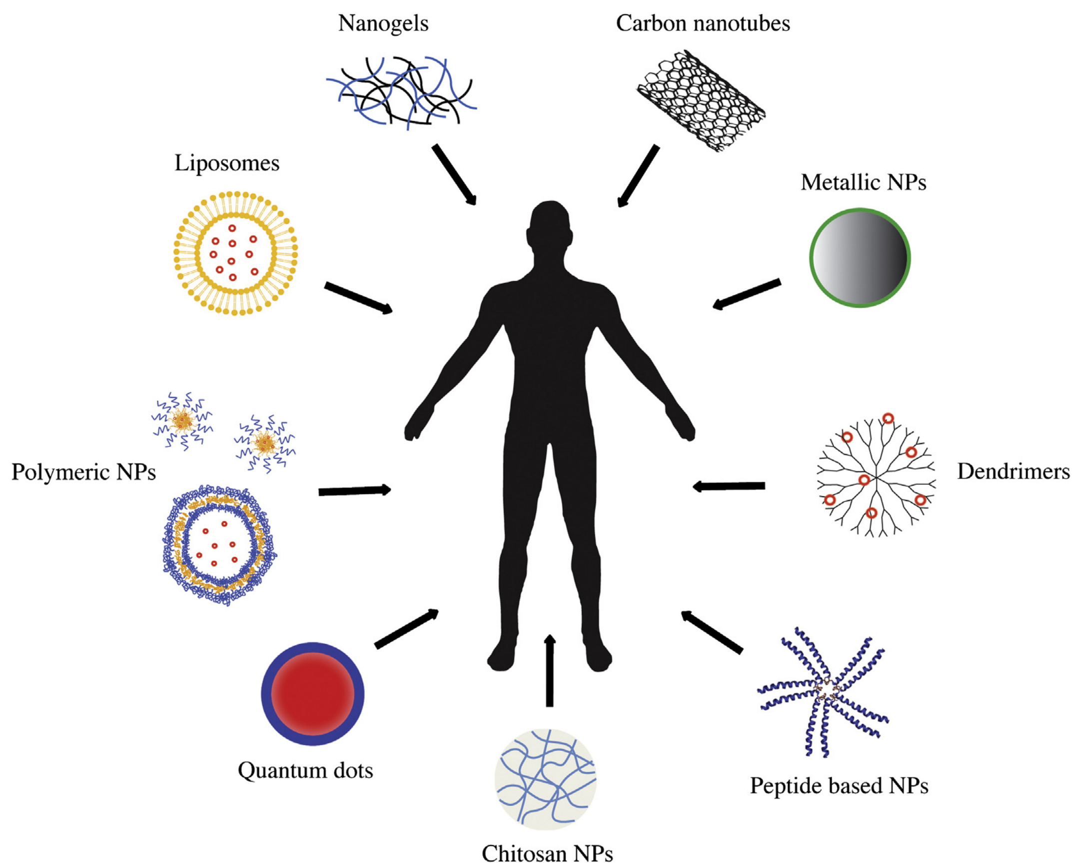

Figure 1. Schematic illustration, showing established therapeutic nanocarrier platforms (NPs) in preclinical development.

Nanotechnology carrier platforms suited for switch functionality

In recent years, major efforts have been devoted to develop suitable nanotechnological platforms to improve drug delivery to tumor tissue. For the development of such platforms, several challenges need to be mastered: (i) the control of the particle size, which can have influence on the NP distribution, clearance by kidney or liver and payload uptake; (ii) biocompatibility, to achieve an optimal benefit/risk relation; (iii) stealth properties, to escape immunological recognition and serum protein interactions; (iv) optimal blood circulation time for a specific application; (v) high target specificity for delivery of drugs or advanced functionality; (vi) controlled release mechanisms, e.g. endosomal escape; (vii) further functionality control through stimuli responsiveness. Multiple nanoscale platforms have been developed for this purpose, of which the most important will be discussed now.

Micelles and liposomes

Micelles are nanosize structures characterized by a hydrophobic core and a hydrophilic coat and form spontaneously from amphiphilic molecules in aqueous environments. Liposomes are self-assembling structures with a spherical shape, composed of a lipid bilayer, which entirely surrounds an aqueous core, able to deliver different kind of biomolecules.[9] Depending on the assembly technique used, the size of the vesicles can range from tens of nanometer to micrometers. Under specific conditions, liposomes of ~ 100 nm in diameter have been successfully used to deliver chemotherapeutic agents to tumors.[10] Drug delivery of poorly soluble molecules can be achieved through micelles using lipid moieties as hydrophobic blocks linked to hydrophilic polymers.[11] Different lipids have different fatty acid chain lengths and different head groups, resulting in a broad range of achievable physicochemical characteristics like minimal micellar concentration or melting temperature, allowing the creation of environment-sensitive (e.g., temperature-, pH-sensitive, mechano-sensitive) liposomes by choosing the specific setup.[12] Several liposome-based cancer drugs have entered the clinical domain, e.g. carrying the anthracyclines doxorubicin (Doxil, Myocet, Caelyx) for treatment of Kaposi's sarcoma, ovarian cancer, multiple myeloma, metastatic breast cancer or daunorubicin (DaunoXome) for treatment of Kaposi's sarcoma.[13,14] Beyond approved agents, liposomal chemotherapeutics are finding their way into clinical trials.[15–17] One important future direction is the development of receptor-specific targeting for cell-specific delivery, which may render liposomes suited for purposes such as siRNA delivery. As reported recently by Gao et al, delivering siRNA by immunoliposomes to epidermal growth factor receptor (EGFR) overexpressing breast cancers in principle is possible.[18] However, early trials have also shown that important challenges, in particular due to interaction of such systems with the immune system, need more work.

Liposomes are interesting carrier candidates for delivery of intelligent switches at the nanoscale because the inner aqueous core offers a "nano-compartment" where processes may take place that require protection from the surrounding body fluids when injected into an organism. Liposomes have already been converted into intelligent nanosystems by incorporating a wide variety of stimuli response functionalities such as temperature, light, pH, ultrasound, enzymatic response or even as drug delivery system for radiation sensitive nanoparticles[19–24] highlighting that liposomes are simple, but effective carriers for multimodal nanoscale trigger and effector functionalities.

Polymeric systems

Polymeric and polymeric-biologic hybrid nanomaterials have gained increasing attention as modifiers of pharmacokinetics of "biologicals" (pharmaceutical products originating from biomacromolecules), as carriers of hydrophobic drugs and in particular as non-viral vectors for nucleic acid delivery. A structurally simple approach is to couple a polymer (typically polyethylene glycol, PEG) with a protein, a strategy that allows the control of pharmacokinetics while maintaining the biological properties of the protein. Self-assembly to nanoparticles is not exploited here. Different types of polymers, biodegradable or non-degradable, synthetic and natural are being used for formation of polymeric micelles and polymersomes (polymeric vesicles) as drug delivery systems.[12,25–27] One difference of certain synthetic polymers compared to natural polymers is their longer lifetime and therefore improved ability to concentrate at a disease location. This increases the possibility of prolonged drug release over several days to weeks.[5] Polymeric micelles can be built by self-assembly of amphiphilic-block copolymers exhibiting a hydrophilic outer shell and a hydrophobic core, which can enable transport of water insoluble drugs to their specific target. Several important anticancer drugs like paclitaxel, tamoxifen or campthotecin[28,29] are highly water insoluble. The relatively small size of polymeric micelles (10–100 nm) compared to polymeric vesicles enhances their ability to accumulate in tumor tissues through the enhanced permeability and retention (EPR) effect.[30] Another important aim is the development of cationic polymers that are able to form complexes with nucleic acids with the goal achieving cell transfection and siRNA delivery, thereby promising new approaches to the therapy of severe diseases including inherited metabolic disease or cancer. For example, Mao et al reported a cationic triblock copolymer for the delivery of siRNA targeting the acid ceramidase gene for cancer therapy. They demonstrated biodegradable micellar triblock copolymer/siRNA complexes that efficiently deliver siRNA into cancer cells and thereby induce gene silencing effects.[31] A number of cationic polymers used for encapsulating siRNAs have shown toxicity, rendering their clinical application challenging.[32] Nevertheless, the positive charge of cationic polymers can facilitate cellular uptake and endolysosomal escape as reported by Park et al, thereby overcoming one major cellular barrier in target-specific drug delivery.[33] Other nucleic acid carrier systems include cationic polymers poly(L-lysine)(PLL), poly(ethyleneimine)(PEI), chitosan and dendrimers.[34] Detailed information of cationic carriers for nucleic acid delivery is given by.[35] Polymeric delivery platforms are excellent carrier platforms for integration of switches at the nanoscale and other complex functionality, due to the large freedom of design, their improved stability compared to liposomes, and their ability to integrate functional biomolecules within the hydrophobic bilayer. However, there is less experience in clinical application of composite polymeric nanosystems and vesicles compared to the well established liposome field, which is a factor to keep in mind when developing commercial products for clinical application that will require thorough insight into pharmacokinetics, metabolism and mass balance, toxicity for the regulatory process and industrial scale-up and production.

Dendrimers

Dendrimers are large and complex molecules with very regular chemical structure, which were pioneered in the early 80s.[36] They are nearly perfect monodisperse macromolecules with a regular and highly branched tree like architecture. Dendrimers are constructed through a repeating sequence of chemical reaction steps, leading to predictable alterations in their size determined by each generation.[12,37,38] In typically used chemical syntheses, dendrimers are structures with a size of 1 to 10 nm and a hydrophobic interior, which enables drug delivery of hydrophobic compounds such as cancer drugs. Poly(amidoamine), PAMAM is the most well known dendrimer for biological applications.[39–41] Cationic PAMAM dendrimers show cytotoxic effects, which have been thought to result from the interaction with negatively charged molecules. To overcome this effect, Jia et al reported the use of phosphorylcholine, a zwitterionic molecular segment found mainly at external surfaces of cell membranes and the end of some lipids.[42] Such zwitterionic PAMAM dendrimers encompassing a phosphorylcholine surface efficiently lower the cytotoxity compared with the native PAMAM dendrimers.[42,43] Dendrimers have been explored clinically as outlined by.[44–46] Dendrimers significantly differ in size from micelles, liposomes and polymeric systems leading to differential distribution behavior after injection into an organism. Forming a single, covalently linked structure, they are mechanically more stable than liposomes. They offer some inner, hydrophobic cavity space for the encapsulation of payloads and switch functionality, but payload/carrier ratio, measured as weight/weight ratio, is lower compared to micelles and lipid/polymer vesicles, which represents a limitation when space-consuming smart sensors or nanoscale switches need to be integrated in the interior of a nanosystem. A challenge for development of intelligent nanoscale systems based on dendrimers as commercial products at the current time is the fact that clinical experience with such systems is still limited. This is associated with a degree of uncertainty for pharmaceutical development, regulatory path and clinical success, which needs to be taken into account in decision making for industrial development.

Carbon nanotubes

Carbon nanotubes are a distinct molecular form of carbon atoms, yielding a hexagonal arrangement. Carbon nanotubes exist as single-walled and multi-walled variants.[47] Their

structure, formed from layered graphite sheets, gives them extreme physical strength, ten times as strong as steel, and unusual heat and conductivity properties.[48] Recently carbon nanotubes have attracted attention due to their use in controlled drug release as well as delivery of nucleic acids, peptides and antibodies. Their inner core and their outer surface allow the insertion of specific payload into the small inner core, while the outer surface can be modified to achieve the necessary biocompatibility within the body or to attach targeting ligands or drug payloads.[49] Gu et al recently published data concerning single-walled carbon nanotubes (SWNT) coated with doxorubicin, exhibiting a pH-responsive drug release behavior.[50] They covalently attached hydrazinobenzoic acid to the SWNTs using hydrazine as linker molecule. Doxorubicin was bound to the modified SWNTs forming hydrazone bonds with hydrazinobenzoic acid. In vitro testing of these SWNTs showed drug release upon pH change. In an acidic tumor microenvironment, this application could provide a specific drug release system. Conflicting results have been reported for cytotoxicity from in vitro and in vivo studies dependent on size, length, and surface topology.[51–53] One challenge in toxicity testing is that due to their hydrophobicity, carbon nanotubes can only be brought into solution in physiologic liquids through detergents or surface modification. Although surface functionalization of carbon nanotubes by attachment of appropriate molecules showed dramatically lowering of their toxicity, more effort has to be put into understanding the cytotoxicity of carbon nanotubes.[54] Animal experiments resulted in specific targeting, tumor binding and drug delivery from which one can envision new therapeutic approaches.[55,56] Clinically however carbon nanotubes have not overcome phase I trials. A number of research groups have reported the functionalization of carbon nanotubes in relation to sensitivity to environmental conditions such as pH, temperature or glutathione. Most SWNT have a diameter of close to 1 nm incorporating the graphene layer, forming an inner core of <1 nm. Compared to the achievable payload volume of other platforms such as liposomes and polymeric vesicles, the much smaller volume to weight ratio of carbon nanotubes is another limiting factor for their therapeutic use. To compete against micelles, liposomes, polymeric systems and dendrimers as platform for clinical development of intelligent nanomaterials, a compelling argument based on their unique characteristic is probably required and not yet achieved.

Metallic nanoparticles

Metallic nanoparticles such as iron oxide, gold and silver have been developed and modified for use in drug delivery, magnetic separation and diagnostic imaging.[57–59] Superparamagnetic nanoparticles (SPION) built from oxide nanoparticles, such as magnetite (Fe_3O_4) and maghemite (Fe_2O_3), exhibit particular features like ultrafine size, biocompatibility and magnetic properties. The superparamagnetic properties become manifest when a magnetic moment is induced through the application of a magnetic field. The large magnetic moment yields a strong signal change in magnetic resonance imaging (MRI) allowing therefore sensitive detection at high resolution. Another application of iron oxide nanoparticles is tumor treatment by magnetically induced hyperthermia.[60]

Thanks to its chemical inertness and suited mechanical properties gold has been used in medicine for teeth implants and is also in use in cancer radiotherapy.[61] Gold nanoparticles can be formed with core sizes ranging from 1 to >100 nm. The initial claim of absence of cytotoxicity has raised enthusiasm as an excellent drug delivery system although increasing recognition of size dependent cytotoxicity needs to be considered before their application.[62] Gold nanoparticles are capable of delivering peptides, proteins nucleic acids or small molecules. When functionalized with quaternary ammonium groups, they can bind negatively charged DNA or RNA and also protect the nucleic acids from enzymatic degradation.[63]

Silver has been used for a long time as a staining agent for glass and as a bacteriostatic agent.[64] Elemental silver and silver salts are well known to be relatively nontoxic to human cells and have been applied in the treatment of burn wounds, diabetic skin ulcers and newborn eye infections.[65–67] Silver nanoparticles have therefore been developed for antibacterial applications including coating of medical catheters to prevent contamination and infections.[68]

The electromagnetic properties of various metallic nanoparticles render them interesting for electromagnetic control, in particular induced hyperthermia; plasmon effects on metallic nanoparticles are another physical effect that can be used to incorporate switches at the nanoscale. Metallic nanoparticles can be surface-functionalized with relative ease, allowing to equip the particles with a wide range of advanced functionalities, including pH sensitivity and redox responsiveness. A potential disadvantage is the weight/weight ratio between functional payload and inert platform, which tends to be low in metallic nanoparticles. The absence of an inner cavity is a disadvantage, and the question of long-term behavior (mass balance, bioaccumulation, elimination pathways) after injection into an organism, and of cell toxicity need to be carefully weighted when their application for clinical trials is considered.

A potential concern of metallic nanoparticles using heavy metals is their release into the environment in a potentially non-recyclable form; a mass balance in the body and an estimation of the environmental mass balance of such materials seems prudent when industrial products are developed.

Other organic nanoparticles: carbohydrate based NPs, chitosan and starch

Certain organic nanoparticles originating from natural materials feature good water solubility, low toxicity, biocompatibility and biodegradability, rendering them interesting candidates for carriers. Chitosan is a polymer obtained by deacetylation of chitin. Chitin itself can be found in the exoskeleton of crustaceans like shrimps and crabs but also in the cell walls of fungi.[69] Studies have shown the versatility of chitosan for drug delivery and stimuli responsiveness in pH- and temperature-triggered applications.[70–73] Starch can be found abundantly in nature in crops such as rice, corn or potatoes. Chemical modification of starch allows a wide range of different applications e.g. biodegradable packaging material or as target

specific drug delivery system.[74] Suyao et al showed preparation of folate-conjugated starch nanoparticles for tumor targeting. They encapsulated the anticancer drug doxorubicin with folate modified starch polymers. Successful cellular uptake followed by loss of tumor cell viability could be shown.[75] The biodegradability and the low potential of toxicity of organic materials render them a competitive nanomedical platform for clinical application. Nevertheless, issues regarding the dissolution of the polymer matrix at low pH as well as poor mechanical strength as seen for chitosan have been raised.[72]

Quantum dots

Quantum dots (QDs) are small sized (1–10 nm) semiconductor nanocrystals which were developed in the early 80s by Alexei Ekimov and Louis E. Brus.[76] They are composed of an inorganic elemental core (e.g. Cd and Se) surrounded by a metallic shell (ZnS), which constitutes a barrier between the optically active core and the surrounding medium. QDs can be modified by ligand attachment or encapsulated with amphiphilic polymers to improve solubility, specificity, size and visualization properties in tissue.[77,78] They are widely used in biological research as fluorescence imaging tool due to their resistance to photobleaching and their excellent quantum yield compared to organic dyes.[79] QDs can also be used as drug carriers or simply as fluorescent labels for other drug carriers. Derfus et al have demonstrated the use of QDs as beacons to track siRNA delivery by PEGylating QD cores, conjugating them with siRNA and tumor-homing peptides necessary for cellular uptake.[80] A second approach is the labeling of common drug carriers such as polymers, liposomes or dendrimers with QDs. Zintchenko et al have introduced a drug nanocarrier called “quantoplex”. Quantoplexes are polyethylenimine nanocarriers incorporating plasmid DNA and QDs, allowing real-time nanoparticle tracking in living animals.[81] QDs are of particular interest when combined with other nano platforms, as one functional building block among other functionalities. For clinical application, their ability to combine molecular imaging and cancer therapy can open new doors in the field of cancer therapy,[82–84] but the toxicity of certain materials used in QD synthesis is an important concern.

Nanogels

The field of nanogels is a new and fast developing domain of research in nanomedical drug delivery. Nanogels are a three-dimensional network either formed chemically (covalent bonds) or physically (hydrogen bonds, Van der Waals and electrostatic interactions) of cross-linked hydrophilic polymers with a size of < 200 nm. Due to their hydrophilic properties, nanogels can swell and encapsulate a high volume of water when added to an aqueous solution. Due to their polymeric nature, a broad range of chemical modifications is possible. Such hydrogels can entrap through spontaneous processes a large amount of biological molecules as e.g. DNA, RNA, proteins and drugs, rendering them well suited for drug delivery. For site-specific tissue targeting within the body, the surface structure of nanogels can be chemically modified with different ligands. Nanogels proved their value as carrier platform for siRNA and for stimuli responsiveness (pH, temperature).[85,86] Wu et al have presented multi-functional core-shell nanogels combining magnetic regulation with biochemical sensing. They formed a core from magnetic Ni and fluorescent Ag surrounded by a pH-sensitive p(EG-MAA) nanogel shell. Combination of a pH-dependent magnetic response with fluorescent pH sensing allows the creation of an intelligent carrier system for diagnostic and therapeutic applications.[87] Nanogels exhibit high stability in vivo, controllability of size, biodegradability, surface functionality and specific drug delivery. Nanogels have found their way into clinical applications.[88,89]

Peptide-based nanoparticles as delivery system

Peptides can have lipophilic or hydrophilic properties based on their amino acid composition and can therefore be used to construct amphiphilic molecules that form nanostructures by self-assembly.[90,91] Design considerations of these biopolymers for drug carriers are similar to other biocompatible polymers, but should take into account that peptides may act as strong immunogens and that the body already contains several lines of defense against foreign proteins and peptidic structures like virus capsids. In addition, a variety of peptidases exist in the body; if a system is preclinically developed for later clinical use in man, species differences in peptidase expression needs to be carefully considered. Virus self-assembly can act as an inspiration to build hollow or solid, peptidic nanostructures.[92,93] Bawa et al showed enhanced cellular delivery and activity of the anticancer drug ellipticine to human lung carcinoma A549 cells using self-assembling peptide-based nanoparticles.[94] Naskar et al presented the formation of multivesicular structures from self-assembling peptides, depicting sensitivity upon exposure to calcium ions leading to vesicular disruption. This intelligent sensing/switching functionality, allows cargo release suited for medically relevant payloads.[95] However, a natural extension of peptide-based systems is the exploitation of biologic peptide functions like their use as receptor ligands or enzymatic activity, naturally leading to nanomaterials with complex or switchable functionalities. Clinically, peptidic systems have entered clinical trials dominantly as nano-platforms for vaccines offering multivalency as a potent immune system stimulant.

Advanced functionality including internal and external stimuli-responsiveness

Many different nanomaterial-based platforms have been developed to improve drug delivery to tumor tissue, expand diagnostic and enhance the therapeutic efficacy while minimizing possible side-effects, but only a few have found their way into clinical application. An important direction of future research is the development of smarter, functional materials that turn nanosize delivery vehicles into “intelligent” nanoparticles through stimuli responsiveness. Insights into the complex biological properties of e.g. a diseased area may be exploited to enhance specific drug release within the diseased area or the cytoplasm of a key cell type involved in pathophysiology, by

taking advantage of unique patterns or multimodal factors of the microenvironment. The design of such intelligent, stimuli responsive nanoplatforms also promises diagnostic opportunities with increased disease specificity.

Switchable nanoparticles can be classified based on the modality of stimulus into locally/internally triggered and externally switchable nanoparticles.

Internal stimuli such as pH, redox potential, enzymatic activity or temperature are increasingly used for the design of intelligent, stimuli responsive nanoplatforms in preclinical studies.[24] They have the advantages of high specificity due to the possible control by a molecular mechanism dependent on a certain disease. Internally switchable nanosystems are expected to exhibit less damage to healthy tissues compared to non-switched materials; as their control signal is tissue specific, bystander organs are spared as well. Nanomaterial switched by internal stimuli have entered clinical trials,[96,97] but the widespread clinical benefit for the patient has not yet been achieved. An important challenge is the fact that not every disease shows a specific internal molecular trigger suitable for exploitation as a trigger for switchable nanosystems.

Externally controlled nanosystems can be divided into temperature-, light-, electromagnetically, radiation- or ultrasound-sensitive delivery systems and have the advantage of external regulative application properties regarding the area of interest.[24] A limiting factor for externally controlled nanomaterials is the tissue penetration of the control signal and the potential for tissue damage between energy source and target organ. Tissue damage may arise upon interaction of certain forms of radiation (heat, light, electromagnetic fields) with biomacromolecules (e.g. membrane proteins), leading to tissue disruption and damage also in healthy bystander tissues. Some externally controlled systems have already proven clinical benefit as seen for iron oxide based NanoTherm® nanoparticles developed by the company MagForce for the treatment of glioblastoma by magnetic hyperthermia[98] or the photodynamic therapy based compounds Visudyne®, Photofrin® and Levulan®Kerastick® for the treatment of age-related macular degeneration, esophageal cancer and actinic keratosis.[21,99] In the year 2000 the U.S. Food and Drug Administration (FDA) approved the first antibody linked drug named Mylotarg®, developed by the Celltech Group and American Home Products, for the treatment of acute myelogenous leukemia based upon redox-sensitivity.[100] Rejected by the European Medicines Agency (EMA) in 2008 due to failure of full remission and an inappropriate cost-benefit ratio for the patient, the FDA also decided to take Mytolarg® off the market.

Engineering cell and tissue interaction at the nanoscale

Targeting strategies

Liposomes and more recently other nanoparticles have found broad application for delivery of diverse payloads. Their small size, biocompatibility, stability and stealth properties render them capable of prolonged circulation half-life, targeting of tumor tissue and cellular uptake (Table 1). Successful targeting implies sufficient stability to avoid premature drug release into the bloodstream and to circumvent degradation of the payload in the circulation prior to target arrival. Of equal importance are adequate stealth properties, which allow nanoparticles to avoid interactions with plasma proteins or the immune system that could lead to early elimination of the material. Cancer therapy is one of the most frequently pursued applications because of the severity of the disease and of the well-known severe side effects of many conventional therapies. Specific targeting of only the diseased tissue while sparing the rest of the body is therefore the Holy Grail for this clinical application. Targeting to tissues and cells can either be passive, through properties of cancer vasculature, or active, through receptor-specific ligands on the nanoparticle surface intended for cell binding. Intraarterial injection into disease-related arteries is a further variant of ‘targeted’ delivery.

Passive targeting

Passive targeting depends on the Enhanced Permeability and Retention effect (EPR), characterized by enhanced accumulation of nanoparticles within tumor tissues.[102–105] Fast growing tumor tissue is characterized by rapid angiogenesis triggered by tissue anoxia and expression of growth factors like the vascular endothelial growth factor (VEGF). Newly formed blood vessels show a higher rate of vascular fenestration and therefore, permeability, in different tumors.[106–108] Nanoparticles extravasate through the walls of such blood vessels and tend to show prolonged retention in the tumor tissue. Nanoparticle extravasation is size dependent.[109–111] In passive targeting strategies, a sufficiently long blood circulation time of the nanoparticles is important to allow a high contrast between tumor accumulation compared to that in liver, spleen and kidney, where particles tend to accumulate in the absence of a suited tissue target. This goal can be achieved through optimal stealth properties, typically by incorporation of polymers like PEG into the shell of the nanomaterial. In addition, premature release of the payload from the nanocarriers (e.g. exchange of hydrophobic drugs with albumin) is an important issue, which needs to be considered. A frequently used animal model of human cancer, the xenograft mouse transplantation model, shows significant differences in vascular biology compared to human cancers,[112] partly explaining why it is much easier to cure human cancer in mice than in humans. For this reason, the development of alternative models to study nanoparticle-tissue interaction before clinical application is desirable.

Active targeting

Active targeting was conceptually introduced in 1906 by Paul Ehrlich, who coined the term “magic bullet” by describing the need for a system able to target specific drug delivery within the body.[113–115] For active targeting, nanocarriers need to first arrive in the target organ; therefore initial design considerations are similar to those for passive targeting. Active targeting then exploits specific mechanisms that increase specific particle-target cell interaction through suited ligands. These ligands (either small molecules like folic acid and carbohydrates, or macromolecules including aptamers, antibodies, proteins, peptides or oligonucleotides) must be carefully chosen to exhibit maximal binding to the target while minimizing binding to

Table 1
Examples of nano-based platforms and their stages in clinical use.

Nanoplatform	Size range	Compound (Trade name)	Application	Target	Status	References
Liposome	<100 nm	Doxorubicin (Doxil/Caelyx)	Cancer therapy	Kaposi's sarcoma, ovarian cancer, breast cancer	FDA approved	In market
		Daunorubicin (DaunoXome)	Cancer therapy		FDA approved	In market
Dendrimer	1–10 nm	(VivaGel)	Microbicide	Cervicovaginal	Phase II	44
		Methotrexate	Cancer therapy	Several different cancers	In vitro/in vivo	142
Polymer	50–200 nm	Pegaspargase (Oncaspar)	Cancer therapy	Acute lymphoblastic leukemia	FDA approved	In market
		Doxorubicin	Cancer therapy	Breast/Lung cancer	Phase II	143
		Paclitaxel	Cancer therapy	Breast cancer	Phase II	144
Micelle	10–100 nm	Paclitaxel (Genexol-PM)	Cancer therapy	Breast cancer	Phase IV	No publication provided
		Paclitaxel (Taxol)	Cancer therapy	Psoriasis	Phase II	145
Carbon nanotubes	1–25 nm diameter		Cancer therapy	Lung cancer	Phase I	146
		Paclitaxel	Cancer therapy	Breast cancer	In vivo	147
Metallic nanoparticles	1–150 nm	Ferumoxides (Feridex)	MRI contrast agent	Liver	FDA approved	In market
		Iron-oxide (NanoTherm)	Cancer therapy	Glioblastoma	EU approved	98
Organic nanoparticles	20–400 nm		Cancer therapy	Liver cancer	Phase II	148
Quantum dots	1–10 nm	Doxorubicin	Cancer therapy	Ovarian cancer	In vivo	149
			Cancer therapy	Breast, prostate cancer	In vivo	150
Nanogels	<200 nm	NB-001	Anti-viral	Herpes labialis	Phase III	No publication provided
		MuGard	Mucositis	Head and neck	Phase IV	No publication provided
CPP	~30aa residue long	Azurin	Cancer therapy	Refractory solid tumors	Phase I	151
	Synthetic peptides	XG-102	Cancer therapy	c-Jun-N-terminal kinases	Phase II	101

healthy tissue.[111,116,117] Popular cancer targets include transferrin, folate[118,119] and epidermal growth factor receptors due to their over-expression in tumor tissues.[120,121] Targeted delivery requires highly specific target recognition and target binding with sufficient affinity, but as stealth properties imparted through polymers tend to compete with specific binding sites, careful titration of these effects, e.g. by optimizing the length of branches carrying a ligand and the number of ligands per nanocarriers is of key importance.[122]

Endocytotic cellular uptake

Once a delivery nanodevice has arrived in a target tissue, several barriers to optimize pharmaceutical activity remain. Cellular uptake is a first step: different routes to enter a cell exist, including the physiological uptake mechanisms like passive diffusion through the cell membrane, channel or transport protein mediated uptake, or various vesicular uptake mechanisms. The endocytotic pathway is the major uptake mechanism of cells for nutrients, but also for therapeutic DNA, siRNA and proteins. There are several distinct endocytotic pathways including clathrin-mediated endocytosis, phagocytosis, caveolar endocytosis and macropinocytosis.[123,124] Detailed information concerning these uptake pathways can be found in excellent reviews.[125–130] Various ligands used in active targeting actually bind to membrane proteins that are associated with endocytosis and thus trigger endocytosis upon receptor binding. Prominent examples are the folate receptor and the transferrin receptor. After cellular uptake, molecules internalized via the plasma membrane will follow the intracellular endocytotic pathway involving early endosomes (with potential receptor recycling to the cell surface) or may progress to late endosomes and lysosomes. If the payload has its cellular target in the nucleus, the nuclear membrane is a second barrier, which is difficult to pass. Endocytotic nanoparticle uptake can be overcome using liposomes as delivery platform or through surface modification using cell-penetrating peptides as discussed below.[123,131]

Cellular uptake by cell penetrating peptides

More than 20 years ago, Frankel and Pabo found that the trans-activating transcriptional activator (TAT) protein of HIV-1 is able to cross mammalian cell membranes and translocate into the nucleus.[132] A few years later, Prochiantz et al reported the same effect for the *Drosophila melanogaster* Antennapedia homeodomain.[133] It was found that a short sequence of 10–16 amino acids was responsible for translocation. Based on this discovery, numerous cell-penetrating peptides (CPPs) have been developed for potential delivery of various biomolecules such as oligonucleotides, DNA, RNA, proteins, peptides and drugs. CPPs are typically cationic or amphiphatic peptides of less than 30 amino acids showing lack of toxicity, and can be grouped into two major classes comprising covalent linkage and non-covalent complexation with cargo molecules.[131] Cationic CPPs are generally composed of positively charged amino acids as arginine, lysine and histidine, whereat amphiphatic CPPs are

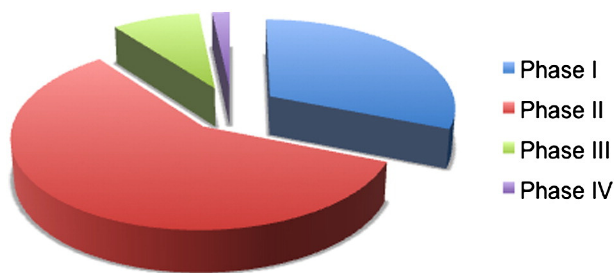

Figure 2. Illustration of nanoparticles in clinical trial phases as seen from www.clinicaltrials.gov, date 25.09.2012.

Table 2
Nanoplatforms in clinical trials.

Platform	Cancer	Global
Liposome	443	590
Polymer	32	204
Gold	171	1110
Silver	104	379

The numbers of ongoing clinical trials involving various nanoplatforms illustrating their application in cancer studies or global clinical application. Data adapted from www.clinicaltrials.gov and www.clinicaltrialsregister.eu (25.09.2012).

comprised out of lipophilic and hydrophilic parts.[134] Cellular uptake can occur through endocytotic (clathrin dependent, macropinocytosis, via caveola) or non-endocytotic pathways although mechanisms are not fully elucidated. Effective CPP-based delivery to target different diseases has been shown using covalently linked cargos such as Tat, transportan, penetratin, polyarginine or VP22.[135–138] Covalent linking the cargo to CPPs can be a limitation by altering the biological activity of the payload as seen by Juliano et al who examined CPPs conjugated to siRNA.[139] In contrast to a covalently bound payload, amphiphatic peptide carriers such as MPG and Pep-1 show successful in vivo and in vitro cargo delivery of siRNA, plasmid DNA or proteins without the need for chemical cross-linking.[140,141]

Endosomal escape

Many examples of endosomal escape by pathogens can be observed. In fact certain bacteria (e.g. listeria) or viruses are able to escape lysosomal degradation through specific interactions with the vesicle membrane in a surprisingly successful manner and are an example where human ingenuity has not yet been able to mimic the capability of biologic organisms.[152] Endosomal escape belongs to the key challenges still to overcome to enable clinical application of therapeutic delivery of nucleic acids to target cells. In endosomes, mild acidification detaches typical ligands from their receptors. The latter are recycled to the cell surface, while the vesicle content progresses to late endosomes and lysosomes. Various strategies have been explored to achieve endosomal escape. Behr et al have proposed a proton sponge effect in 1995, suggesting that specific agents become protonated at low pH in endosomes, leading to an influx of ions and water, causing swelling and rupture of the endosome with release of the payload. A prominent example for this effect is the use of polyethylenimine (PEI) for transfection of plasmid DNA or siRNA, which becomes protonated due to its amino groups at endosomal pH.[153,154] Another approach for endosomal escape is incorporation of fusogenic peptides into the endosomal membrane. These peptides are derived from viruses and show conformational changes upon changes in pH, which allow them to fuse with the endosomal membrane by inducing membrane perturbations. This has been well documented for haemagglutinin, an influenza virus derived fusogenic peptide.[155,156] A strategy called photochemical internalization has been described by Bert et al Payload release was achieved through incorporation of photosensitizers into the endosomal or lysosomal membrane. The membranes are disrupted through chemical action of singlet oxygen, which is formed upon light exposure of the photosensitizers.[157,158] Several photosensitizers as $TPPS_4$, $TPPS_{2a}$ or $AlPcS_{2a}$ have already been used to this end in different studies.[153,159–162]

Clinical application of nanomaterials

The clinical trial landscape: an emphasis on cancer

On Sep 25, 2012, there were 116 clinical trials registered at http://www.clinicaltrials.gov mentioning the keyword “nanoparticle”. Most of them are Phase I and Phase II trials in the status of ‘recruiting’ or ‘active and not recruiting’ (Figure 2). Within the last 10 years, the number of clinical trials has increased continuously and it is likely that this trend will continue in the future due to innovative research. Most nanomaterials that have entered clinical trial are currently devoid of switch functionality (Table 2). The majority of the mentioned trials are focusing on cancer treatment applying the cancer drug paclitaxel in a liposomal or nanoparticulate carrier, or its albumin bound formulation Abraxane®. According to world health organization (WHO) estimations, cancer is a leading cause of death around the world, accounting for 7.6 million deaths in 2008. Even though cancer treatment has evolved significantly within the last few decades, surgery, radiation and chemotherapy are still the main pillars for cancer therapy. While some spectacular successes have been achieved in specific malignant diseases (e.g. in certain leukemia types), most current chemotherapies for solid cancers are not curative and prolong life for a few months or years rather than for decades. Advances in cancer management with the goal of prolonging lifespan and eventually curing cancer depend on early cancer detection, prevention of recurrence and metastatic spread and effective management of established metastases. All factors together play a key role, and this is where novel nanomaterial-based strategies could have the highest impact.

Early stage cancer diagnosis is desirable to prevent metastasis, but remains challenging because clinical symptoms usually become manifest only in advanced cancer stages. Nanoparticles have specific features which render them interesting for imaging in cancer as well as for therapy of solid cancer because of the propensity of nanoscale materials to distribute preferentially to vessels with altered permeability (enhanced permeation and retention EPR effect) and the

Table 3
Clinically approved nanoparticle-based therapeutics and their benefits and risks.

Nanoplatform	Constitution	Trade name	Application	Target	Benefits through platform implementation	General possible risks regarding platform
Liposomes	Lipsomomal amphotericin B	Abelcet	Antifungal	Fungal infections	Decrease in toxicity by factor 20,[163] lowering of nephrotoxicity of 58%, reducing risk of mortality by 28%[164]	Hypersensitivity reactions to liposomal drugs emerging from complement activation-related pseudoallergies (CARPA) as seen for Doxil, AmBisome, DaunoXome, can lead to life-threatening reactions in first time treated patients especially with a cardiovascular disease background[165–167]
		AmBisome	Antifungal	Fungal and protozoal infections	Lowering of nephrotoxicity of 30%[163,168] and infusion related toxicity of 30-40%,[163,169] treatment success increase of 24%[170]	
		Amphotec	Antifungal	Fungal infections	Decrease of mortality of 10% and renal toxicity of 25%[171]	
		Amphocil	Antifungal	Fungal infections	Decrease in nephrotoxicity[172]	
	Liposomal daunorubicin	DaunoXome	Cancer therapy	HIV-related Kaposi's sarcoma, Leukaemia, Non-Hodgkin lymphoma	Increase of drug concentration at the site of KS lesions,[173] decrease in alopecia 30% and neuropathy 30% in Kaposi's sarcoma treatment,[174] escape multi drug resistance[175]	
	Liposomal doxorubicin	Myocet	Cancer therapy	Combinational therapy with cyclophosphamide in metastatic breast cancer	Decrease of cardiotoxicity of 16%,[176] total retention of activity[177]	
	Liposomal-PEG doxorubicin	Doxil, Caelyx	Cancer therapy	Metastatic breast and ovarian cancer, HIV-related Kaposi's sarcoma	Increase of circulation half-life of 74h vs. 10min, less frequent dosing schedule and reduction in cardiotoxicity of 20% for breast cancer[178]	
	Liposomal verteporfin	Visudyne	Neovascularization	Age-related macular degeneration, pathologic myopia, ocular histoplasmosis	First and only drug therapy approved for treatment of macular degeneration[99]	Skin toxicity in areas of vitilgo related to pegylated liposomal doxorubicin[179]
	Liposomal vincristine	Onco TCS	Cancer therapy	Non-Hodgkin's lymphoma	Reduction of neurotoxicity,[180] increase of circulation half-life of 72h vs. several minutes, less frequent dosing schedule[181]	
	Liposomal cytarabine	DepoCyt	Cancer therapy	Neoplastic meningitis and lymphomatous meningitis	Less frequent dosing schedule and delaying neurological progression[182]	
	Liposomal IRIV vaccine	Epaxal/Inflexal V	Anti-viral	Hepatitis A, Influenza	Achievement of 100% seroprotection in infants and children, increase in immunogenicity of 10%[174]	
	Liposomal morphine	DepoDur	Postsurgical analgesia	Treatment of postsurgical analgesia	Increase in analgesia for hip arthroplasty up to 48h[183]	
	Liposomal propofol	Diprivan	Anesthetic			

Polymers	PEGylated formations	Oncaspar	Cancer therapy	Acute lymphoblastic leukemia	Increase of circulating half-life, less frequent dosing schedule,[184] decrease of immunogenicity[185]	PEGylation is well tolerated and has not been associated with any adverse events regarding Pegasys, PegIntron, Neulasta, Somavert[203]
		Somavert	Cancer therapy	Acromegaly	Increase in half-life of clearance[187]	
		Neulasta	Cancer therapy	Cancer chemotherapy associated with neutropenia	Increase of serum half-life of 40h vs. 3h[188]	PEG can generate complement activation products in human serum, leading to possible infusion-related reactions[190]
		Adagen	Immunosuppression	Severe combined immune deficiency		
		Mircera	Anemia	Anemia treatment with associated chronic kidney disease	Increase in half-life, less frequent dosing schedule[191]	PEGylated proteins possibly trigger antibody formation against PEG, leading to accelerated clearance[186]
		Pegasys	Anti-viral	Hepatitis B, C	Longer half-life, reduced clearance[192]	
		Macugen	Macula treatment	Age-related macular degeneration	First available opthalmic anti-VEGF agent[195]	Detection of PEG antibodies of 22–25% in patients treated with PEG-asparaginase[193,194]
		Cimzia	Inflammation	Crohn's disease	Increase in half-life, less frequent dosing[196]	
	Glu-Ala-Tyr copolymer	Copaxone	Immunomodulator	Multiple sclerosis		
	Poly(alylamine hydrochloride)	Renagel		Chronic kidney disease	Reduction of 20-35% in mortality for elderly people, decrease in bone disease, vascular calcification[197]	
	Colesevelam hydrochloride	Welchol	Anti-diabetic	Type 2 diabetes		
Iron nanoparticlesik	SPIONS	Feridex	Imaging	Liver		Dextran-coated superparamagnetic iron oxide nanopartcles (SPIO) as Feridex show interactions with plasma proteins, which can lead to complement activation and further clearance by the liver or spleen[198,199]
		Gastromark	Imaging	Gastrointestinal		
		Lumirem	Imaging	Gastrointestinal		
		Resovist	Imaging	Liver		
	Iron oxide based	Venofer	Anemia	Chronic kidney disease		
		Cosmofer	Anemia			
		Ferrlecit	Anemia			
Other platforms	Albumin-bound paclitaxel	Abraxane	Cancer therapy	Metastatic breast cancer	Increase in overall response efficacy of 14%, reduction of risk for death by 28%, decrease of toxicity permitting administration of 50–70% higher doses[200,201]	
	Nanocrystalline	Emend	Antiemetic	Brain	Increase of bioavailability[202]	
		Tricor	Anti-hyperlipidemic	Vascular endothelium	Increase of bioavailability[202]	
		Triglide	Anti-hyperlipidemic	Vascular endothelium	Increase of bioavailability[207]	
		Rapamune	Immunosuppressant	Binding of mTOR complex1	Increase of bioavailability 21%[202]	
		Megace ES	Antianorectic	Pituitary	Increase of bioavailability[202]	
	Emulsions	Estrasorb	Menopause treatment	Estrogen receptor		
		Elestrin	Menopause treatment	Estrogen receptor		

additional possibility to apply active tissue targeting through targeting ligands or physical means. Conventional, small molecular chemotherapeutics often have a short circulation half-life due to rapid kidney filtration or liver metabolism, have a small therapeutic index limiting their dose and exhibit a relative lack of specificity for cancer tissue compared to other fast growing cells, which leads to the well known, undesirable side effects of cancer therapies. Many clinical cancer treatment protocols are dose-limited due to side effects and thus of suboptimal efficacy. The emergence of drug resistance is another challenge in conventional cancer chemotherapeutics. The combination of chemotherapy with nanoparticle based drug delivery systems has shown to improve tolerability and there is some evidence for improved efficacy in specific contexts, but challenges for clinical applications still remain.

Potential risk factors regarding approved nanoparticle based therapies

A number of nanoparticle-based therapies have been approved by the U.S. Food and Drug Administration (FDA) for clinical use, highlighting the benefit that can be achieved through nanomedicine strategies (Table 3). Although all mentioned approved drugs have fulfilled the safety requirements of the FDA, the European Medicines Agency (EMA) or other agencies, clinical observation has led to heightened awareness of potential side effects. In particular, possible interactions involving complement activation and antibody formation against PEG that may lead to hypersensitivity reactions or preterm drug clearance by the liver and kidney[186] are a concern, although PEGylation has been claimed to be safe in particular if applied to proteins.[203] The fact that such complement activation related pseudoallergic reactions (CARPA)[165,166,189,204,205] have been observed only in a minority of patients, but in various liposomal drugs including Doxil, AmBisome, DaunoXome and others, points to individual predisposition. This effect might be related to direct complement activation on the particle surface or to reactive antibodies against polymers such as PEG as triggers of complement activation, infusion reactions, and might also lead to altered particle clearance through opsonization. Garratty et al observed a 22–25% occurrence of PEG antibodies in 350 healthy blood donors[193] whereas in 1984 a value of 0.2% had been described.[206] It has been hypothesized that such an increase in the occurrence of PEG antibodies in the last 30 years could be due to a higher appearance of PEGylated compounds within pharmaceuticals, cosmetics and processed food products, as being suggested by Armstrong et al,[194] though major methodological differences preclude firm conclusions.

Conclusions and outlook

Medicine needs new solutions to achieve highly effective, side effect free, and cost efficient solutions for major diseases.[208] The emerging field of intelligent nanomaterials for medical diagnosis, therapy and their combination ‘theragnostics’ is based on a range of well studied carrier platforms, a number of targeting strategies each offering advantages and challenges and a “smart” payload. This combination has the goal of increasing specificity and efficacy in the diseased tissue, while abolishing toxicity largely. Although pioneering work has been done in oncology, both in preclinical development and clinical application, it is widely felt that the added capabilities of intelligent systems beyond “target cell killing” may render intelligent nanosystems a shaping force for a much broader range of diseases. To advance in such applications, a thorough know-how about clinical challenges, material properties, physicochemical properties, nano-bio interactions, toxicity, regulatory pathways and clinical trials results is required.

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Review

Designing *switchable nanosystems* for medical application

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A B S T R A C T

Within the last decade, nanotechnology has had a major impact on preclinical development in medicine, shaping the emerging scientific field of nanomedicine. Diverse nanomaterial platforms have been introduced as a carrier systems for the delivery of a variety of payloads (e.g. drugs, proteins, peptides, nucleic acids) but additional improvement by stimulus responsiveness would be of tremendous significance. The design of intelligent, stimuli responsive nanosystems promises to expand diagnostic and therapeutic options in medicine by making available an array of highly effective, well tolerated platforms that go beyond simple delivery of drugs or imaging agents. Controlled by internal triggers which may be characteristic for a disease or by external devices that permit tight spatiotemporal control of activity, enhancement of desired therapeutic effects and further suppression of side effects in remote organs may be possible. This review focuses on the toolbox of available internal and external switches suited for the integration into nanoscale carriers and on the clinical experience with stimuli-responsive nano-platforms. A substantial body of evidence shows that internal stimuli including pH, redox potential, enzymatic activity and temperature are suited to trigger nanosystems. For some such systems, clinical trials are in progress, but solid clinical proof of significant patient benefit will be required next. Externally controlled systems include electromagnetically-, temperature-, light-, radiation- and ultrasound triggered systems, and for certain clinical indications, such systems have already proven clinical benefit.

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1. Brief introduction

Nanomedical approaches are a major transforming factor in medical diagnosis and therapies. Based on important earlier work in the field of liposomal drug delivery and inorganic nanomaterials, the last decade

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has brought a broad array of new and improved nanoscale carrier platforms such as biodegradable and non-degradable polymers, dendrimers, carbon nanotubes, metallic and organic nanoparticles, quantum dots, nanogels or peptidic nanoparticles. These systems are not only suited to deliver drugs and imaging agents but also to carry advanced functionality including internal and external stimuli-responsiveness in a highly targeted fashion to a diseased area. Improvements in efficacy, safety, target specific non-viral drug delivery, cellular uptake, as well as intracellular stability and activity, are major reasons for the design of multifunctional, intelligent and switchable nanosystems. Such switchable nanosystems can be classified into pH-, redox potential-, enzymatic-, temperature-, light-, ultrasound-, mechano- and electromagnetic-/ radiation sensitive nanoparticles [1]. First insights in the variety of existing stimuli responsive nanosystems and their impact to therapy and diagnosis have been given in the last years [2,3]. With the aim to prepare the ground for a new generation of smart, highly active, well tolerated nanoscale therapeutic systems, this review considers advantages and disadvantages of nanoscale carrier platforms for further functionalization with nanosize switches. The focus is on the toolbox of internal and external stimuli that can be used to incorporate switch functionality into such nanocarriers and review the clinical experience with various nanosize carrier systems as a basis for the design of new, improved, functional and "intelligent" nanosystems for benefit to patients. As prior reviews on multifunctional and stimuli responsive nanocarriers have put their focus mainly on pH and temperature sensitivity, a further aim of this review is to include and compare a broad range of stimuli suited for switchable nanosystems.

2. Engineering stimulus responsiveness into nanomaterials

To enhance therapeutic efficacy while minimizing side effects, and to allow molecular imaging in patients, a large number of nanomaterial-based platforms have been developed that allow simple delivery of drugs or diagnostic agents. A next frontier is to achieve more complex functionality of nanoparticles through stimuli responsiveness. This will allow the exploitation of specific biologic properties of a diseased area for more specific, functional diagnosis to trigger a therapeutic response in an intelligent fashion. Switches at the nanoscale are expected to play a key role in progressing from "dumb" nanosize delivery vehicles to "intelligent" nanosystems, reacting at specific spatiotemporal locations. Switchable nanoparticles can be classified based on the type of stimulus as internally and externally controllable materials. Internal stimuli (e.g. activation by pH, redox potential, enzymes) might be controlled by a molecular mechanism highly specific for a disease and therefore excel in targeting properties. However, absolutely disease-specific internal molecular triggers are difficult to find for certain diseases. External stimuli like light, ultrasound, electromagnetic fields or ionizing radiation have the advantage of being focusable on certain body areas. This may be a significant advantage where a target cell is strongly involved in pathogenesis at one location (e.g., cancer stem cells in a cancer tissue), but of vital importance in other locations (e.g., stem cells in the bone marrow). A key challenge in externally controlled nanomaterials is tissue penetration and avoidance of undesired tissue damage in the radiation path from radiation source to target tissue. The ease of temporal control in external stimuli may represent a particular advantage for certain applications.

2.1. Internal stimuli

2.1.1. pH-switchable nanoparticles

Tumor and inflammatory tissues show differences in pH-levels compared to healthy tissue. In many tumors of a certain size, pH decreases from physiological conditions ($pH \approx 7.4$) to acidic levels ($pH \approx 6.5$) [4], although considerable differences even between healthy organs and between different tumor types exist [5]. An acidic pH can also be found in endosomal and lysosomal compartments of cells ($pH \approx 5–6$) used for the degradation of undesired internalized molecules. This lower pH can be utilized for the preparation of a potential switch for drug release or gene delivery from a pH sensitive nanoparticle [3,6–9]. Successful application has already been demonstrated in the mid 1990s by Geisert et al., using immunoliposomes for delivery of dyes, drugs, proteins and DNA [10]. A variety of polymeric pH-sensitive components with pH-cleavable bonds have been described to self-assemble to nanoparticles, permitting release of entrapped drugs at compartments with lowered pH [11]. Kim et al. have published an L-histidine based polymeric micelle system, which can be triggered by an early endosomal pH of 6.0 [12]. The advantage of this system is the effectiveness of high dose drug delivery into the cytoplasm with minimal drug loss [4]. Another approach for exploiting the acidic pH is activation of a payload enzyme upon arrival at the cellular target as published by Broz et al. by encapsulating a pH-sensitive enzyme and a water-soluble substrate with synthetic triblock copolymers [13]. To enable a controlled substance transport across the impermeable polymer membrane, the porin OmpF was integrated into the synthetic bilayer. OmpF is known to remain fully functional in acidic pH and allows passive diffusion of molecules smaller than 600 Da [14,15]. The pH-switchable functionality could be demonstrated upon variation of the surrounding pH, whereas the enzyme hydrolyzed the water-soluble substrate into a water-insoluble fluorescent molecule [13]. Further development of this system enabled the successful encapsulation of the pH-triggerable enzyme acid phosphatase in combination with glucose-1-phosphate and gadolinium chloride, which are being proceeded into insoluble gadolinium phosphate precipitates upon lower pH related enzyme activation, describing a novel gadolinium nanoparticle-based contrast agent for imaging [16]. Mok et al. have recently published a pH-sensitive siRNA nanovector for targeted gene silencing to C6 glioma cells. They created a nanoparticle with an iron oxide core, coated with polyethylenimine (PEI), siRNA and chlorotoxin. The covalently bound PEI is cleaved after cellular internalization by the acidic microenvironment but also enhances the biocompatibility of the nanoparticle at physiological conditions. Chlorotoxin reacts as a tumor-specific targeting ligand while effective gene silencing could be detected upon siRNA delivery [17]. Sethuraman et al. reported a cell-penetrating peptide (CPP) based delivery system that exploits the pH difference between inflammatory and healthy tissue [18]. The system consisted of two parts, a polymeric micelle forming a hydrophobic core with a TAT peptide sequence attached to it, enabling drug incorporation combined with an ultra pH-sensitive anionic block copolymer and shielding the positive charges of the TAT peptide. There was no binding or cellular uptake at physiological conditions, whereas de-shielding of the anionic copolymer at the tumor site in the slightly acidic microenvironment could be observed. Kale et al. used pH-sensitive TAT-modified pegylated liposomes to show transfection enhancement in tumor bearing mice, attaching PEG via a pH sensitive bond to the liposome. Detection of transfection efficacy has been done using plasmid DNA encoding for the green fluorescent protein (GFP) [19]. Up to now, pH-sensitivity has been the internal trigger that has attracted most attention [3].

2.1.2. Redox potential sensitive nanoparticles

Redox-sensitivity is another switch functionality of major potential impact in intelligent nanomaterials. In vivo, a large difference in redox potential by factor of 100–1000 between the oxidizing extracellular and reducing intracellular space has been reported [1,20]. The high intracellular redox potential is due to a high intracellular concentration of thiols, in particular glutathione (GSH), free cysteine and homocysteine. In an oxidative environment, thiol groups form disulfide bonds, which will be cleaved in a reductive environment. The formation and reduction of disulfide bonds in oxidized and reduced environments allows to the construction of nanoscale switches. Wang et al. have shown that such redox-responsive copolymer based nanoparticles can overcome multidrug resistance in cancer cells [4,21]. Multidrug resistance (MDR) is a major drawback within chemotherapy based

cancer treatment [5,21]. MDR is cancer cell phenotype characterized by over-expression of efflux pumps, which are actively removing chemotherapeutic drugs from the cytoplasm. Therefore, they designed redox-responsive nanoparticles based on disulfide-bond bridged copolymers PCL-SS-PEEP loaded with doxorubicin (DOX), showing a high internalization rate by cells. After cellular uptake, the disulfide-bridged copolymers were reductively cleaved and the nanoparticles disintegrated, leading to enhanced release of DOX and enhanced cytotoxicity to MDR cancer cells. Zhao et al. have shown successful protein delivery into several human cancer cell lines and release through redox-responsive nanoparticles [9]. They demonstrated effective encapsulation of caspase-3 (CP-3) with positively charged, disulfide containing cross-linker polymers followed by internalization into cancer cells. Subsequent release into the cytosol induced cancer cell apoptosis due to active CP-3 in HeLa, MCF-7 and U-87 MG cells. Mesoporous silica nanoparticles have been studied as a redox-sensitive drug delivery system, characterized by a high surface area, diversity in surface functionality, uniform and tunable pore size and biocompatibility [9,10,22–24]. Liu et al. used a polymeric cross-link network on the surface of mesoporous silica nanoparticles as a gatekeeper for controlled release of the payload [11,22]. After loading the silica nanoparticles with dye, the pores surrounded by the covalently linked polymers were blocked via addition of cystamine, forcing disulfide cross-linking of the polymers. The pores could be reopened by the reducing agent dithiothreitol (DTT), mirroring glutathione-based reductive cleavage in the cytosol. Kim et al. demonstrated a similar gate-keeping effect using mesoporous silica nanoparticles covalently linked through disulfide bridges with cyclodextrin instead of polymers and loaded with DOX [12,23]. Cyclodextrin blocked the pores and inhibited undesired DOX release. *In vitro* studies using A549 cancer cells showed reduced cancer cell survival after application of DOX loaded mesoporous silica nanoparticles with GSH induced drug release. Liposomes are also under investigation for redox-sensitive drug delivery [4,23,25–28]. In the year 2000, FDA approved the anti-CD33 antibody linked drug Mylotarg®, developed by Celltech Group and American Home Products and based on disulfide bond and acylhydrazone linkers, for the treatment of acute myelogenous leukemia [13,29]. As confirmatory trials failed to confirm a clinical patient benefit, the FDA decided in 2010 to take Mylotarg® from the market.

2.1.3. Enzyme responsive nanoparticles

Enzyme responsive systems exploit differential enzyme expression in a diseased area for site-specific drug release. Much effort has been put into investigation of enzymes secreted to the extracellular matrix such as matrix metalloproteases (MMPs) [14,15,30]. MMPs are endopeptidases containing zinc and are responsible for the remodeling of the extracellular matrix (ECM) by degradation of extracellular matrix proteins. Different MMPs are over-expressed in specific locations, e.g. the arteriosclerotic plaque or certain tumor tissues (lung, breast, skin, ovarian), where they degrade the ECM and appear to contribute to invasive tumor growth [13,31,32]. This differential expression and the relevance of the enzyme for the disease process render MMPs highly interesting as triggers for intelligent nanomaterials. Garipelli et al. have synthesized MMP-sensitive thermogel polymers by incorporation of MMP2-sensitive peptides into amphiphilic co-polymers. Upon *in vivo* injection, the polymers form a gel structure due to their thermosensitivity, which can act as drug reservoir for hydrophobic cancer drugs like paclitaxel. Degradation of the polymer due to cleavage of the MMP2-sensitive peptide with subsequent release of paclitaxel could be shown by addition of MMP2 [17,33]. Singh et al. published bovine serum albumin (BSA) based nanoparticles with MMP-2 sensitive peptide coating. They investigated two different peptides, revealing differential sensitivity to MMP-2 cleavage, and allowing regulation of drug release. Similar results were also published by Elegbede et al., using liposomes and MMP-9 instead of polymers and MMP-2, with drug release triggered by cleavage of the lipopeptide [18,34]. Over-expressed MMPs in a tumor can be exploited for molecular imaging. Jiang et al. introduced activatable cell-penetrating peptides (ACPPs) incorporating an inhibitory domain with negatively charged residues, which disables cellular translocation of the ACPPs. Upon arrival at sites of MMP over-expression, cleavage of the linker by proteases allows the CPPs to regain their capacity to bind and enter cells [3,35]. This allows an effective imaging of MMP activity within different cancer types as shown by Olson et al. [36]. Park et al. reported the synthesis of silica nanoparticles modified with cyclodextrin as gatekeeper molecule responding to α-amylase and lipase, as both enzymes are related to acute pancreatitis. Silica nanoparticles were loaded with calcein, a fluorescent dye, which could be detected upon cleavage of the gatekeeper by the enzymes [37]. Bernardos et al. used lactose and starch as gatekeeping molecules, synthesized on silica nanoparticles and demonstrated selective cleavage by β-D-galactosidase with proof of cellular uptake by HeLa cancer cells [38,39]. Patel et al. reported the synthesis of ‘snap-top’ silica nanocontainers blocked by α-cyclodextrin, where enzymatic activation could be achieved by addition of liver esterase resulting in release of the cargo molecules [40]. Another approach using enzyme responsive hydrogels was published by Thornton et al. [41]. Hydrogel particles were functionalized with peptide-based actuators composed of enzyme cleavable sites and positive charged amino acids able to entrap proteins. Addition of thermolysin resulted in cleavage of the peptide and therefore release of anionic fragments, rendering the residual hydrogel particles positively charged, which induces particle swelling and drug release. Exploiting over-expressed enzymes in cancer tissues for drug release is thus a promising option awaiting successful clinical application. A step beyond exploitation of native local enzymes is the design of artificial, receptor targeted nanosize organelles as reported by [42] that deliver an enzymatic pathway to a target cell, thus expanding the metabolic repertoire of the target cell and, by choosing suited substrate molecules, allow to exploit such “designer pathways” to run a pharmacologic or biologic reaction of choice at a target.

2.2. Internal/external stimuli

2.2.1. Temperature sensitive nanoparticles

A spontaneous temperature difference between diseased and healthy tissue is a hallmark of various diseases, and producing local hyperthermia or hypothermia by physical means as a therapeutic strategy is widely utilized in trauma, inflammation and other diseases. Nanoscale triggers sensitive to spontaneous and induced local temperature change are therefore of interest. Temperature-sensitive polymers exhibit a phase transition at a certain temperature. Polymers which become soluble upon heating have an upper critical solution temperature (UCST) whereas those who turn insoluble upon heating, have a lower critical solution temperature (LCST) [43]. LCST polymers exist in an extended conformation below the phase transition temperature and turn into a collapsed state above LCST. This feature has been used in tumor research. Certain tumor cells are more sensitive to heat-induced damage than normal cells, rendering a combination of tumor hyperthermia and heat responsive nanomaterials promising [3]. Liposomes and dendrimers have been rendered temperature sensitive by incorporation of LCST polymers. Upon an increase in temperature above the LCST, the polymers collapse, the liposomal membrane bursts and a drug can be released [11,44]. A well-known representative of this class is poly(N-isopropylacrylamide) (PNIPAM) [45]. Wei et al. have synthesized a star-block copolymer based on PMMA and PNIPAAM for controlled drug delivery. PNIPAAM is highly soluble in water at temperatures below about 32 °C, while it becomes dehydrated and therefore insoluble in water beyond that temperature [46]. Lee et al. have recently shown cytosolic delivery of siRNA followed by gene silencing through a temperature sensitive cationic polymer nanoparticle [47]. They used pluronic poly(ethylenimine), which exhibits a thermo-sensitive and reversible volume phase transition in a temperature range of 20–37 °C [48]. At 37 °C, pluronic/PEI2K nanoparticles were in a collapsed state

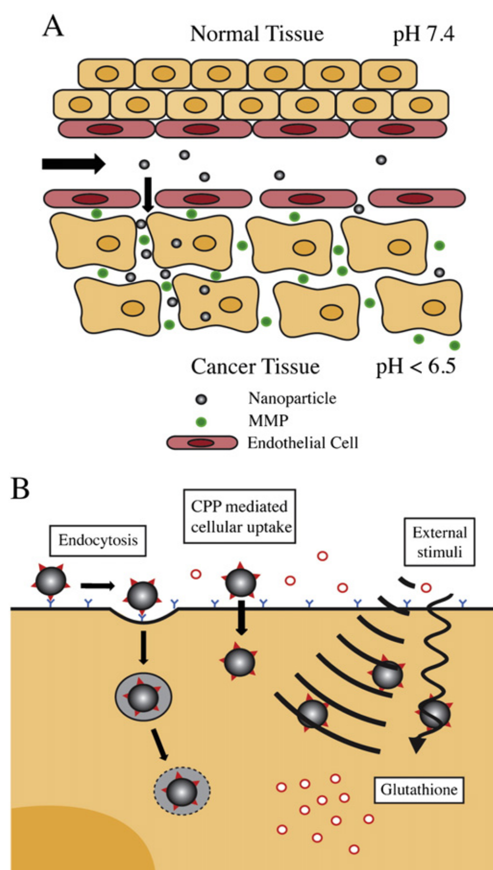

Fig. 1. A) Representative illustration, showing perfusion of normal and cancerous tissue. Nanoparticles predominantly permeate through the vascular endothelium within cancerous tissue due to larger fenestration. Cancer tissue showing decrease of pH and overexpression of MMPs (green) compared to healthy tissue, which can be used as internal stimuli for responsive nanoparticles (gray). B) Schematic overview of a cancer cell, presenting internal (glutathione) and external stimuli (e.g. magnetic field, ultrasound, light, radiation) used for imaging, drug release and therapeutical treatment. Targeted cellular uptake can be achieved via endocytosis and non-endocytotic cellular uptake through cell penetrating peptides (CPPs).

with an average diameter of 120 nm compared to a diameter of 350 nm at room temperature. After uptake via endocytosis, a short cold shock induced disruption of the endosome (150–200 nm) due to an abrupt swelling of the nanoparticles, with siRNA delivered into the cytoplasm leading to effective gene silencing. Temperature sensitive liposomes (TSLs) can release their payload near their phase transition temperature (Tm), where the lipid membrane shows a transition from a gel to a liquid crystalline phase [49,50]. Several studies have shown delivery of doxorubicin by thermosensitive liposomes triggered by induced tumor hyperthermia [51,52]. DPPC/MSPC/DSPE-PEG2000, a so-called low-temperature-sensitive liposome (LTLS) is currently undergoing clinical phase III trials for liver cancer and phase II for breast cancer [53] (Fig. 1).

2.3. External stimuli

2.3.1. Light switchable nanoparticles

Light is an attractive form of energy for triggering switchable drug delivery systems. Therefore it is not surprising that major efforts have been invested into the development of light responsive nanoparticles, including polymers, liposomes and dendrimers as drug delivery systems [54–58]. Light-responsive materials can be built through covalent incorporation of specific light-sensitive chemical groups with the aim to release a payload by illumination [59]. Cabane et al. have reported the synthesis of a photocleavable amphiphilic block copolymer. They used an o-nitrobenzyl linker as a photosensitive molecule, which they incorporated between the hydrophobic and hydrophilic blocks. Through self-assembly of the copolymers in buffer media, the formation of vesicles and micelles could be achieved. Rapid structural changes in size of the vesicles were observed in electron microscopy and dynamic light scattering after irradiation with UV-light, demonstrating successful photocleavage [60]. UV damage to the surrounding tissue is a major limitation to broad clinical application of this chemistry, however.

In photodynamic therapy, activation of photosensitizers via light generates highly reactive singlet oxygen radicals, which are known to have damaging effects on biological macromolecules such as membrane lipids and proteins [61]. Built into a nanocarrier system, such nanoscale photodynamic approaches have found their way into clinical applications such as photodynamic eye therapy for treatment of neovascularization or abnormal endothelial proliferation [62]. Photodynamic therapy using photosensitizers can also be used for cancer treatment due to its cytotoxic effects and direct cellular damage [63]. Tissue penetration of light is the limiting factor, which has to overcome for successful drug release in the depth of a tissue. Near-infrared (NIR) light at wavelengths of 700 to 1000 nm can penetrate up to several centimeters deep into tissue without causing any damage [64,65]. This renders NIR much more attractive than UV-light with its potential for severe tissue damage [57]. Babin et al. published a two-photon-sensitive block copolymer, incorporating a coumarin chromophore, which absorbs NIR at 794 nm. They used Nile red, a hydrophobic dye, to detect micelle formation and disruption by NIR irradiation by analysis of the changes in the fluorescence spectra [56]. Sun et al. reported a two-photon-sensitive, carbohydrate targeted dendritic nanocarrier for NIR-triggered doxorubicin release. An NIR-induced DOX release proportional to irradiation time proved their concept. Carbohydrate coating of the dendritic micelles allows for specific carbohydrate–target receptor interaction of such phototriggered nanosystems [57]. Parak et al. showed an effective NIR light triggered delivery of macromolecules such as proteins into the cytosol [66]. Nucleic acids (DNA, siRNA) are an attractive payload for light-triggered release, and can be achieved by caging [67]. Caging involves incorporation of a photolabile group into a molecule of interest, which inhibits its biological function. Mikat et al. caged siRNA such that it became inactive and could be successfully reactivated upon irradiation with UV light. They introduced a photolabile group into a single nucleotide, located in the central part of an siRNA, leading to a bulge in the double helix with disruption of RNA interference [68]. Several photolabile groups have been used to cage biomolecules since caging of ATP by Kaplan was first reported in 1978 [25,69]. An alternative form of controlling gene silencing through caging of DNAzymes was demonstrated by Young et al. DNAzymes are enzymatical active desoxyoligonucleotides capable for site-specific cleavage of RNA. The caging group inhibits hybridization of DNAzymes with mRNA, permitting mRNA translation by the cell [70]. Yamaguchi et al. reported caging of DNA with a biotinylated photolabile protection group for light activated gene expression [71]. Caged plasmid DNA has been reported by several groups, but inducing effective gene expression by a low dose of light remained a major challenge [72,73]. This could be explained by the caging method used which inserts a photolabile protecting group at random positions in the plasmid DNA phosphate backbone, resulting in various differently caged plasmid DNAs; another detrimental factor is the high level of light required for activation of gene expression, which also can cause phototoxicity [74]. By site-specific labeling of the promoter region, using a biotinylated photolabile group, Yamaguchi et al. could show a more effective activation of gene expression in HeLa cells by a low dose of light, rendering this method a more promising candidate for light induced gene expression within living cells [71].

2.3.2. Ultrasound sensitive nanoparticles

Ultrasound has a number of attractive features as a trigger for drug delivery. It can penetrate deep into the body, is non-invasive and can

be applied in a precise and focused controlled manner with millimeter precision [75]. Energy-based tumor treatment requires tumor imaging, often performed by MR or CT before treatment. Ultrasound allows combination of dual-modality imaging and therapy using high intensity focused ultrasound (HIFU) [76–78]. Two main physical mechanisms arise from focused ultrasound: heat and pressure. By applying long pulsed signals with high energy, the temperature can be raised in order to release an encapsulated drug in addition to the pressure effects. Using shorter pulsed signals with lower energy, pressure-mediated release without hyperthermia can be achieved [79]. Ultrasound induces partial drug release from micelles as shown by Rapoport and Husseini et al. using a triblock ABA-type polymer encapsulating Doxorubicin [80,81]. In 1968, Gramiak and Shah pioneered the field of ultrasonic microbubble imaging by documenting improvement of aortic wall delineation through injection of saline containing tiny air bubbles [82]. Currently, microbubble-based contrast agents are made from polymers or phospholipids and filled with inert gas. Microbubbles are in the micro- rather than in the nano range, as the stable incorporation of gas into smaller objects is challenging; the gas content is important for function because it is the cause of strong nonlinear resonance effects required for imaging and ultrasound-mediated destruction. The delivery of drugs and gene delivery by such microbubbles is an active field [79,83–86]. Compared with other drug delivery systems, therapeutic ultrasound has the advantage of potentially delivering payload into the cytosol without the endocytosis pathway due to pore formation in the cell membrane by oscillating or disrupting microbubbles [87]. Takashi et al. reported successful transfer of therapeutic genes into the spinal nerve system using ultrasound and microbubbles [88]. Desphande showed a 200-fold efficiency increase in DNA transfection by combining ultrasound and PEI [89]. Ultrasound has also been used for delivery of siRNA. Negishi et al. reported the development of bubble liposomes entrapping siRNA. Efficiency could be proven by down-regulation of luciferase activity [90]. Otani et al. have published the delivery of siRNA into mesenchymal stem cells, indicating that this technique could be a useful strategy for genetically modifying stem cells for therapy [91]. Using the systems described above, it would be possible to specifically deliver drugs, genes, therapeutic proteins and diagnostic agents into deep sites for example heart or pancreas, which will be exposed to ultrasound [92,93].

2.3.3. Electromagnetically sensitive nanoparticles

As electromagnetism has been a valuable biomedical principle for imaging and energy transmission, magnetic nanoparticles have quickly raised the interest as diagnostic and therapeutic agents. Magnetic nanoparticles are widely used in drug delivery, cell labeling, magnetic resonance imaging, cell separation, magnetic hyperthermia and as magnetic sensors for metabolites [94–101]. Magnetic nanoparticles are typically made from iron oxides with diameters of 1 to 100 nm. The two main forms for iron oxide particles have a magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3) core, which is surrounded by a biocompatible material [102]. Due to their relatively low toxicity, such superparamagnetic iron oxide nanoparticles (SPIONs) have attracted significant attention. “Naked” SPION's are not stable at physiological conditions but aggregate quickly. Therefore, a hydrophilic, biocompatible coating is necessary for biomedical application [11]. Usage of PEI polymer to coat SPIONs can increase the efficiency of gene delivery and can also protect DNA from further degradation by nucleases [103]. Al-Deen et al. have reported *in vitro* data about the usage of PEI complexed, superparamagnetic nanoparticles for the triggered delivery of malaria DNA vaccine. Application of an external magnetic field resulted in major improvement of the transfection efficiency [104]. For magnetically targeted therapy, magnetic nanoparticles are injected into the bloodstream and are then concentrated at the target, through application of a magnetic field, although deposition of particles at non-targeted sites still must be taken into account [105]. Critical parameters for targeting include field strength, magnetic properties of the particle, blood flow rate or tissue depth [95]. Pouponneau et al. proposed the concept of magnetic resonance navigation (MRN), which enables tracking and targeting of deep tissue within a weak magnetic field, using an upgraded magnetic resonance imaging scanner [106,107]. Recently they reported the development of biodegradable microcarriers, loaded with magnetic nanoparticles and doxorubicin, for *in vivo* treatment of hepatocellular carcinoma with control of the microcarriers to a depth of 4 cm below the skin [105]. Cancer treatment can be achieved via induced hyperthermia. To this purpose, magnetic nanoparticles have to be dispersed within the target tissue. Through the application of a magnetic field of sufficient strength and frequency, the particles can be heated to the therapeutic threshold of 42 °C for cancer destruction [108,109]. The advantage of magnetic hyperthermia treatment is that healthy tissue will not be affected but only the designated tissue is heated [104]. The first clinical applications have been realized by the company MagForce, using their iron oxide based NanoTherm® nanoparticles for glioblastoma. A number of diagnostic platforms based on nanoparticles with magnetic or superparamagnetic properties have been developed for biomolecule measurements to enable early detection of different diseases. Magnetic nanoparticles coupled to affinity ligands can function as sensitive biosensors, also known as magnetic relaxation switches [110]. Superparamagnetic nanoparticles, conjugated with a specific ligand such as nucleic acids, peptides, small molecules, proteins or antibodies, form stable nano-assemblies upon their interaction, which leads to a corresponding decrease in the spin–spin relaxation time of neighboring water molecules [111–113]. This feature has been recently used with aptamer-functionalized gold-coated iron oxide nanoparticles, for detection of thrombin by Liang et al.: gold-coated iron oxide nanoparticles with conjugated aptamers corresponding to human α-thrombin were synthesized. Through addition of a solution containing human α-thrombin, the nanoparticles switched from a dispersed into an aggregated state, leading in a change in the spin–spin relaxation time as well as the UV–vis absorption spectra. Therefore it was possible for the first time to develop a magnetic relaxation and colorimetric switch [110]. Alexiou et al. showed effective treatment of squamous cell carcinoma in rabbits by injection of magnetic particles (ferrofluids) bound to mitoxantrone with no signs of toxicity [114].

2.3.4. Radiation sensitive nanoparticles

Neutron Capture Therapy (NCT) is another interesting approach for stimulus responsive nanoparticles, which can be used for enhanced cancer therapy. The concept of NCT for cancer treatment was first introduced in 1936 by Locher [115]. In NCT, the target is irradiated with neutrons, which are then captured by atoms characterized by a large absorption of neutrons, measured by the neutron cross-section. Typically used high cross-section materials are ^{10}B with 3830 barn, ^{155}Gd with 55000 barn and ^{157}Gd with 255000 barn (1 barn = 10^{-24} cm^2) [116]. Neutron energy ranges typically used for NCT are thermal (≈0.025 eV) or epithermal (1.0 eV–10 keV). Using thermal neutrons just allows low tissue penetration (about 1.5 cm) whereas epithermal neutrons show deeper penetration depth (about 3–4 cm). Epithermal neutrons are slowed down during tissue penetration to thermal energies, allowing them to be captured by the boron or gadolinium neutrons [117]. Irradiation of ^{10}B with thermal neutrons yields formation of lithium-7 ions (^7Li) and helium-4 nuclei or alpha particles (^4He), which have a high energy of 2.31 MeV and can kill cells through various mechanisms. The ions path lengths are roughly 10 μm, which is comparable with the diameter of one cell, therefore destroying only ^{10}B containing cells without causing any damage to normal cells [116,118,119]. Successful treatment of cancer requires about 20–30 μg of ^{10}B per g of tissue or 10^9 atoms/cell [89,120]. In addition, irradiation of gadolinium with thermal neutrons leads to generation of gamma rays up to 7.8 MeV and Auger electrons in the energy range of 41 keV. Gamma rays can travel several centimeters through the tissue, therefore destroying tissue in a less focused area, whereas low energy Auger electrons only have path lengths of several nanometer, which is

sufficient to impart significant DNA damage when the emitting nucleus is in close proximity to the DNA helix [90,121]. Up to now, most research has been done in the field of boron neutron capture therapy (BNCT), but targeting of boron labeled drugs into cancer tissue in sufficient concentration still is a challenge with the use of current BNCT agents used clinically, *p*-boronophenylalanine (BPA) and disodium-mercaptoundecahydrododecaborate (BSH) [91,122–124]. Nanosize delivery systems have been introduced to neutron capture therapy as potential drug delivery system to increase target concentration of the BNCT agents, using liposomes as drug delivery systems functionalized with antibodies, folate, transferrin or epidermal growth factor (EGF) as ligands [92,93,125–130]. Yanagie et al. used liposomes as drug delivery systems for BNCT. They conjugated a murine monoclonal antibody with liposomes, forming immunoliposomes which could deliver high amounts of ^{10}B to the tumor cells [92,94–101]. Shirakawa et al. have synthesized a novel liposome containing BPA-peptide conjugate able to increases the number of boron up to 12 or 15, therefore overcoming the need of high boron concentration for successful BNCT [131]. Ueno and his group have developed new vehicles for boron delivery based upon dodecaborate lipid liposomes [132]. The unique feature within these vesicles is that their shell itself possesses cytocidal potential in combination with neutron irradiation. Hawthorne et al. described encapsulation of polyhedral boron compounds by liposomes made from distearoylphosphatidylcholine (DSPC) and cholesterol [133]. Dendrimers can also be used as boron delivery system as reported by Shukla and Backer et al. [134,135]. Gadolinium could combine diagnostic (MRI) and therapeutic properties (NCT) in a single material. Watanabe et al. investigated the biodistribution of Gd lipid nanoemulsion in tumor tissue by intravenous injection as an alternative application to intraperitoneal injection [136]. Oyewumi et al. compared the cell uptake, biodistribution and tumor retention of folate- and PEG-coated gadolinium nanoparticles [137]. Significantly enhanced cellular uptake and tumor retention was shown in the case of folate–coated nanoparticels over PEG-coating. Encapsulation of a gadolinium compound into liposomes and further addition into a thermo-sensitive polymeric gel was reported by Le et al. [138]. This system showed extended retention of the Gd compound in tumors, rendering it a potential system for transport of cytotoxic chemotherapeutic agents.

3. Challenges for switches at the nanoscale in medicine

A key challenge for switches at the nanoscale is complexity [163]. Added complexity requires more sophisticated engineering, adds new pitfalls for behavior prediction and probably most important, tends to increase costs in manufacturing. These downsides must be counterbalanced by a significant improvement in efficacy and safety to render more complex systems viable in clinical application. Cancer therapy with switchable nanosystems will serve as a test case in this regard: Even though cancer treatment has evolved significantly within the last few decades, surgery, radiation as well as chemotherapy still are the main pillars of cancer therapy. While some spectacular successes have been achieved in specific malignant diseases (e.g. in certain leukemia types), most chemotherapies for solid cancer are not curative and prolong life for a few months up to a few years rather than for decades. Advancement in cancer management with the goal of prolonging lifespan and eventually cure of cancer depends on early cancer detection, prevention of recurrence and metastatic spread and effective management of established metastases. All factors together play a key role, and this is where novel nanomaterial-based strategies could have the highest impact.

3.1. Challenges for internal stimuli response

Functionalization of nanoparticles for response to *internal* stimuli exploit biologic characteristics of the tumor cell or the tumor-specific microenvironment to improve specificity and efficacy, and at the same time to reduce side effects on bystander tissues. Tissue acidosis reflected in differential pH values in tumor tissues, presence of high amounts of glutathione in the cytosol and over-expression of matrix metalloproteases are internal mechanisms which can be used for targeting and drug release. The feasibility of pH switchability within tumor tissue has been shown in principle, but at the same time has exposed problems with this approach. The application of pH-sensitive nanoparticles as drug delivery systems profits from active targeting whereby receptor mediated endocytosis can be achieved. In contrast, passively targeted long circulating PEGylated drugs are associated with only low intracellular uptake [121–124,164,165]. A combination of PEGylation, pH responsiveness and active targeting may thus be a preferred approach. The over-expression of matrix metalloproteases into the tumor microenvironment may provide an opportunity for PEGylated nanoparticles to have their stealth providing surface groups removed in the extracellular matrix, allowing further cellular uptake. When exploiting the gradient in redox potential between the exterior and interior environment of cells is chosen, the presence of low concentrations of reducing agents as GSH and cysteine in the blood stream needs to be taken into account, which may lead to premature reduction of disulfide bonds and loss of the desired effect [92,125–130,166]. Internal triggers in complex biological systems like an entire organism are inherently difficult to control. Temperature sensitive nanoparticles combine multiple external and internal control properties, including hyperthermia associated with inflammation, local application of ultrasound and electromagnetic fields and are therefore of particular promise.

3.2. Challenges for external stimuli response

Switchable nanomaterials responsive to external stimuli can be controlled and regulated by an external control apparatus, allowing a wider choice of parameters. The ‘control’ does neither require nanosize scale nor introduction in the body and therefore offers more freedom in design. The advantages of light as external trigger are availability (although the protection of the patient from excess ambient light for a certain period may be a downside) and the “biocompatibility” of light, in particular for wavelengths longer than ultraviolet. The major drawback of light is tissue penetration depth, which severely restricts the applications of caged compounds and therapies based on nano-photodynamic therapies. The use of near infrared light and the exploitation of two-photon effects and upconverting schemes is a solution, albeit with significant challenges to today's science and engineering. Most published data using NIR use irradiation times in the range of hours, therefore complicating practical application in trials and clinical practice. Ultrasound has deeper penetration depth, but tissue damage in high intensity ultrasound applications does occur and requires particular care in design of materials with optimal ultrasound sensitivity to allow reduction of transmitted energy. Electromagnetically and magnetically controllable nanosystems require ingenious designs, in particular if the field needs to be focused in deep tissue or if the field is used to control the trajectory of the materials. Neutron capture depends on a strong source of neutrons within a defined range, which are only available in a few locations. Chemical elements present in normal tissue, like nitrogen and hydrogen, have a lower neutron capture cross section, but are present in extremely high concentrations. Therefore achieving a sufficient contrast between tissue and neutron capturing nanomaterials is often difficult (Table 1).

4. Conclusions and outlook

Nanomedicine, the emerging field of applying nanoscience methods, tools and materials to the benefit of human health, has evolved into a significant driver of medical progress, in particular in the area of drug delivery. Different platforms have been established to overcome major

Table 1
Illustrative overview of stimuli responsive nanoparticles and their current clinical state.

Application	Stimuli	Origin	Nanocarrier	Target	Drug/Trade name	Status	Ref.
Internal	pH	Decreased pH in tumor and inflammatory tissue, endosomal and lysosomal compartments	Liposome	MCF-7, A-549 cells	Docetaxel	In vivo	[139]
			Liposome	K562 cells	Mastoporan	In vitro	[140]
			Micelle	Ovarian cancer	Doxorubicin	In vitro	[12]
			Iron nanoparticle	Glioma cells	siRNA	In vitro	[17]
			CPP	Breast cancer	no available data	In vitro	[18]
			Polymer	Breast cancer	Paclitaxel	In vivo	[141]
			Polymer	Murine colon cancer	Doxorubicin	In vivo	[142]
Internal	Redox-potential	Presence of different thiol based compounds such as glutathione, free cysteine and homocysteine in the intracellular lumen compared to its extracellular concentration	Antibody–drug conjugate	Multiple myeloma	Maytansine	Phase I	[143]
			Antibody–drug conjugate	Breast cancer	Trastuzumab-DM1	Phase II/III	[144]
			Cationic polymer	Different cancer cell lines	Doxorubicin	In vitro	[145]
			Copolymer	J7741A-1	no available data	In vitro	[146]
			Polymer	A-549, MCF-7, Hela cells	Paclitaxel	In vitro	[147]
			Dendrimer	BV-2 microglial cells	N-Acetyl-l-cysteine	In vitro	[148]
Internal	Enzyme-responsive	Tumor associated over-expressed enzymes	Liposome	MT1-MMP expr. cell lines	Docorubicin	In vivo	[149]
			Polymer	HT1080 cells	Paclitaxel	In vitro	[33]
			Polymer	Glioma cells	Adriamycin	In vitro	[150]
			Micelle	Lewis lung carcinoma	Doxorubicin	In vivo	[151]
Internal/ External	Temperature	Internal through pathological induced hyperthermia	Liposome	Liver/Breast cancer	Doxorubicin	Phase II/III	[53]
			Liposome	Squamos cell carcinoma	Doxorubicin	In vivo	[51]
			Liposome	B16F10 cells	Doxorubicin	In vitro	[152]
		External through locally applied ultrasound or high frequency causing oscillation and further heat release	Liposome	Liver cancer	ThermoDox	Phase III	[131]
			Liposome	Murine adenocarcinoma	Doxorubicin	In vivo	[153]
External	Light	UV, two-photon laser, NIR	Liposome	Neovascularization	Visudyne	FDA approved	In market
			Liposome	Gastric cancer	Photofrin	In vivo	[154]
External	Ultrasound	High or low pulsed ultrasound signals	Micelles	Breast cancer	Doxorubicin	In vitro/in vivo	[76]
			Micelles	HL-60, MCF-7 cells	Doxorubicin	In vitro	[155]
			Liposome	Mouse ascites tumor cells	Plasmid DNA	In vivo	[156]
			Liposome	Lymph cancer	Cisplatin	In vivo	[157]
External	Electro-magnetic	Magnetic field application	Ferrofluid	Glioblastoma	NanoTherm	EU approved	[158]
			Iron nanoparticle	Hepatocellular carcinoma	Doxorubicin	Phase I/II	[159]
			Iron nanoparticle	Lewis lung carcinoma	Doxorubicin	In vivo	[160]
External	Radiation	Neutron capture therapy	Boronphenylalanine	Glioblastoma	Boron	Phase I/II	[161]
			Liposome	Colon cancer	Boron	In vivo	[132]
			Polymer	Neoplastic tissue in the rat jejenum	Boron	In vivo	[162]

bottlenecks such as biocompatibility, stealth properties, specific targeting and controlled drug release, where a number of products have found their way into the market. However, the progress in drug delivery systems for specific clinical applications is still a challenging task. One important avenue for the future evolution of nanomaterials is the development of stimuli responsiveness and of multi-functional nanosystems that permit progressive optimization of diagnosis and therapy and can be adapted to the needs of individual patients. Such nanosystems are expected to be a key enabling technology for personalized medicine [167].

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Review

Why not just switch on the light?: light and its versatile applications in the field of nanomedicine

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Abstract

Over the last decade, the emerging field of nanomedicine has undergone rapid progresses. Different internal and external stimuli like pH, temperature, radiation, ultrasound or light have been introduced to expand the diagnostic and therapeutic options of various applications within the field. This review focuses on the novel application of light in the field of nanomedicine as a mechanism to control drug delivery, release and biochemical and genetic functionality at the target. The field of functional nanomaterials for medicine, and in particular of light responsive nanocarriers, polymers and biomolecules offer new therapeutic options but also requires substantial further research to render this approach broadly applicable in clinical practice.

Keywords: light; nanoparticles; photodynamic therapy; photothermal therapy.

Introduction

A variety of new nanomaterials such as polymers, liposomes, micelles, dendrimers or metallic nanoparticles have shaped the constant and rapid progressing field of nanomedicine within the last decade. Today, nanoparticles have found their way into the clinical domain as drug delivery systems, for imaging, sensing and therapy. To provide specific characteristics, nanoparticles can be tailored into "intelligent" nanoparticles through stimulus responsiveness. Stimuli responsive nanoparticle for medicine can be classified based on the type of stimuli into locally/internal triggered systems responding to their close environment, and externally triggered stimuli-responsive nanoparticles that can be remote-controlled even from outside the body. Various internal stimuli such as pH, redox potential, enzymatic activity, temperature and external stimuli like ultrasound, magnetic field, temperature and light are being intensively investigated. Out of all these stimuli, light shows particularly attractive features such as high sensitivity, ease of controllability and a range of physical properties (e.g., light intensity, wavelength, exposure time) that allow in principle to design selective and multiplexed activities to be programmed into a material. Therefore, it is not surprising that a significant effort is currently invested into the development of light responsive nanoparticles, oligonucleotides or peptides.

This review presents a brief overview on light and its applications within the field of nanomedicine. It will describe mechanisms of light-controlled drug delivery, controlled drug release, light-controlled activity switching for biochemical mechanisms, gen expression and gene silencing at the target. The aim of this paper is to identify opportunities, describe gaps, and thus to stimulate further research, such that light- controlled nanomedical therapies develop into well tolerated, highly effective interventions to the benefit of the patient.

Application of light in nanomedicine

Light for triggered release and activation of drugs and biomolecules

Despite the efforts in drug delivery design and developments, major obstacles such as endosomal escape and efficient payload release within the diseased tissue and cell have to be overcome for efficient clinical application. Light can be used to enhance drug delivery and payload release by applying light sensitive moieties to drug delivery platforms and of photolabile protecting groups to biologically active molecules by a strategy called caging (Figure 1).

Caging is an attractive way of turning biological molecules e.g., nucleic acids (DNA, RNA), proteins or peptides light sensitive for the investigation of biological processes. Caged biomolecules incorporate a light-removable protecting group, so-called "caging group", which aborts its native biological or biochemical activity. Since caging of ATP was first reported in 1978 by Kaplan et al., several different photolabile groups have been introduced to turn biomolecules temporarily inactive (1–3). Examples of caged biomolecules are neurotransmitters (4), nucleotides (4), peptides (5, 6), siRNA (7) or DNA (8). The most widely used caged neurotransmitter so far is glutamate for which different protecting groups have been applied (9). RNA interference is a mechanism able to inhibit protein translation by gene silencing. Nguyen et al. caged a 1-(2-nirophenyl)ethyl (NPE) group to the 5′ terminal phosphate of the siRNA antisense strand, which inactivates

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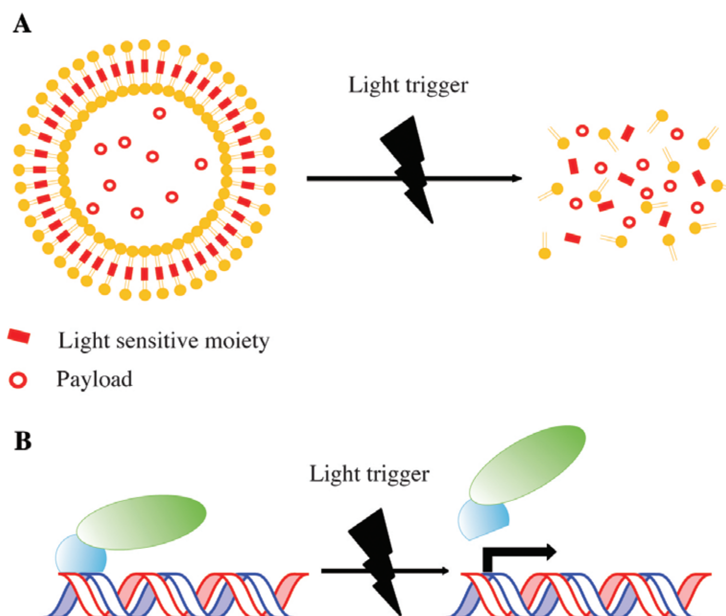

Figure 1 (A) Light responsive drug delivery system built through covalent incorporation of specific light-sensitive chemical groups (red rectangles) with the aim to locally release cargo (red circles) by illumination. (B) Site-specific caging of DNA can be used for light-activated gene expression.

the siRNA activity (10). They could demonstrate an approximately 70% efficient light induced RNA interference using wavelengths between 345 and 385 nm. An alternative form to siRNA mediated control of gene silencing has been reported by Young et al. (11). They introduced a caging group to DNAzymes to inhibit hybridization with mRNA. DNAzymes are enzymatically active desoxyoligonucleotides, which can cleave RNA in a site-specific manner. Translation of mRNA can be aborted upon illumination with UV-light to photorelease the caging group. Caging of DNA has widely been studied as seen from several publications (8, 12, 13). To render those approaches suitable for future clinical application, extension of the work towards longer wavelengths and therefore reduced toxicity should be accompanied by identification of suitable in vitro and in vivo disease models of human disease.

Light-responsive materials for drug delivery can be constructed by the covalent incorporation of specific light-sensitive chemical groups with the aim to locally release cargo by illumination. The synthesis of a photocleavable amphiphilic block copolymer has been demonstrated by Cabane et al. (14). As photosensitive molecule they introduced an o-nitrobenzyl linker between the hydrophobic and hydrophilic blocks, which form vesicles or micelles upon self-assembly in aqueous solution. Successful disruption of the vesicles could be demonstrated after irradiation with UV-light by electron microscopy and dynamic lights scattering data. The design of photocleavable liposomes for drug delivery using different photolabile groups has been reported in several publications (15, 16). Dvir et al. presented a simple proof of concept by carboxylated polystyrene nanoparticles labeled with the unspecific amino acid sequence YIGSR, which adheres to β1 integrins present on most cell surfaces (4, 17). The peptide was caged with a nitrobenzyl group, which could be removed via illumination, leading to nanoparticle binding to the cells. Another approach of light sensitive nanoparticles currently being investigated uses nano-impellers. Nano-impellers are nanomechanical systems allowing the spatiotemporal drug release upon illumination, turning them into an attractive application for clinical trials (5, 6, 18, 19). A clear disadvantage of many published systems is the requirement for light energy in the UV range, limiting their application due to phototoxicity and the very limited penetration range of short wavelength light in biological tissues.

Light induced gene expression and control of gene silencing

Light-mediated control of gene expression and silencing is a powerful and fast growing field in the areas of systems biology, functional genomics and biotechnology. Spatiotemporal and precise gene expression represents the most fundamental level of further complex biological processes such as the control of thousand of proteins and the associated control of metabolic processes. Therefore, light represents a suitable stimulus for in vitro as well as in vivo studies as it is non-invasive, sensitive and allows the spatiotemporal and precise application without interfering with metabolic conditions. Light-induced gene expression can either be achieved using caged biomolecules such as plasmid DNA (12, 13), transcription factors (8, 20, 21) or via photoreceptors harboring a chromophore (9, 22, 23). Several reports focused on caged plasmid DNA's have been published, whereas effective gene

expression remained a major challenge due to ineffective random backbone modifications (8, 10). In addition, successful uncaging and activation of gene expression required high levels of light that can cause phototoxicity (24). A more effective approach for light controlled activation of gene expression was shown by Yamaguchi et al. using a site-specific labeling of the promoter region with a biotinylated photolabile group, leading to effective activation of gene expression in HeLa cells even under low levels of light (12). A successful gene regulation system combining light-sensitive proteins and programmable zinc finger transcription factors has been published by Polstein et al. (14, 20). The system is based on two light-inducible fusion proteins from Arabidopsis thaliana, GIGANTEA (GI) fused to a Zinc finger protein leading the complex to the target DNA sequence and the LOV domain of FKF1 fused to the transcriptional activation domain VP16. Illumination with light leads to fusion of the GI and LOV domain, which guides the LOV-VP16 domain to the target gene and enables gene expression.

Beside light induced gene expression, the focus of photochemical control of gene function has been directed to RNA interference. RNA interference represents one of the major approaches leading to gene silencing/such as that occurring in embryogenesis) and is being extensively explored as a therapeutic strategy for different kind of diseases, including cancer. Two primary approaches for photochemical regulation have been developed. The caging groups are either covalently attached to the phosphate backbone or terminal phosphates or on the nucleotide bases to inhibit the further process of RNA induced silencing (Figure 2). The first report of caged siRNA has been described by Shah et al. using 1-(4, 5-dimethoxy-2-nitrophenyl)ethyl (DMNPE) attached to the phosphate backbone which only showed a 3% caging efficiency (15, 16, 24). Caging of guanosine and thymidin bases by attaching 2-(2-nitrophenyl)propy (NPP) groups has been reported by Mikat and Heckel (25). The modifications have shown knockdown efficiency of about 75% after light irradiation. Jain et al. designed a siRNA caged at the terminal phosphates with a cyclo-dodecyl DMNPE, which is more bulky and therefore shows higher steric hindrance (11, 26). In contrast to the DMNPE, which has been introduced to the phosphate backbone, siRNA terminally caged with cyclo-dodecyl DMNPE showed an efficiency of 89%.

Photodynamic therapy

The therapeutic effect of light has been known for thousands of years and was applied by the Egyptians, Indians and Chinese (8, 12, 13, 27). Its therapeutic relevance to cancer treatment and further development into the photodynamic therapy (PDT) was reported at the beginning of the last century by Oscar Raab, a German medical student and his professor Hermann von Tappeiner (28). The principle of photodynamic therapy involves the administration of a photosensitizer, which will form highly reactive singlet oxygen radical (ROS) from molecular oxygen after illumination with light (Figure 3). Singlet oxygen radicals are known to cause severe damage to biological macromolecules such as membrane lipids and proteins (29). After absorption of light, photosensitizers will change from a ground state into a relatively long-lived excited triple state and a short-lived excited single state. The excited single state can return to the ground state by emitting fluorescence that can be used for clinical detection. In the excited triple state, the photosensitizer molecule can transfer its energy via a type-I or -II reaction. In the type-I reaction, the photosensitizer can react directly with a surrounding substrate to form radicals, which then can further interact with oxygen to produce oxygenated products. In the type-II reaction the energy of the excited photosensitizer can be directly transferred to oxygen to form highly reactive singlet oxygen (30).

Photodynamic therapy has found its way into clinical applications using nanocarrier platforms as delivery system such as photodynamic eye therapy for the treatment of neovascularization, abnormal endothelial proliferation or for different cancer treatments (bladder, skin, head and neck, esophageal, or endobronchial cancer) (31, 32). A number of nanoparticle-

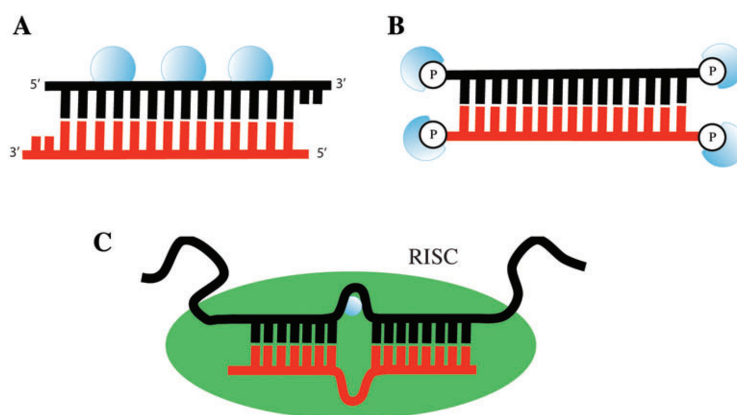

Figure 2 Schematic illustration of caged siRNA strategies. (A) Caged phosphate backbone and (B) caged terminal phosphates of siRNA. (C) Introduction of a caged base into a siRNA antisense strand to inhibit RNA interference.

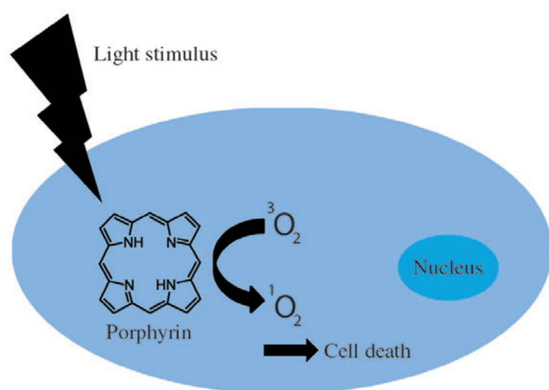

Figure 3 Light activation of a photosensitizer leads to the formation of highly reactive singlet oxygen for selective cell killing.

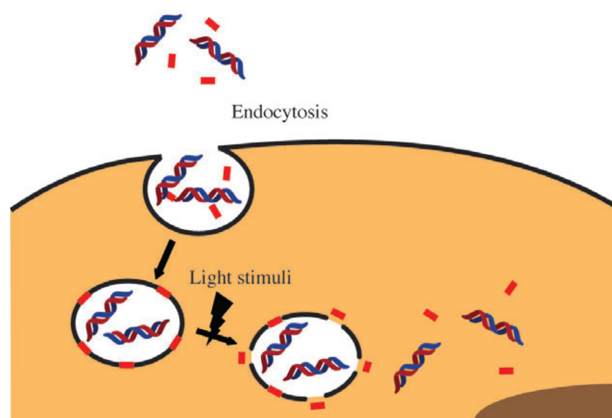

Figure 4 Intracellular drug delivery induced by endolysosomal escape based on photostimulation. Light-irradiation after endocytotic uptake leads to endolysosomal membrane burst upon highly reactive singlet oxygen.

based photodynamic therapies have been approved by the U.S. Food and Drug Administration (FDA) such as e.g., Visudyne®, Photofrin®, Levulan® Kerastick® opening doors for future applications and new possible approaches for future therapies (31). There are several advantages of PDT as a clinical application including a single dose requirement for treatment followed by illumination compared to radiotherapy and chemotherapy, which both depend on a treatment over several weeks or months. Further, it is a local treatment without interfering with the whole organism and retreatment can be simply done in the case of recurrence of a tumor without severe healthy tissue damage. However, further development in the direction of controlled drug release, as well as improved payload capacity of nanoparticle-based delivery systems is warranted.

Photochemical internalization (PCI)

One of the key challenges that still needs to be overcome in order to enable the clinical application of therapeutic delivery of different payloads is endosomal escape. Various strategies have been developed to achieve endosomal escape and these are either based on the characteristic endosomal property of a lower intracellular pH compared to the cytoplasm, incorporation of fusogenic peptides into the endosomal membrane or a strategy called photochemical internalization (PCI). PCI is a site-specific method for intracellular drug delivery by induced endolysosomal escape based on photostimulation. The principle behind PCI relies on photodynamic therapy targeted to endosomes or lysosomes, whereas the vesicular membrane bursts after coming into contact with highly reactive singlet oxygen after illumination of the photosensitizer (Figure 4). In comparison to conventional photodynamic therapy, where the intracellular localization of the photosensitizer does not play an important role because of its complete cellular destruction, PCI is based on the specific accumulation of the photosensitizer in the endolysosomal compartment to achieve endosomal escape without harming the rest of the cell (33).

A fate that may be a consequence to nanocarriers after endocytotic uptake, is the accumulation in the endolysosome, whereas PCI offers a good solution. Lai et al. have demonstrated the effective delivery of doxorubicin and saporin by photochemical internalization using a poylamidoamine (PAMAM) dendrimer (34, 35). Recently, Lu et al. reported the overcoming of doxorubicin drug resistance in vivo by applying dendrimer phtalocyanine-encapsulated polymeric micelles combined with doxorubicin into doxorubicin-resistant bearing mice (36). It has also been shown by Nishiyama et al. that PCI can mediate gene transfection, using a combinational system including polymeric micelles incorporating pDNA and a dendrimer-based photosensitizer (37). Both polymeric micelles are assumed to be taken up by the cells at the same time. After illumination, a remarkable enhancement of transgene expression could be detected while retaining cell viability. Beside enhancement of gene expression, PCI can also be used for siRNA mediated gene knockdown studies. The first application of PCI to facilitate endosomal escape of siRNA was reported in 2007 by Oliveira et al. (38). They used $TPPS_{2a}$ as photosensitizer together with a siRNA able to knockdown epidermal growth factor receptor (EGFR) expression. Complexes of EGFR siRNA and Lipofectamine were applied to the cells. A 10-fold increased efficiency in EGFR knockdown could be detected after illumination compared to siRNA treatment alone. A recently published study by Varkouhi et al. presents PCI mediated enhancement of gene silencing using a polymer-based nanocarrier platform consisting out of cationic polymethacrylates and N,N,N-trimethylated chitosan (39). Furthermore, PCI can enhance the effect of targeted protein toxins that have reached the tumors cells (40). Targeted protein toxins consist of a protein toxin moiety, initiating cytotoxicity and a cell binding moiety, which targets the protein actively to the cell. Denileukin diftitox is the first FDA approved protein toxin for treatment of cutaneous T-cell lymphoma.

Photothermal therapy

Hyperthermia is a non-invasive approach for cancer treatment based on the principle of spatiotemporally increasing the temperature to promote selective destruction of cancer cells, which

are more sensitive to hyperthermia than normal cells due to their higher metabolic rates. Several different approaches have already been applied for delivery of thermal energy such as ultrasound, microwaves or radiofrequency pulses (41–43). A disadvantage is their dispersive property with the result that high fluences (high amount of particles that intersect an area at a specific timepoint) are needed, which lead to undesirable hyperthermic effects on surrounding tissues. Within the last few years, gold nanoparticles have received increasing attention due to their versatile applications such as imaging, cancer therapy, drug delivery and especially because of their unique surface plasmon resonance (SPR) absorption at visible or Near-infrared (NIR) wavelengths (44). The use of NIR is desirable due to its deep penetrating capacity and minimal interference with water and biomolecules in tissues. The principle of photothermal therapy is the combination of light and gold nanoparticles (e.g., gold nanospheres, nanorods, nanoshells, nanocages) for clinical treatment. Illumination of gold nanoparticles leads to conversion of absorbed light into thermal energy, the resulting heat causes cell and tissue destruction (Figure 5). El-Sayed et al. have shown the use of gold nanorods labeled with an anti-EGFR antibody for selective photothermal treatment of cancer cells (45). A dual-modality approach for photodynamic and photothermal therapy has been recently published by Kuo et al. (46). They used gold nanomaterials conjugated with the hydrophilic photosensitizer, indocyanine green, to achieve photothermal therapy (PTT) and photodynamic therapy (PDT). The combination of PTT and PDT showed enhanced destruction of cancer cells in contrast to their single application effectiveness. Photothermal tumor ablation in mice could be proven by O'Neal et al. using gold nanoshells (47). They subcutaneously injected murine colon carcinoma cells into immune-competent mice, followed by injection of gold nanoshells. After 6 h of circulation, tumors were illuminated with NIR. All treated mice looked healthy and tumor free after more then 90 days post-treatment.

Photoswitchable fluorescent nanoparticles

Over the past decades a huge number of nanoparticles made of different materials have been developed and these have biological and medical applications. Whereas many of those platforms have been developed for the purpose of improved drug delivery and therapy another promising direction, which has attracted considerable interest is molecular imaging. Nanoparticle-based imaging offers a non-invasive and quantitative detection method of biomolecules, while at the same time improves sensitivity and specificity of diagnostic imaging as a tool for e.g., early cancer detection. Fluorescence spectroscopy is a powerful method used for molecular imaging of living cells, allowing very sensitive measurements at high resolution. Fluorescence imaging is based on the principle of the absorption of light by a fluorescent dye (e.g., fluorophore or fluorochrome), which emits fluorescent light at a longer wavelength than that absorbed. Fluorescent nanoparticles such as polymer NPs, silica NPs, gold NPs or quantum dots (QD) gained intensive interest during the last years. They can be produced by doping the material with suitable fluorescent dyes or luminescent metals while quantum dots can directly be applied due to their intrinsic fluorescence properties (48). The advantages of fluorescent nanoparticles compared to normal organic dyes are higher brightness due to the fact that a nanoparticle can carry several dye molecules, increase in photostability because the dyes are entrapped within the nanoparticles, higher specificity upon their functionalization properties and their long-term-tracking ability.

Understanding cellular networks is the essential key factor to understand the complex structure of certain diseases. To achieve this goal, significant progress has been made in the development of quantum dots for cellular sensing which have been recently reviewed (49). Sensing quantum dots are based on the principle of the recognition of an analyte, which acts as a fluorescence quencher, by a receptor or chemosensor causing changes upon emission of the fluorophore. Various quantum dots based on overcoating of the core with ZnS or CdSe to improve their fluorescence quantum yield and additional modification of the surface properties to increase their emission have been reported (50–52). Furthermore, this concept can be used to prepare glucose or maltose sensing systems, whereas a photoinduced electron transfer (PET) from the coating molecules to the valence band of an excited quantum dot results in emission quenching as shown by Cordes and Sandros et al. (53, 54).

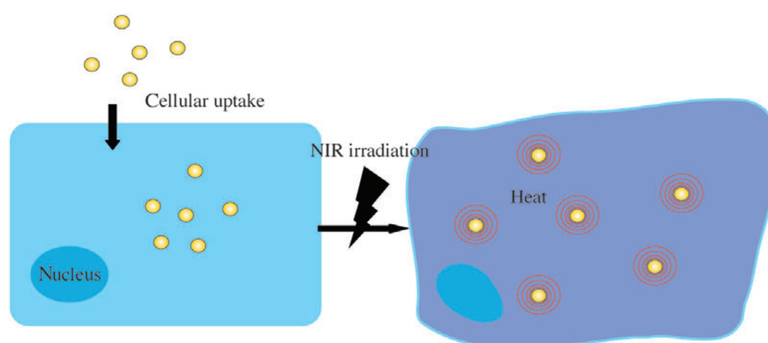

Figure 5 Photothermal therapy is based on intracellular uptake of gold nanoparticles, which after irradiation with near-infrared light convert absorbed light into thermal energy for specific destruction of cancer cells.

Beside the mentioned applications, the most largely exploited photoswitchable fluorescent sensing mechanism is by fluorescence resonance energy transfer (FRET) by which an energy transfer from a QD to a fluorophore will be determined. Until now, several strategies allowing generic on/off photoswitching based on FRET have been reported (55–57). Another more complex approach for photoswitching has been explored by dye doped nanoparticles allowing coactive triggering of multiple processes. These dye doped sensing nanoparticles were first introduced by Kopelman in the late 1990s and are called photonic explorers for bioanalysis with biologically localized embedding (PEBBLEs) (58). The insertion of chemosensors into nanoparticles shows several advantages as minimization of interaction with other biomolecules within the cells or the introduction of multifunctional sensing schemes as for example by pH sensitivity. A wide variety of PEBBLEs have been reported since their development, whereas further literature can be found here (59–61).

However, the ability of QDs to combine molecular imaging and therapy can open new doors for clinical application, but the toxicity of especially heavy metals used in QD synthesis such as cadmium is an important concern (62).

Limiting factors of light

Light as external stimuli for enhanced drug delivery, cargo release, imaging and therapy offers some attractive features such as high sensitivity and spatiotemporal control. However, the major drawback of light is tissue penetration depth, restricting its applications. Solutions to overcome this problem have been made by development of near infrared (NIR) light sensitive photochemical compounds. Near-infrared light at wavelengths of 700–1000 nm can penetrate up to several centimeters deep into tissues without causing any damage (63). This renders NIR much more attractive than the often used UV-light regarding its potential for severe tissue damage. The use of two-photon excitation systems as well as application of upconverting nanoparticles, both provide possible solutions of how to overcome the problem of tissue penetration depth. Two-photon excitation depends on the principle of exciting a caged group by absorption of two photons induced via a pulsed laser. This method allows the usage of caged groups, which absorb light in the UV range but can be excited via pulsed NIR. Upconverted nanomaterials are able to convert NIR into UV light, which generates the same benefit as seen for the two-photon excitation. Practical application of such systems into clinical trials might still need some time due to the fact that most published data using NIR, show irradiation times of hours, which might interfere with clinical practice (64).

Conclusion

The use of light as an external stimulus is a promising approach for a wide range of applications within the field of nanomedicine based on its attractive properties such as sensitivity and biocompatibility, in particular for wavelengths longer than UV. Furthermore, it has shown advantages regarding its high spatial and temporal precision. However, the major drawback of light is tissue penetration depth, which severely restricts the applications of caged compounds, light sensitive drug delivery systems and light-based therapies into clinical application. Thus, approaches like the usage of NIR linked to two-photon uncaging and up-converting systems seem to be promising but further optimization of these methods is needed to increase the chance of further application in clinical trials.

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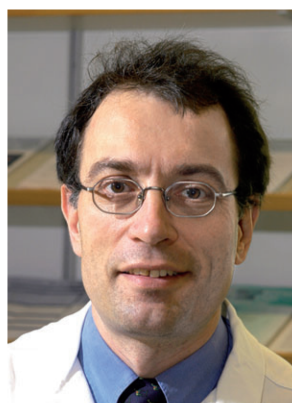

Patrick Hunziker has studied Medicine at the University of Zurich, Switzerland. He received a doctoral degree based on thesis work in experimental immunology from the University of Zurich and did further research in experimental hematology at University Hospital in Zurich, Switzerland. He earned specialist degrees in Internal Medicine, Cardiology and Intensive Care Medicine. As a fellow of the Massachusetts General Hospital, Harvard Medical School, he worked on cardiac imaging in a joint project with the Massachusetts Institute of Technology, Cambridge. His professional activities in Europe, the U.S., Africa and China gave him a broad insight into the needs for the medicine of the future in a variety of settings. Hunziker became involved in medical applications of Nanoscience in the late 1990s and has been the pioneer physician in Nanomedicine in Switzerland since then. With improved prevention, diagnosis and cure of cardiovascular disease as his main research topic, he worked in the nanoscience fields of atomic force microscopy, nano-optics, micro/nanofluidics, nanomechanical sensors and polymer nanocarriers for targeting. He is the founding president of the European Society of Nanomedicine, cofounder of the European Foundation for Clinical Nanomedicine and coinitiator of the European Conference for Clinical Nanomedicine and is clinically active as deputy head of the Clinic for Intensive Care Medicine at the University Hospital Basel, Switzerland. In November 2008 Patrick Hunziker became professor for Cardiology and Intensive Care Medicine at the University of Basel.

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Original Article

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Plasmid linearization changes shape and efficiency of transfection complexes

Abstract: The ability to efficiently transfect plasmid DNA (pDNA) into eukaryotic cells has exerted major impact on scientific research in recent years, and translation to clinical application is ongoing, but challenging. In addition to the choice of the delivery vector, the topology of the DNA seems to be a key factor for efficient transfection. The nanostructured DNA/Vector complexes may differ in size, charge, and shape, for example. This study therefore investigated the transfection efficiency of circular versus linearized plasmid DNA using a GFP expressing vector with Lipofectamine2000 and linear 25 kDA polyethylenimine (PEI). Transfection efficiency and cytotoxicity were measured by flow cytometry and fluorescence microscopy. Shape was determined by transmission electron microscopy. Transfection agent concentrations were chosen below the toxicity level. We determined the optimal N/P ratio over 48 h by using two different concentrations of plasmid DNA. With the increase of DNA concentration and increasing N/P ratio, transfection efficiency also increased. Our results showed a better transfection efficiency with the circular compared to the linearized DNA, under the same experimental conditions for both Lipofectamine and PEI. In electron microscopy, there was a notable difference in the shape of the complexes: circular DNA had random coil appearance in well compacted, roughly spherical shape, while linearized DNA appeared as worm-like strands, both, when complexed with Lipofectamine or with polyethyleneimine. This generates the hypothesis that the shape of the transfection particle may be an important factor for successful gene transfer.

Keywords: cytotoxicity; DNA transfection; polyethylenimine.

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Introduction

The development of transfection techniques of DNA into eukaryotic cells has had a drastic impact on basic scientific research within the past decades such as to study the function of genes or gene products, by enhancing or inhibiting specific gene expression in cells and to produce recombinant proteins. Different purposes have even led to various clinical applications such as gene therapy (1) and DNA immunization (2). Application of nanotechnology in medicine, the emerging scientific field of nanomedicine, has yielded new synthetic transfection vectors for nucleic acids, leading to significant expansion of the potential for clinical applications. For the delivery of DNA to the target cell, several different delivery vehicles have been developed, including liposomes (3), polymers (4), dendrimers (5) or magnetic nanoparticles (6), and the advent of receptor-targeted delivery (7), smart nanomaterials (8, 9) and multifunctional smart nanosystems (10) expands our toolbox further. In addition to the gene delivery system, the structure of the DNA segment to be transfected plays also a key role for transfection efficiency. While DNA in the form of circular plasmids is often used even if the transfection target cell is eukaryotic, such circular plasmids are not the naturally occurring form of eukaryotic DNA, which typically is rather structured as a linear expression segment within a chromosome. While circular bacterial plasmids are easily produced in large quantities in bacterial culture, alternatives like linear expression systems or microcircle DNA (11) are thought to confer advantages including improved nuclear translocation (linear constructs) or absence of sequences that might lead to side effects, like non-methylated CpG, or antibacterial resistance. Shape and charge of the DNA/Vector construct may also play a role for cell uptake.

The focus of this study was first, to compare transfection efficiency of circular versus linear plasmid DNA, using cancer cells as target cells, with a cationic lipid system (Lipofectamine) or a cationic polymer (polyethyleneimine), and second, to develop hypotheses for potential differential efficiency. Lipofectamine is a standard pDNA

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transfection agent usable only for in vitro transfection (12, 13). Polyethylenimine (PEI) is a highly cationic polymer proven to be efficient and versatile for gene delivery in vitro and in vivo, and developed also with the goal of clinically applications (14). PEI is a highly cationic polymer available at different molecular weights and different molecule structures such as branched and linear versions. High molecular weight PEIs (800 kDa) have shown increased toxicity compared to low molecular weight and linear PEI (25 kDa), rendering the latter the preferred transfection agent (15). We therefore compared circular and linearized forms of a plasmid DNA regarding transfection efficiency with either Lipofectamine®2000 or linear 25 kDa PEI.

Materials and methods

Preparation of plasmid DNA

The pEGFP-C1 plasmid, encoding green fluorescent protein (Clontech) was used in this study for transfection. To linearize the plasmid, the restriction enzyme *AseI*, (New England BioLabs) was used for digestion (Figure 1). Twenty units of enzyme were used to cleave 2 µg of pDNA in 37°C for 15 min. The cut DNA was analyzed by 1.0% agarose gel and stained in ethidium bromide solution (0.5 µg/mL). Electrophoresis was carried out with a current of 80 V for 1 h in TAE running buffer.

Preparation of PEI solution

Linear 25 kDa PEI was purchased from Polyscience (Warrington, PA) and used to prepare a 1 mg/mL stock solution. To dissolve PEI, deionized H_2O was heated to ~80°C and mixed with 1 mg of PEI. In addition the solution was cooled down to room temperature. The pH was adjusted to pH 7.2 and the solution was filtered using a 0.22 µm filter (Merck Millipore). Stock solution of linear PEI were stored at –20°C and thawed and stored at 4°C while in use.

Cell culture

HeLa cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; catalog number ACC-57) and adapted to grow in Roswell Park Memorial Institute 1640 Medium (RPMI 1640 Medium; Invitrogen catalog number 31870-025). All cells used in the experiment were cultured in RPMI containing 10% fetal calf serum (FCS), 1% GlutaMax™, 1% P/S and 1% NEAA. Cultures were maintained at a temperature of 37°C in a humified 5% CO_2 atmosphere.

Transfection procedures

All transfections were carried out in 24-well plates (Corning) using either Lipofectamine®2000 (Invitrogen) or linear 25 kDa PEI. HeLa cells were seeded at 3×10^4 cells/well in 0.5 mL RPMI 1640+10% FCS, 24 h prior to transfection.

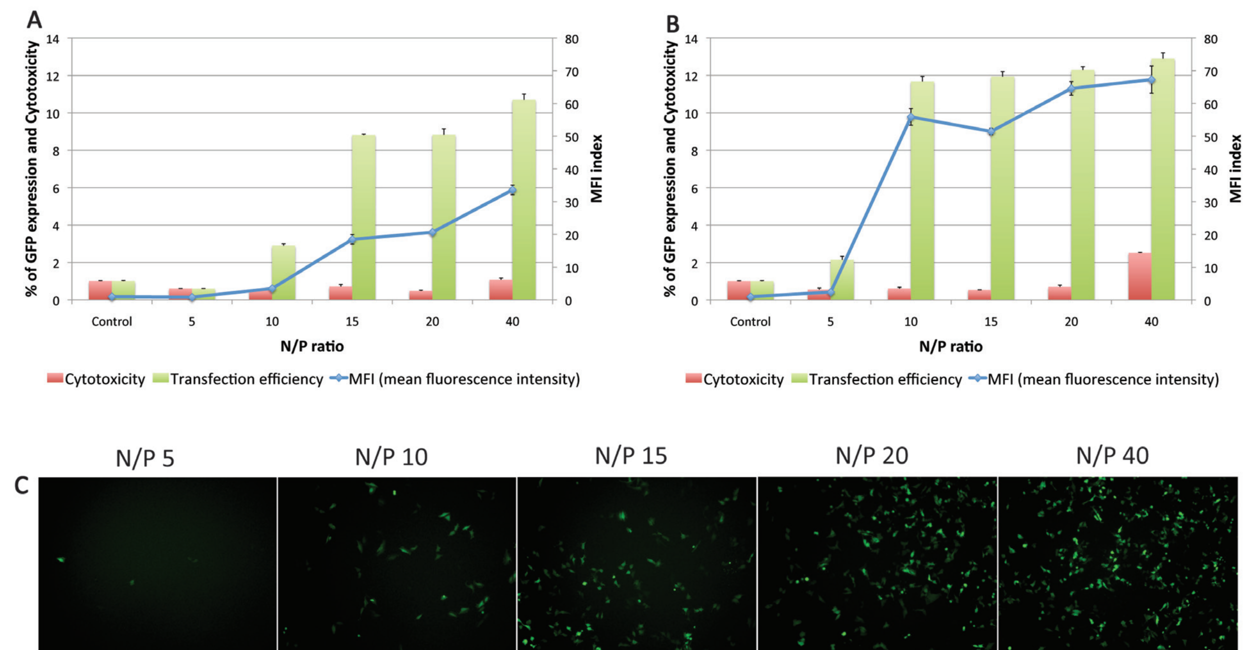

Figure 1 Transfection efficiency and cytotoxicity of linear 25 kDa PEI at varying N/P ratios and different DNA concentrations. pEGFP-C1 plasmid DNA 250 ng (A) and 500 ng (B) was complexed with the exact amount of PEI depending on the N/P ratio. (C) Fluorescence microscopy images showing PEI transfection at different N/P ratios.

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For the transfections with linear PEI, circular and linearized plasmid DNA (0.25 µg or 0.5 µg/well) and the exact amount of PEI depending on the N/P ratio was separately added to 50 µL OPTI-MEM® Reduced-Serum Medium, briefly vortexed and kept at room temperature for 5 min. The 50 µL of plasmid DNA was added to the 50 µL PEI solution, briefly mixed and kept at room temperature for 10 min. For the transfections with Lipofectamine, circular and linearized plasmid pEGFP-C1 DNA (0.25 µg or 0.5 µg/well) and 0.5 µL of Lipofectamine was separately diluted into 50 µL OPTI-MEM and left at room temperature for 5 min. The 50 µL of plasmid DNA was added to the 50 µL Lipofectamine solution, briefly mixed and kept at room temperature for 5 min.

The culture medium was removed from each well containing PEI/DNA complexes. The cells were washed with phosphate buffered saline (PBS) and fresh RPMI medium without antibiotics was added. The 100 µL of PEI/DNA and DNA/Lipofectamine complexes was added drop-wise to the wells and the cells were incubated at 37°C. The cells treated with PEI were washed after 5 h with PBS and fresh medium was added following incubation at 37°C for additional 43 h.

Transfection efficiency measurements

Transfection efficiency was determined by flow cytometry analysis 24 and 48 h post-transfection. Briefly, transfected cells were washed twice with ice-cold PBS and harvested by trypsinization. Cells were collected by centrifugation at 1000 rpm for 5 min at RT, the supernatant was removed, and the pellet was re-suspended in PBS containing 1% BSA at a concentration of 1×10^6 cells/mL. Percentage of GFP-expressing cells and mean fluorescence intensity (MFI) were detected by flow cytometry equipped with BD Accuri C6 (Becton Dickinson, San Jose, CA). Cytotoxicity was analyzed by adding 0.05 µg/mL 7-AAD (559925, BD pharmingen) to the samples 10 min before flow cytometry analysis.

Transmission electron microscopy

Transmission electron microscopy (TEM) was employed as imaging technique to visualize the circular and linearized plasmid DNA/Lipofectamine and DNA/PEI complexes in aqueous environment. Complexes using 500 ng DNA were placed on a copper grid covered with a nitroglycerin film coated with carbon. A staining agent was added (2% uranyl acetate).

Statistical analysis

The data for flow cytometry analysis were analyzed by FlowJo X. Standard statistics including calculation of means and standard deviations (SD) and Student's t-test for group comparisons. In all experiments, $p<0.05$ was considered statistically significant.

Results

Transfection efficiency and cytotoxicity of linear 25 kDa PEI at varying N/P ratios

To determine the optimal N/P ratio of linear 25 kDa PEI regarding transfection efficiency, 250 ng as well as 500 ng of circular pEGFP-C1 DNA was complexed with the appropriate amount of PEI at N/P ratios of 5, 10, 15, 20, and 40. Transfection efficiency measurements were done by flow cytometry analysis. As shown in Figure 1, the percentage of GFP expressing cells increased with increasing N/P ratios and higher DNA concentration, reaching the highest value of 13% after 48 h at N/P ratio 40 using 500 ng DNA. The mean fluorescence intensity (MFI) significantly increased with the increase of N/P ratio and the higher DNA concentration. The maximal amount of nonviable cells was 2.5% after 48 h, revealing very low toxicity of the transfection agent.

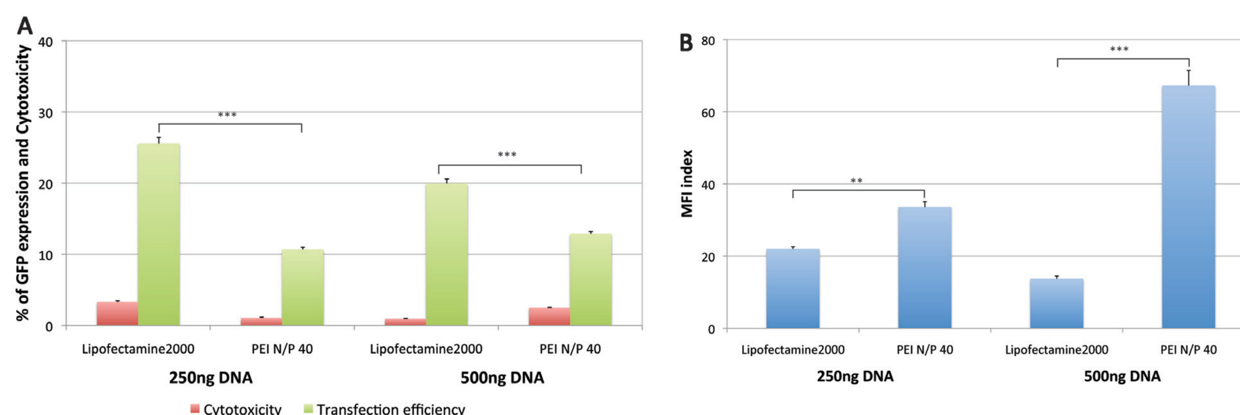

Figure 2 Comparison of transfection efficiency and cytotoxicity of Lipofectamine and linear 25 kDa PEI. HeLa cells were plated at the density of 3×10^4 cells and transfected with 250 ng or 500 ng GFP plasmid DNA, complexed with Lipofectamine or PEI at N/P ratio 40. (A) GFP expression and cytotoxicity of transfected cells. (B) Mean fluorescence intensity of transfected cells. The data shown are the mean and SD from three different experiments. Two asterisks indicated $p<0.01$ and three $p<0.001$.

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Comparison of transfection efficiency and cytotoxicity of linear 25 kDa PEI and Lipofectamine

For the further comparison of PEI with Lipofectamine, the N/P ratio 40 was chosen due to the highest transfection efficiency. The optimal transfection condition for Lipofectamine was determined by seeding in a 24-well plate 2×10^4, 3×10^4, 4×10^4, and 6×10^4 cells per well and complexing 250 and 500 ng circular plasmid DNA with 0.5, 1.0, and 1.5 µL Lipofectamine. The highest transfection efficiency was detected using 3×10^4 and 0.5 µL Lipofectamine for both 250 and 500 ng pDNA (data not shown).

After 48 h Lipofectamine displayed transfection efficiency superior to PEI (26% with 250 ng and 20% with 500 ng vs. 11% with 250 ng and 13% with 500 ng plasmid DNA, respectively). Although the percentage of GFP expressing cells was higher with Lipofectamine, the MFI decreased with increasing amount of DNA. The comparison of MFI of Lipofectamine with PEI N/P ratio 40 revealed much lower values at both DNA concentrations (Figure 2). Comparison of cytotoxicity showed no significant difference between the transfection agents.

Comparison of transfection efficiency and cytotoxicity of circular and linearized plasmid DNA

The circular DNA showed very efficient GFP expression for both Lipofectamine and PEI. For Lipofectamine the GFP expression of linearized pDNA compared with circular dropped 6.5 times down with 250 ng DNA after 48 h. An eight times higher GFP expression was detected in the case of PEI with 500 ng circular plasmid DNA. The MFI of linearized plasmid DNA transfected cells decreased for Lipofectamine approximately 6 times and 40 times for PEI (Figure 3).

Shape of the transfection nanoparticle in transmission electron microscopy

Figure 4 shows the transmission electron microscopy of the resulting transfection nanoparticles created from Lipofectamine and PEI, respectively, with circular and linear plasmids. Circular plasmids display, in the case of both complexing agents, a random coil structure in an approximately spherical, well compacted shape. In contrast, the linear construct appears with both complexing agents as worm-like strands, i.e., an apparently differential tertiary structure.

Discussion

To advance clinical application of gene transfection/delivery, progress in two key technical aspects, namely improved nanomaterials-based vectors and optimal DNA cargo structure needs to be achieved. For transfection vectors, nanomaterials are seen as a promising route to the required optimal balance between nucleic acid binding and release at the target site. In addition, such vectors combine stealth properties (protecting the cargo

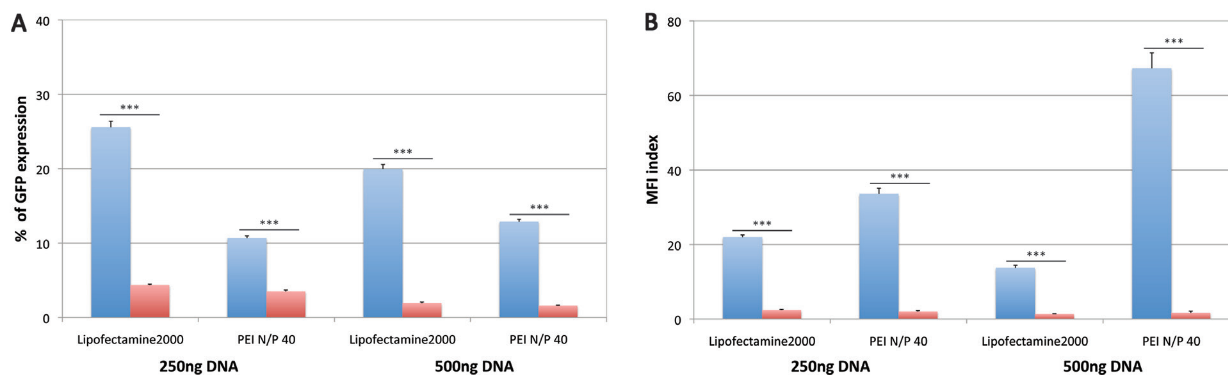

Figure 3 Comparison of transfection efficiency and cytotoxicity of circular and linearized plasmid DNA. HeLa cells were plated at the density of 3×10^4 cells and transfected with 250 ng or 500 ng GFP circular or linearized plasmid DNA, complexed with Lipofectamine or PEI at N/P ratio 40. (A) GFP expression of transfected cells. (B) Mean fluorescence intensity of transfected cells. Blue columns indicate circular DNA, while red columns correspond to linear DNA. The data shown are the mean and SD from three different experiments. Three asterisks indicate $p<0.001$.

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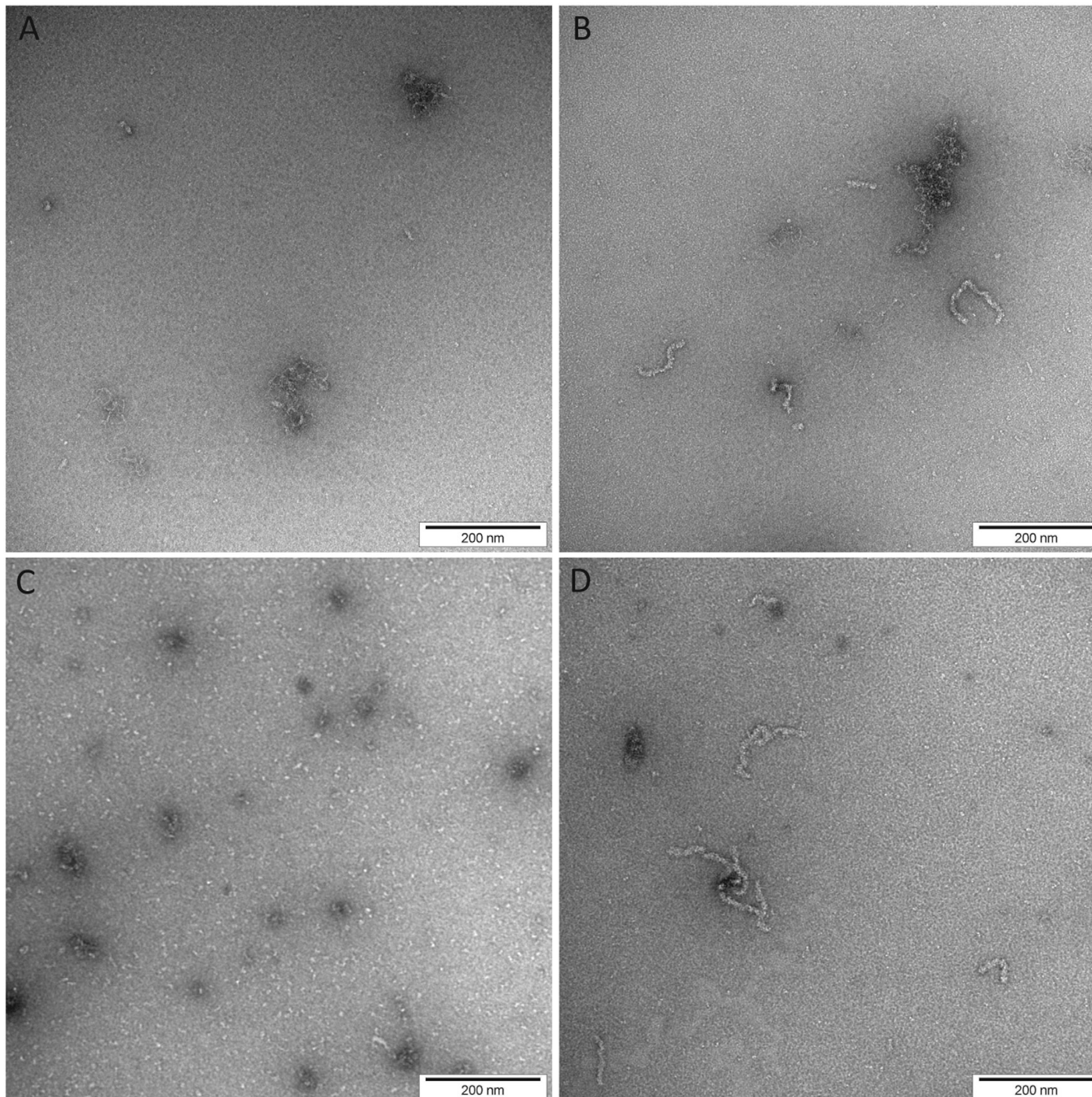

Figure 4 Transmission electron microscopy images of circular plasmid DNA (A) and linearized DNA (B) complexed with Lipofectamine and 25 kDa PEI complexed with circular plasmid (C) and linearized DNA (D).

from premature scavenging by the immune system) with targeting capabilities to the target organ and target cell type. Optimally structured nucleic acid cargo is not less important, as this study shows that the topology of the expression vector has a substantial impact on expression success in vitro. In this study we performed comparative analysis of the transfection efficiency and cytotoxicity of linear 25 kDa PEI with the commercially available non-viral vector Lipofectamine in HeLa cells. Our study supports that a circular plasmid offers advantages in terms of average expression intensity and expression homogeneity among cancer cells, while the linearized version led to expression in a limited number of cells. The findings were consistent for both the fluorescence microscopy as well as flow cytometry. The results showed that the transfection efficiency of linear 25 kDa PEI is dependent on the DNA concentration and N/P ratio. The highest GFP expression could be detected at N/P ratio 40 as already seen by others (16). It is known that the N/P ratio plays a crucial role for maximization of the transfection efficiency by influencing the size and the charge of the PEI/DNA complexes (17). Therefore, higher N/P ratios increase DNA condensation and endocytotic uptake by the cells (18). Although as seen in numerous studies, high transfection efficiency with PEI is mostly combined with high cytotoxicity whereas our study revealed very low toxicity values. The high amount

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of free PEI at higher N/P ratios could be a key factor for the disruption of the endosomal membrane leading to higher expression efficiency from the PEI-transfected DNA.

Lipofectamine is a lipid-based transfection agent, which is known to be highly efficient for the transfection of a variety of cells. Our study revealed that transfection efficiency of Lipofectamine was up to two times higher than PEI. Although Lipofectamine shows better transfection efficiency, the MFI of PEI was higher than for Lipofectamine and increased with increasing DNA concentration. Moreover, we also investigated the transfection efficiency of circular and linearized plasmid DNA. Our results showed a reduced percentage of GFP expression and MFI from linearized plasmid DNA after 48 h for both Lipofectamine and PEI compared with circular DNA.

In principle, differential expression may be due to different composition of the vectors, e.g., different promoters etc. Differential expression may be a consequence of differential vector-cell binding and uptake as we believe that in non-receptor targeted transfection used in this work, positive charge, i.e., a high N/P ratio facilitates nonspecific binding and uptake. Differential expression may be a consequence of intracellular processes like degradation by nucleases, of nuclear translocation or even integration of a vector into chromosomal DNA. Not all these factors could be assessed in this study, but future work will focus on an in-depth understanding of the differential impact of these factors.

From the electron microscopy data, we found a notable difference in the shape of the complexes: circular DNA had random coil appearance in well compacted, roughly spherical shape, while linearized DNA appeared as worm-like strands, both, when complexed with Lipofectamine or with polyethyleneimine. While the intrinsic persistence length of a double stranded DNA coil is known to be approximately 50 nm, defining the dimension of a DNA random coil, the binding of additional molecules to a DNA strand has the potential to alter its properties: polyethyleneimine, as a polycation, will bind through electrostatic interaction at multiple sites on a DNA strand, has an intrinsic persistence length below 1 nm in suited buffer and behaves, if stretched to a different shape, like an elastic rubber. At the scale of an individual polyethyleneimine molecule, a compacting molecule may therefore impart an additional compacting force on the DNA. Charge of the complex may also significantly influence the overall shape: if complexation imparts an overall charge on the complex, electrostatic forces tend to stretch a segment within the Debye radius (i.e., the electrostatic screening distance by the acqueous electrolyte). The N/P ratio was â« in our experiments because transfection rates were low at N/P below 10. Interestingly, at N/P ratios of 1.0, an impact on circular plasmid tertiary structure has been reported using a different polycationic polymer (19). Particle size and shape are important variables in the cell biology of endocytosis and phagocytosis (20). This suggests that the shape of the transfection particle may be an important factor for successful gene transfer.

Thus, for successful transfection of DNA into eukaryotic cells, and in particular for clinical in vivo applications (21), we hypothesize that control of shape of the DNA/cationic vector is an important design variable to be understood and controlled.

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Roman Lehner is a final year PhD student in the group of Prof. P. Hunziker, working within the SNF project “Intelligent Materials”. Roman studied molecular biology at the University of Basel from where he received his MSc in structural biology in 2009 where he was involved in the crystal structure determination of the membrane protein Kdgm, a porin, which is involved in the pathogenicity of the bacteria. After his master diploma, he worked as a scientific associate at the Institute of Anatomy in Basel and for the spin-off company BioVersys. His work is focused on the design of a polymer based drug delivery system, which can be functionalized to target particular tissue and combined with a stimulus responsiveness to light to explore activation of caged compounds.

Xueya Wang studied molecular biology at the University of Basel from where she received her diploma in 2004 and compiled her PhD thesis entitled “Antigen – presentation by vascular endothelium: its role for CTL – mediated vascular injury” under the guidance of Prof. Dr. Barbara Biedermann in 2008. Afterwards Xueya joined the group of Prof. Patrick Hunziker as a senior scientist. Her research interests are nanoscale polymer delivery systems for the safe and specific delivery of therapeutic molecules and intelligent materials to target tissues and cells in humans focusing on atherosclerosis and cancer therapy.

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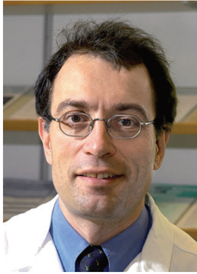

Patrick Hunziker has studied Medicine at the University of Zurich, Switzerland. He received a doctoral degree based on thesis work in experimental immunology from the University of Zurich and did further research in experimental hematology at University Hospital in Zurich, Switzerland. He earned specialist degrees in Internal Medicine, Cardiology and Intensive Care Medicine. As a fellow of the Massachusetts General Hospital, Harvard Medical School, he worked on cardiac imaging in a joint project with the Massachusetts Institute of Technology, Cambridge. His professional activities in Europe, the US, Africa and China gave him a broad insight into the needs for the medicine of the future in a variety of settings. Hunziker became involved in medical applications of Nanoscience in the late 1990s and has been the pioneer physician in Nanomedicine in Switzerland since then. With improved prevention, diagnosis and cure of cardiovascular disease as his main research topic, he worked in the nanoscience fi elds of atomic force microscopy, nano-optics, micro/nanofl uidics, nanomechanical sensors and polymer nanocarriers for targeting. He is the founding president of the European Society of Nanomedicine, cofounder of the European Foundation for Clinical Nanomedicine and coinitiator of the European Conference for Clinical Nanomedicine and is clinically active as deputy head of the Clinic for Intensive Care Medicine at the University Hospital Basel, Switzerland. In November 2008 Patrick Hunziker became professor for Cardiology and Intensive Care Medicine at the University of Basel.

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5.2. Plasmid DNA condensation, size, molecular morphology and gene delivery efficiency by cationic diblock copolymers

Introduction

Within the past few years, a strong interest has arisen regarding the development of gene therapy as a potential cure for several diseases (1,2). A key challenge for gene therapy is the effective protection of the therapeutic DNA from enzymatic degradation and the transport to a specific target. Due to its polyanionic characteristic, the large size and the sensitivity regarding nuclease-induced degradation, naked DNA is unable to cross the cellular barriers by passive diffusion (3). Therefore, it is important to design suitable carrier systems to efficiently and safely carry therapeutic DNA into the target cells as well as release. Several non-viral gene delivery systems (e.g polymers, lipids) have been developed to condense and carry large nucleic acid constructs (4-7). Amongst all these materials, cationic polymers as polyethylenimine (PEI), poylamidoamine (PAMAM) or poly-L-lysine (PLL) are widely used due to their long-term safety, biocompatibility and strong condensing properties (8-10). PEI attracted great attention for gene delivery due to its highly cationic properties. However, PEI itself is not suitable as a drug delivery system for clinical application because of its cationic properties having the general tendency to bind to serum properties as well as increased cytotoxicity (11). Our approach was to synthesize two diblock copolymer, consisting of a hydrophilic poly-2-methyl-oxazole (PMOXA) block and a poly-2-(4-azidobutyl)-oxazole (PABOXA) block, with either terminal primary or tertiary amine groups. It is known that primary amines are especially responsible for DNA binding, while tertiary amines provide good buffering capacity to the system (12). The transfection efficiency of the two polymers was tested using HeLa and HEK293T cells at two different DNA concentrations over a time period of 48 h. In addition to the gene delivery system, comparison studies between circular and linear DNA demonstrated the structure dependency of the DNA segment to be transfected related to the transfection efficiency (13-15).

The goal of this study was first the comparison of the newly synthesized diblock copolymers consisting of primary and tertiary amines with polyethylenimine (PEI) and the influence of DNA concentration concerning transfection efficiency using cancer

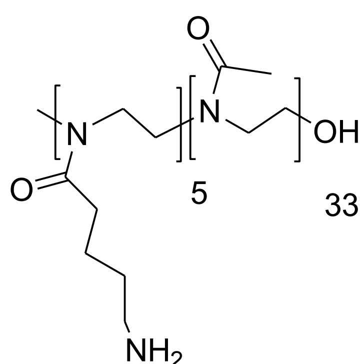
cell lines as target cells and second to investigate the molecular morphology of the condensed plasmid DNA by Transmission Electron and Atomic Force Microscopy.

Material and Methods

Synthesis of copolymers $PABOXA_5$-b-$PMOXA_{33}$-PA and $PABOXA_5$-b-$PMOXA_{33}$-TA

The novel polymers $PABOXA_5$-b-$PMOXA_{33}$-PA (Figure 1A) and $PABOXA_5$-b-$PMOXA_{33}$-TA (Figure 1B) were synthesized by Dr.Kegang Liu.

A)



B)

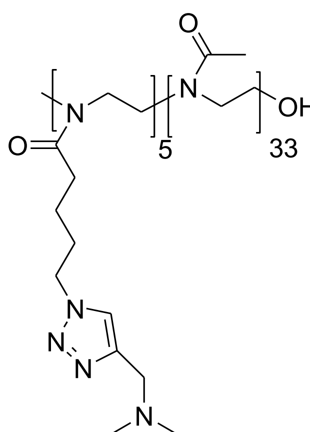


Figure 1: Structures of the cationic diblock copolymers investigated. $PABOXA_5$-b-$PMOXA_{33}$-PA (A), $PABOXA_5$-b-$PMOXA_{33}$-TA (B)

DNA transformation, isolation and purification

For transformation, DH5α competent cells were thawed on ice for 30 minutes. After 10 minutes, 1ug of pEGFP-C1 was added to the cells. The cells were heat-shocked for 90 seconds in a 42° C water bath and put back on ice. Next, 800 µl pre-heated super optimal broth with catabolite repression (SOC) was added following an incubation at 37° C for 1 hour. Cells were then streaked out on LB agar plates containing 40 µg/ml kanamycin and put on 37° C overnight. A single colony from a freshly streaked selective plate was picked and a starter culture of 2 ml LB medium containing 40 µg/ml kanamycin was inoculated for ~ 8 hours at 37° C with vigorous shaking (250 rpm). The starter culture was then added into appropriate amounts of LB media and inoculated for additional 12 – 16 hours to let the cells grow to a late growth phase at 37° C with vigorous shaking (250rpm) Plasmid DNA from DH5α

competent cells was extracted using Qiagen Plasmid Maxi Kit columns (Qiagen, Hilden, Germany) according to the manufacturers instructions.

Preparation of PEI solution

Linear 25 kDa PEI was purchased from Polyscience (Warrington, PA) and used to prepare a 1mg/ml stock solution. To dissolve PEI, de-ionized H_2O was heated to ~80° C and mixed with 1mg of PEI. In addition the solution was cooled down to room temperature. The pH was adjusted to pH 7.2 and the solution was filtered using a 0.22 μm filter (Merck Millipore). Stock solution of linear PEI were stored at -20° C and thawed and stored at 4° C while in use.

Preparation of Polyplexes

The plasmid DNA/polymer complexes were prepared by aqueous self-assembly. The diblock copolymers were dissolved in 10mM PBS and mixed accordingly to the N/P ratio, vortexed for 2 min followed by gentle stirring for 30 min at room temperature.

Cell culture

HeLa cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; catalog number ACC-57) and adapted to grow in Roswell Park Memorial Institute 1640 Medium (RPMI 1640 Medium; Invitrogen catalog number 31870-025). All cells used in the experiment were cultured in RPMI containing 10 % fetal calf serum (FCS), 1 % GlutaMax™, 1 % P/S and 1 % NEAA. HEK293T cells were cultured in D-MEM containing 10 % FCS, 1 % GlutaMax™, 1 % NEAA and 1 % P/S Cultures were maintained at a temperature of 37° C in a humified 5 % CO_2 atmosphere.

Transfection procedures

All transfections were carried out in 24-well plates (Corning) using either pDNA/polymer complexes or linear 25 kDa PEI. Cells were seeded at 3×10^4 cells/well in 0.5 ml growth medium, 24 hours prior to transfection.

For the transfections, circular plasmid DNA (0.5 or 1.0 μg/well) and the exact amount of $PABOXA_5$-b-$PMOXA_{33}$-PA, $PABOXA_5$-b-$PMOXA_{33}$-TA or PEI depending on the N/P ratio was added to 250 μl OPTI-MEM® Reduced-Serum Medium, briefly vortexed and kept at room temperature for 30 min. The culture medium was removed from each well, the cells were washed with phosphate buffered saline (PBS) and the

complexes were added to the wells and the cells were incubated at 37° C. The cells treated with PEI were washed after 4 h with PBS and fresh medium was added following incubation at 37° C.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was employed as imaging technique to visualize plasmid DNA condensation by cationic diblock copolymers in aqueous environment. Samples were placed on a copper grid covered with a nitroglycerin film coated with carbon. A staining agent was added (2 % uranyl acetate).

AFM Imaging

A Bruker MulitMode V atomic force microscope was used for imaging in tapping mode with MPP-11100-10 cantilevers (Bruker AFM Probes; RTESP) with a nominal tip radius of 8nm. The scan frequency was typically 1.0 Hz. All samples were imaged in air.

Preparation of Poylmer/pDNA complexes

Plasmid DNA was first imaged in the absence of polymer. A 1.7 ug/ml in water stock solution was further diluted to 0.5 µg/ml in 5 mM $MgCl_2$. Muscovite mica was used as a substrate for all AFM observations. Pretreatment of mica was necessary to promote electrostatic immobilization between the DNA/polymer condensates and mica. The mica sheet was pretreated with $NiCl_2$ and 10 µl of the DNA solution was added and incubated for 1 min. Next, the mica sheet was thoroughly rinsed with 0.02 % uranyl acetate and then dried with a filter paper. Solutions with polymer/DNA complexes at NP ratio of 40 were treated in the same way.

Flow cytometer measurements

Transfection efficiency was determined by flow cytometry analysis 24 and 48 h post-transfection. Briefly, transfected cells were washed twice with ice cold PBS and harvested by trypsinization. Cells were collected by centrifugation at 5000 rpm for 5 min at RT, the supernatant was removed, and the pellet was re-suspended in PBS containing 1 % BSA at a concentration of 1 x 10^6 cells/ml. Percentage of GFP-expressing cells was detected by flow cytometry equipped with BD Accuri C6 (Becton Dickinson, San Jose, CA).

Statistical analysis

The data for flow cytometry analysis were analyzed by FlowJo X. Standard statistics including calculation of means and standard deviations (SD) for group comparisons.

Results

Gel retardation study

Agarose gel electrophoresis was carried out to determine the plasmid DNA/polymer complexation at different N/P ratios. Polyplexes were formed at charge ratios of 10, 20 and 40 and gel electrophoresis was subsequently carried out. Naked plasmid DNA migrates towards the anode, while complete retardation occurs at high charge ratios for PEI, PABOXA$_5$-b-PMOXA$_{33}$-PA and PABOXA$_5$-b-PMOXA$_{33}$-TA indicating formation of condensed nanostructures.

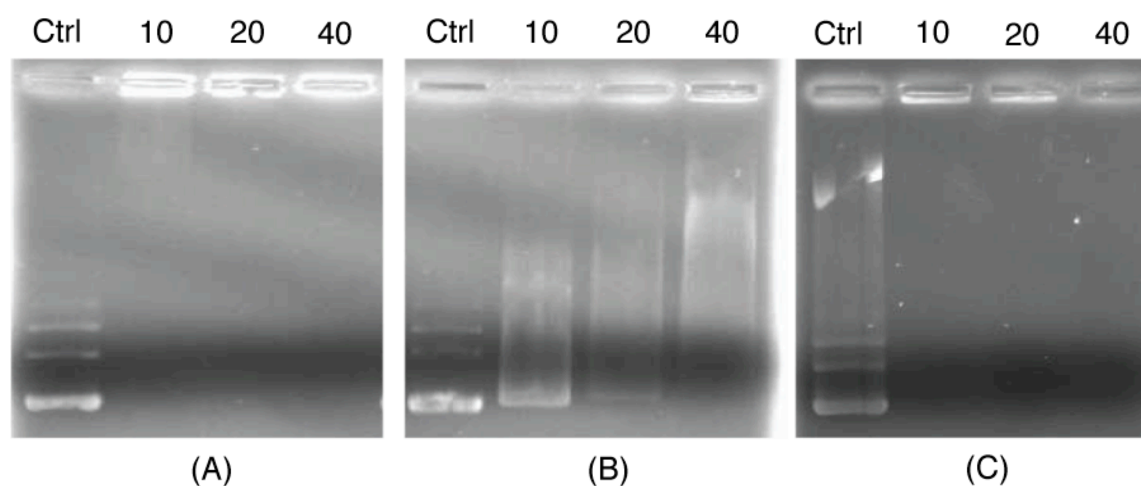
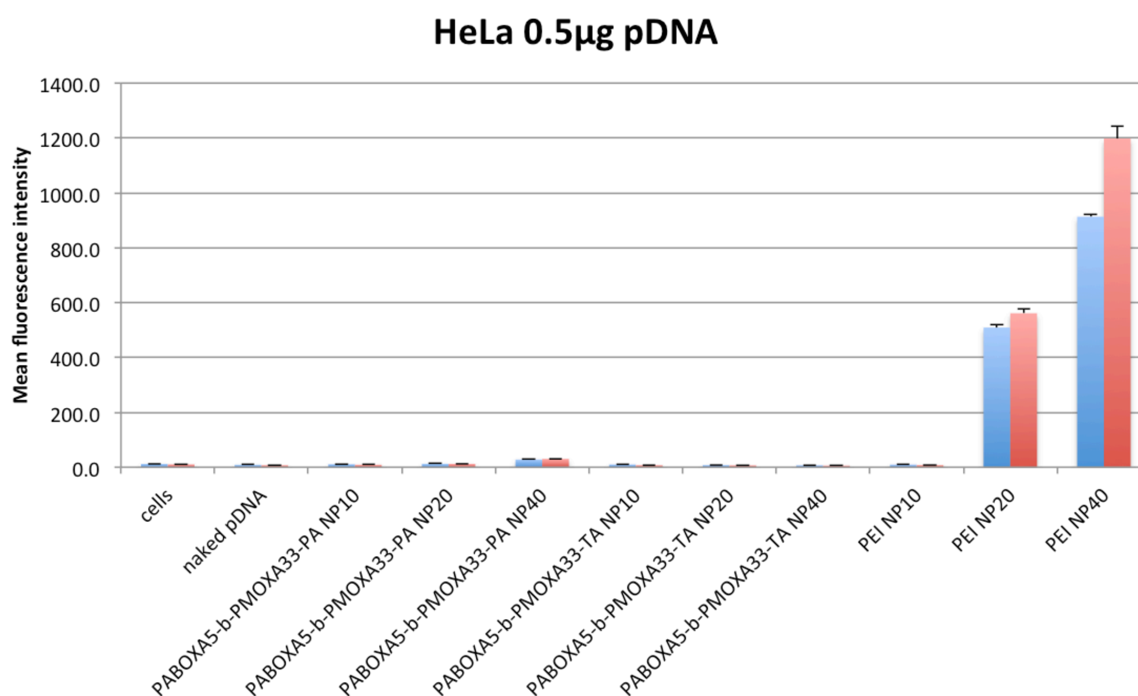


Figure 2: Electrophoretic mobility shift assay of PABOXA$_5$-b-PMOXA$_{33}$-PA, PABOXA$_5$-b-PMOXA$_{33}$-TA and PEI plasmid DNA complexes on 1% agarose gels (A - C). Lane 1 – Ctrl (only plasmid), lane 2 – 4 are loaded with polyplexes formed at N/P ratios of 10, 20 and 40.

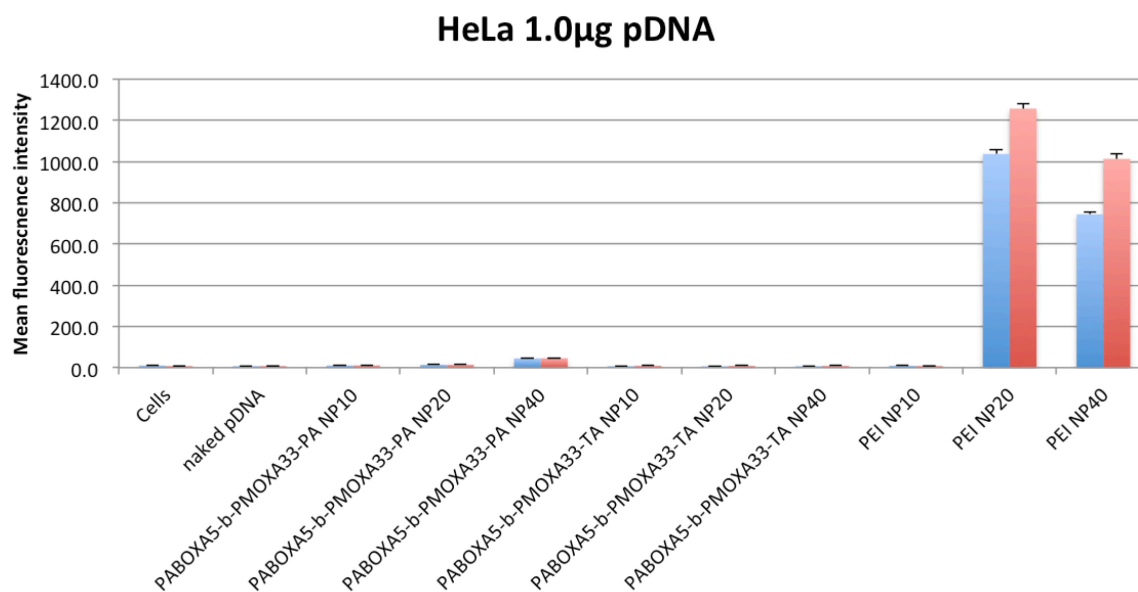
Transfection efficiency of PABOXA$_5$-b-PMOXA$_{33}$-PA and PABOXA$_5$-b-PMOXA$_{33}$-TA at varying N/P ratios

Plasmid DNA showed very efficient increasing GFP expression for the experiments with PEI for both HeLa and HEK cells and after 24 as well as 48 h. Comparison of the dependency of the DNA concentration revealed a concentration based increase of GFP expression for PEI for the HEK cells. The diblock copolymer PABOXA$_5$-b-PMOXA$_{33}$-PA revealed minimal transfection efficiency with the highest values detected at N/P 40. For the HeLa cells, the transfection efficiency of PABOXA$_5$-b-PMOXA$_{33}$-PA decreased after 48 h with increasing DNA concentration (36 times transfection efficiency decrease to 40 times for 0.5 µg DNA and 23 times decrease to 28 times for 1.0 µg), whereas the HEK cells showed an increase after 48 h (13 times transfection efficiency increase to 10 times for 1.0 µg DNA). Transfection studies of PABOXA$_5$-b-PMOXA$_{33}$-TA resulted in no protein expression for both cell lines even after 48 h.

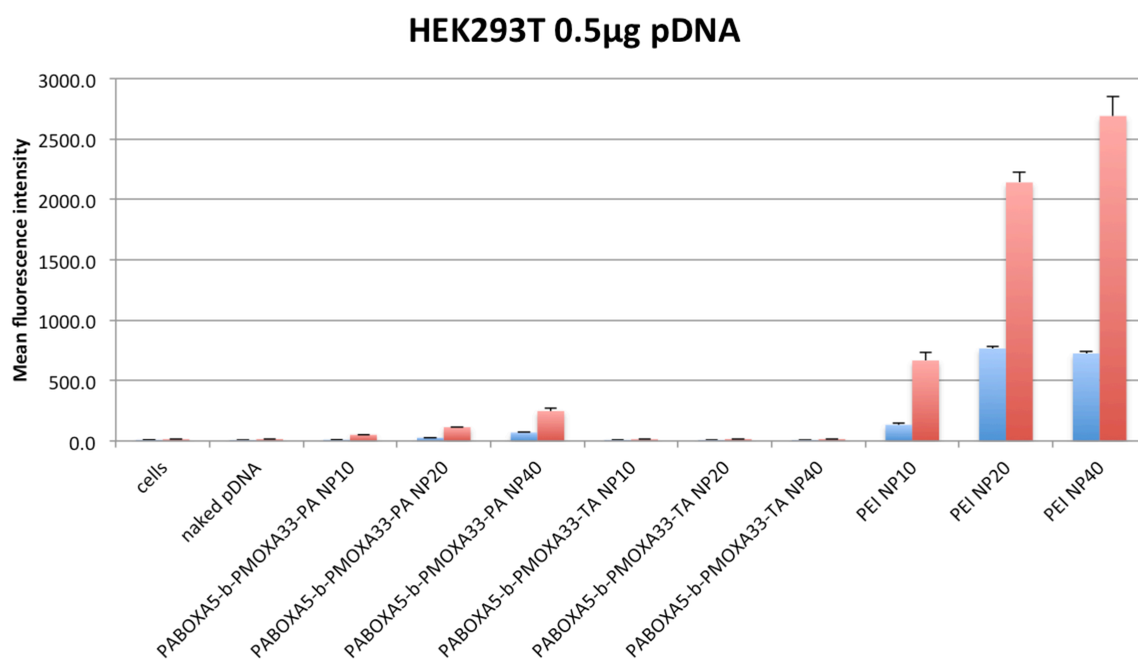
A)



B)



C)



D)

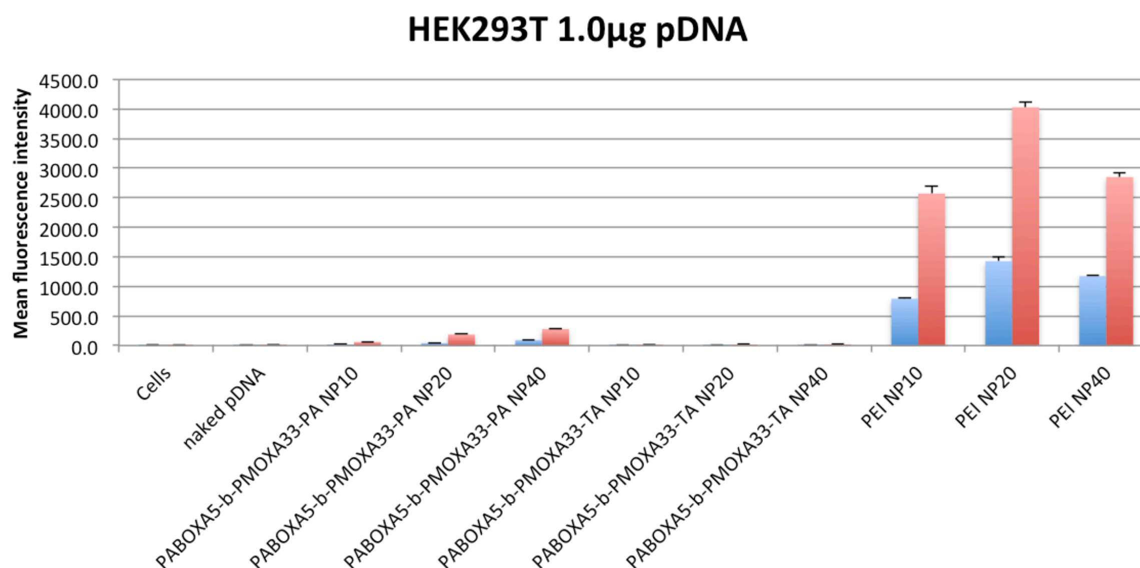


Figure 3: Comparison of transfection efficiency of circular plasmid DNA. HeLa (A, B) and HEK293T (C, D) cells were plated at the density of 3 x 10^4 cells and transfected with 0.5 or 1.0 µg GFP plasmid DNA complexed with PABOXA$_5$-b-PMOXA$_{33}$-PA, PABOXA$_5$-b-PMOXA$_{33}$-TA or PEI at N/P ratios of 10, 20 and 40. Blue columns represent 24 h, while red columns 48 h measurements. The data shown are the mean and the SD from three different experiments.

Shape of the transfection nanoparticle in TEM and AFM

Figure 4 shows the transmission electron microscopy of the resulting transfection nanoparticles created from PABOXA$_5$-b-PMOXA$_{33}$-PA, PABOXA$_5$-b-PMOXA$_{33}$-TA and 25 kDa PEI, respectively. Circular plasmids display, in the case of PEI a random coil structure in an approximately spherical, well compacted shape. In contrast, PABOXA$_5$-b-PMOXA$_{33}$-PA forms flowerlike structures, whereas PABOXA$_5$-b-PMOXA$_{33}$-TA shows the similar structure as naked pDNA.

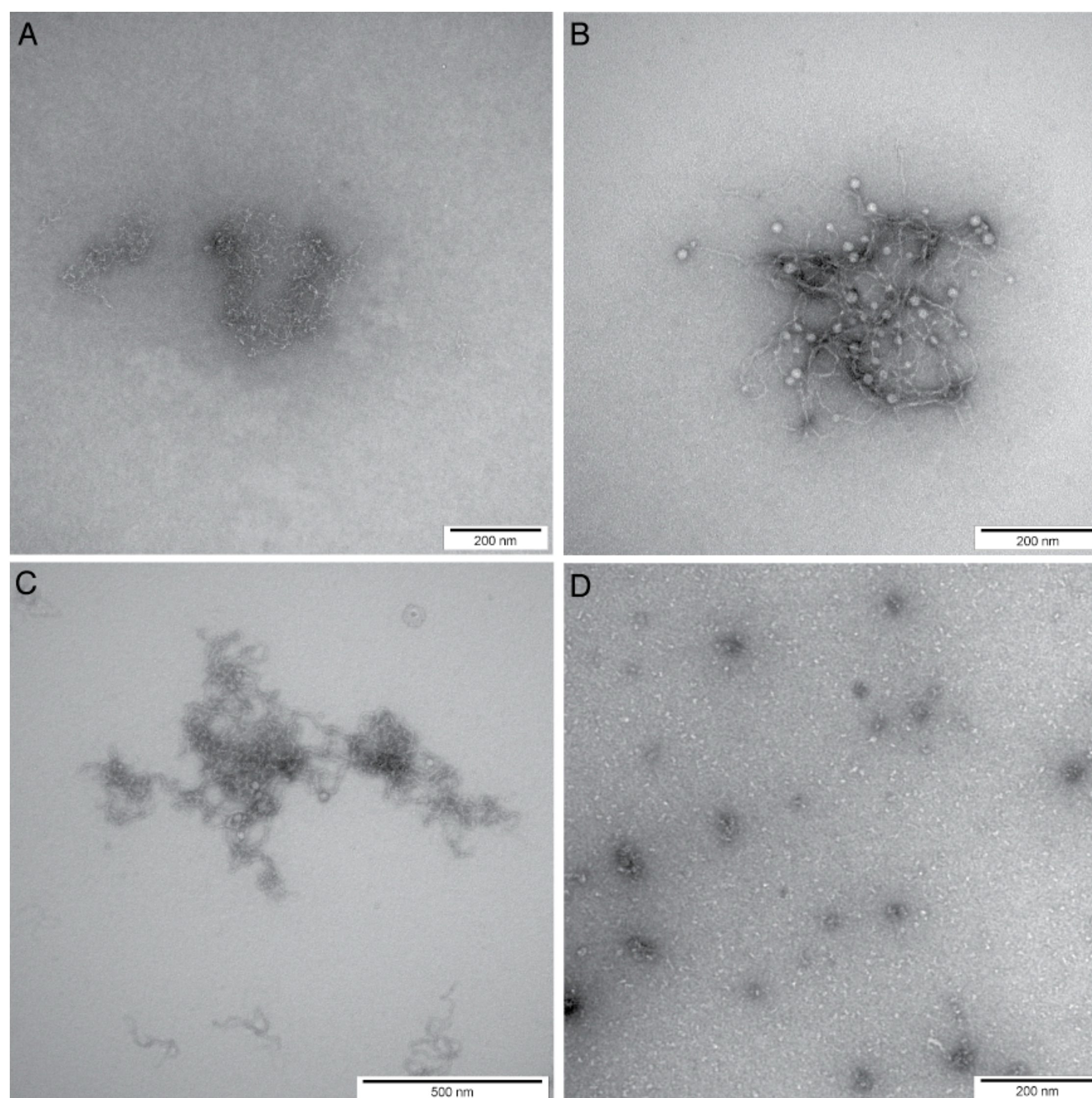


Figure 4: Transmission electron microscopy images of free circular plasmid DNA (A) and complexed with PABOXA$_5$-b-PMOXA$_{33}$-PA (B), PABOXA$_5$-b-PMOXA$_{33}$-TA (C) and 25 kDa PEI (D).

Figure 5 presents the atomic force microscopy images of free plasmid DNA, PABOXA$_5$-b-PMOXA$_{33}$-PA, PABOXA$_5$-b-PMOXA$_{33}$-TA and PEI. Naked plasmid DNA was first imaged displaying clearly visible double stranded DNA with closed loop structures forming twists, which are typical for uncondensed DNA (16). AFM images of PABOXA$_5$-b-PMOXA$_{33}$-PA reveal, that the polymer forms large aggregates. In contrast, PABOXA$_5$-b-PMOXA$_{33}$-TA showed two kinds of structures: a similar morphology like naked pDNA alone and plasmid molecules together with higher core structures indicating condensation start. PEI showed strong condensation of the DNA leading to spherically shaped structures.

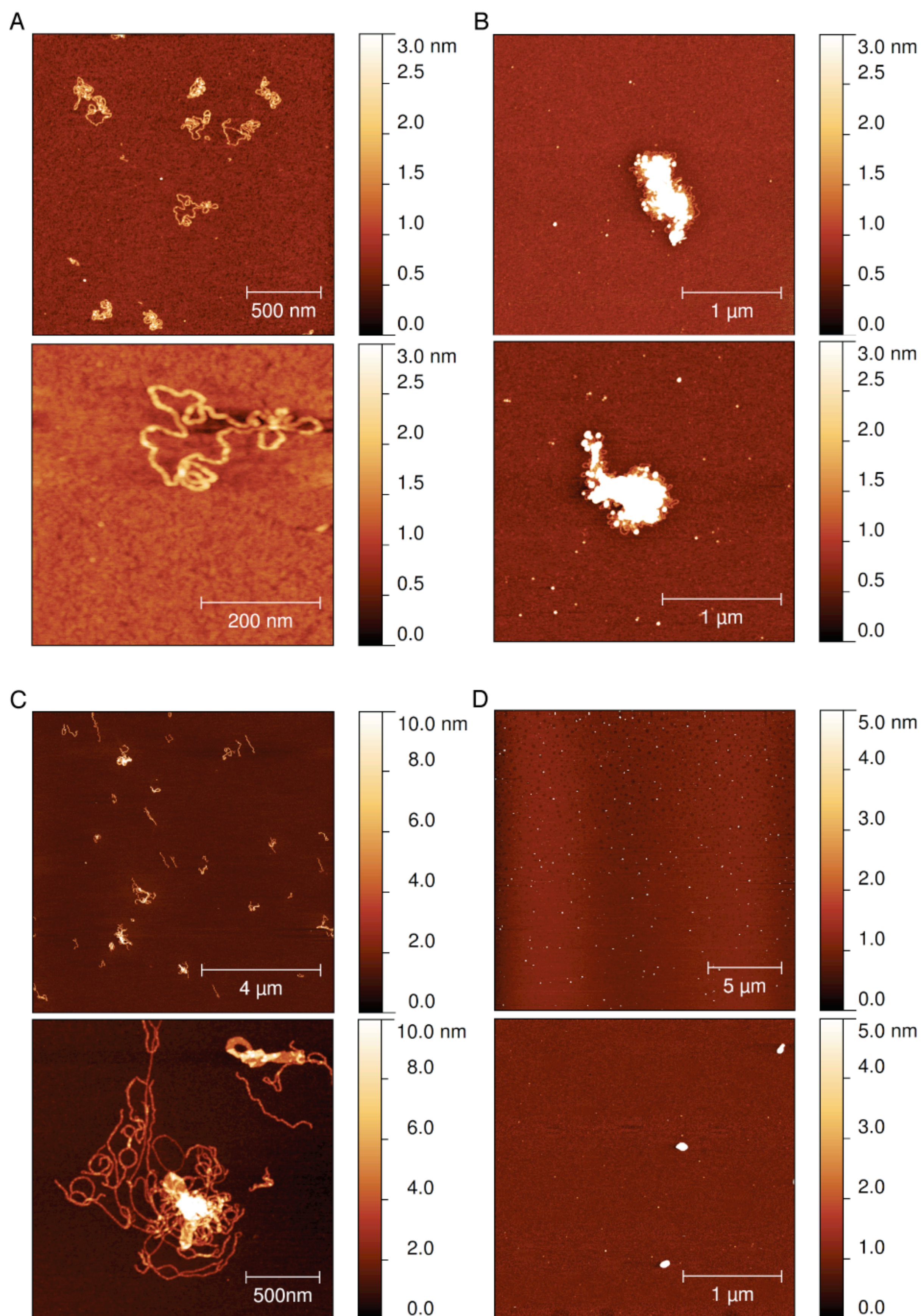


Figure 5: Atomic force microscopy images of circular plasmid DNA and DNA complexed with PABOXA$_5$-b-PMOXA$_{33}$-PA, PABOXA$_5$-b-PMOXA$_{33}$-TA and 25 kDa

PEI. Naked pDNA (A), $PABOXA_5$-b-$PMOXA_{33}$-PA/DNA complexes (B), $PABOXA_5$-b-$PMOXA_{33}$-TA/DNA complexes (C) and PEI/DNA complexes (D).

Discussion

Gene delivery is a complex process involving several steps and a challenging task to face all the barriers, such as proper DNA condensation, cellular uptake, gene expression and cytotoxicity, which have to be overcome. Our approach was the synthesis of diblock copolymers, comprising primary and tertiary amine groups. The first step, which has to be addressed, is to understand some of the factors that control DNA condensation such as DNA or salt concentration and material properties. Those parameters might affect DNA condensation as well as efficiency, size or aggregation of the nanomaterial.

Therefore, the emphasis of this study was to investigate three parameters (DNA concentration, targeted cell line, chemical condensation properties) and investigate their influence on DNA condensation and transfection efficiency. The transfection efficiency of the cationic diblock copolymers was measured in HeLa and HEK293T cells, using a plasmid DNA containing a reporter gene encoding enhanced green fluorescent protein (GFP) and compared with the commercially available transfection reagent polyethylenimine. PEI is a highly cationic polymer proven to be efficient and versatile for gene delivery in vitro (17). This study supports the fact that primary amines compared to tertiary are showing much stronger binding to DNA (12). $PABOXA_5$-b-$PMOXA_{33}$-TA showed no transfection efficiency for all tested conditions (Figure 3 A - D). This can be explained by the fact that tertiary amine groups show weaker binding with DNA and therefore lower condensation, as also approved by TEM and AFM images. The results of $PABOXA_5$-b-$PMOXA_{33}$-PA compared with PEI showed that the transfection efficiency is dependent on the time, the cell line as well as the DNA concentration used (Figure 3 A – D). Increase in the N/P ration lead to higher transfection efficiency due to an increase in DNA condensation and endocytotic uptake by the cells, which is known by the literature (18,19). In principle different expression between $PABOXA_5$-b-$PMOXA_{33}$-PA and PEI may be because of the high amount of charges of PEI, helping to efficiently condense DNA into small spherical structures, which are favourably been taken up by endocytosis due to their cationic characteristic allowing interaction with negatively charged sugar moieties on

the cell surface. The study revealed, that the best transfection efficiency of $PABOXA_5$-b-$PMOXA_{33}$-PA was 10 times lower compared with PEI, when using HEK cells (Figure 3 D).

The microscopy data showed clear differences in the shape of the complexes. PEI complexed plasmid DNA showed random coil appearance in well compacted, roughly spherical shape while $PABOXA_5$-b-$PMOXA_{33}$-TA showed flowerlike structures, implying that the condensation process has started (Figure 5 B – D). This was also been seen by Lidgi-Guigui et al (20). Condensation might lead either to monomolecular condensation or multimolecular aggregation as it can bee seen with $PABOXA_5$-b-$PMOXA_{33}$-PA, forming multimolecular aggregates (Figure 5 B). The size of the condensed DNA/nanoparticle plays a crucial role for efficient transfection, since smaller particles are more likely to be taken up the cells then larger particles, which can only enter the nuclear pore in dividing cells (21). This might explain the weak gene expression since the polyplexes are still being taken up by cells but due to the aggregate structure are not capable to enter the nuclear pore and therefore successfully start gene expression. In contrast, the compacted PEI polyplexes show strong transfection efficiency, which can be correlated to the highly condense and cationic structures formed, simplifying cellular uptake. A difference can be seen for $PABOXA_5$-b-$PMOXA_{33}$-PA by TEM (Figure 4 A) and AFM (Figure 5 B). TEM shows clear spherical structures, sticking to DNA and therefore forming a mesh like structure whereas AFM shows highly condensed multimolecular aggregates. This difference may be explained by the different sample preparation methods for the specific microscopic observations.

Thus, the obtained results demonstrate proof of concept. It supports the fact that primary amines compared to tertiary are showing much stronger binding to DNA and better transfection efficiency. Furthermore, it gives insights into DNA concentration and cell line dependency regarding transfection efficiency.

Conclusion

The aim of this study was first to synthesize and understand the influence of primary and tertiary amines in regard to transfection efficiency of plasmid DNA. Furthermore, it attempted to give insights into the condensation efficiency of the diblock copolymers by visualization and comparison of the condensates via transmission

electron and atomic force microscopy. Transfection efficiency studies showed weak transfection with diblock polymers containing primary amines, whereas there was no significant signal detectable for polymers containing tertiary amines. Electron microscopy data revealed flowerlike structures of the diblock polymer consisting out of primary amines, whereas the AFM images showed strong aggregation formation. Diblock polymer consisting out of tertiary amines revealed for both, TEM as well as AFM analysis, showed similar morphology like naked pDNA alone**,** which is in agreement with the observed flow cytometer data of $PABOXA_5$-b-$PMOXA_{33}$-TA compared with naked pDNA**.**

The data observed showed proof of concept and gives stimulation for further experimental investigations coupling in other factors like improvements of polymer design, pH, intracellular barriers etc, which need to be further explored.

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Efficient receptor mediated siRNA delivery in vitro by folic acid targeted pentablock copolymer-based micelleplexes.

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Keywords: nanoparticles, siRNA delivery, targeting, block copolymer, serum stability

Synthesized copolymers presented in this manuscript are currently under patenting process. Manuscript ready for submission.

Abstract

Since its discovery, small interfering RNA (siRNA) has gained a lot of attraction as a promising biological strategy for treatment of several diseases. Due to its instability and poor cellular uptake efficiency, several delivery systems have been developed to overcome these problems. In this study we describe the design and characterization of novel pentablock based polyplexes, based on the combination of cationic pentablock copolymers with folic acid functionalized copolymers for target specific siRNA delivery. The resulting pentablock based micelleplexes spontaneously formed polymeric micelles of about 21 nm with a hydrophobic core surrounded by a cationic poly-2-(4-azidobutyl)-oxazole (PABOXA) and hydrophilic poly-2-methyl-oxazole (PMOXA) layer. The micelleplexes described form highly stable particles even in complete serum after 24 h compared with other cationic polymer and nanoparticles,

which show aggregate formation in serum containing buffer solution. Targeted siRNA delivery and gene knockdown could be shown using GFP expressing HeLa cells, resulting in ~ 31% and ~ 8% suppression of the gene (GFP) for targeted and non-targeted micelleplexes, respectively. Comparison studies of folic-receptor positive HeLa cells with normal folic-receptor-negative HEK293 cells revealed clearly specific receptor mediated cellular uptake of fluorescently labeled siRNA.

The new designed pentablock polymer based material showed no cytotoxicity during in vitro experiments, hence turning it into a promising candidate for further in vivo applications.

Introduction

Since the discovery of RNA interference (RNAi) there has been a considerable interest in the field of biology and medicine due to its possible huge capability for personalized treatment of several human diseases (1,2). The potential to selectively interfere and stop the development of gene-dependent diseases turns siRNA into an ideal therapeutic agent to suppress cancer growth. However, naked siRNA have a very short half-life when applied to systemic circulation because of rapid enzymatic degradation and clearance by kidney or liver due to its small molecular size, in average 21 base pairs and a weight of about 13.3 kDa versus a molecular renal cut-off of 5-70 kDa for glomerular filtration (3-5). Naked siRNA molecules can also cause toxic effect, by stimulation of the immune response, off-target effects and saturation of the RNAi machinery (6).

Due to their polyanionic characteristics, siRNA molecules are not able to spontaneously cross the cell membrane and thus require a delivery platform, which allows cellular uptake as well as cytosolic release for efficient gene knockdown. In regard to this given fact, numerous non-viral delivery platforms have been developed because of their low immunogenic effects, tunable size and targeting properties such as cationic lipids, polymers, dendrimers and inorganic nanoparticles (7). However, a major drawback of non-viral delivery platforms still is their cationic nature. The positive charge of the delivery platforms is relevant to complex negative charged siRNA molecules and is often involved in endosome disruption but positive charged nanoparticles often show increased cytotoxicity, cellular uptake by unspecific binding and binding to serum proteins, which inhibits cellular uptake of the particles (8-10).

To improve the biodistribution, the specificity of cellular uptake and actively target the cell of interest, ligands can be incorporated that specifically bind to receptors on target cells to induce receptor-mediated interaction (11). Folic acid (FA) has been shown to be a favorable ligand to increase cellular uptake via receptor mediated endocytosis using polymer based delivery systems as seen by (12-14). Folate receptors are up-regulated in more then 90% of ovarian carcinomas and expressed at higher levels in lung, brain, kidney and breast carcinoma, whereas in most normal tissue it occurs at very low levels (15). However, the introduction of ligand molecules on the surface of polymer drug delivery systems can also have an adverse effect on the stability of micelles in aqueous solution.

Promising candidates for in vivo therapeutic use are carrier systems composed from poly(2-methyloxazoline)-*b*-poly(dimethylsiloxane)-*b*-poly(2-methyl-oxazoline), PMOXA-PDMS-PMOXA, enabling the design of synthetic organelles with intelligent sensor-effector functionality (16,17). These copolymers form highly stable micelles or closed vesicles with a controlled diameter upon self-assembly in aqueous solution (18). In addition, they don't show cytotoxicity, limited nonspecific plasma protein binding due to their hydrophilic outer layer and avoid detection by the immune system and therefore prolonging blood circulation lifetime (19).

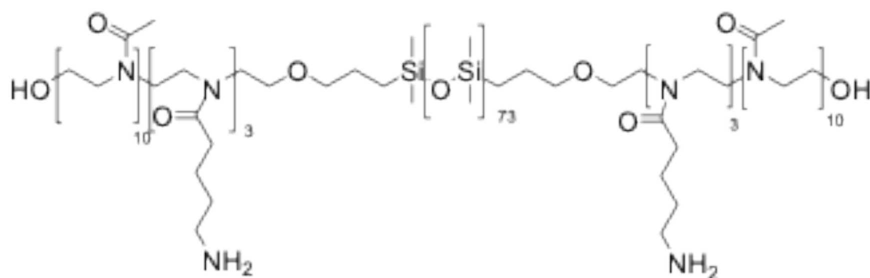
The concept of this study was the design of a siRNA drug delivery system, based on PMOXA-PDMS-PMOXA block copolymers, combining cationic pentablock copolymers with folic acid functionalized copolymers for target specific interactions. The synthesized pentablock copolymer based siRNA delivery system was studied in the present manuscript and achieved outstanding colloidal stability in complete serum, siRNA condensation, targeted delivery, endosomal escape and neutral overall surface charge preventing undesired immunological interactions. To our knowledge, no related report exists dealing with the rational design of a pentablock copolymer siRNA delivery system for successful targeted siRNA delivery, based on PMOXA-PDMS-PMOXA block copolymers.

Material and Methods

Synthesis of block copolymers

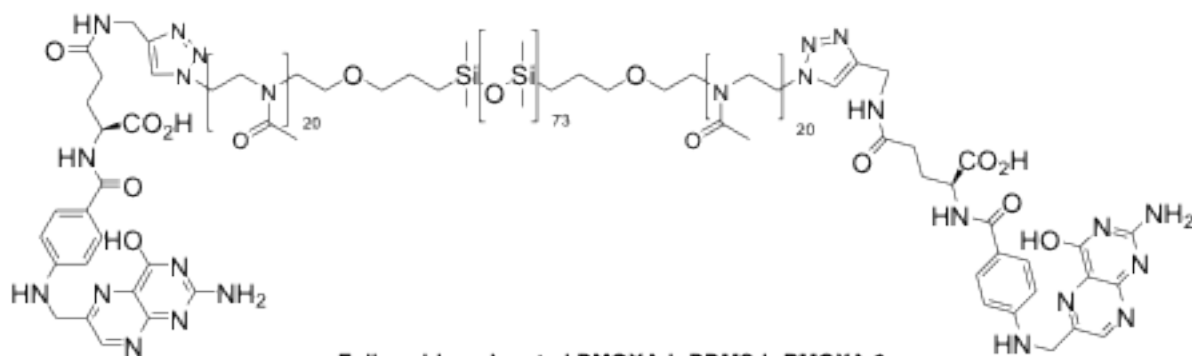
The novel block copolymers discussed here were synthesized by Dr. Kegang Liu and are currently under patenting process.

Scheme 1:



PMOXA-b-PABOXA-b-PDMS-b-PABOXA-b-PMOXA 3

Scheme 2:



Folic acid-conjugated PMOXA-b-PDMS-b-PMOXA 6

Preparation of siRNA loaded pentablock based micelles

The siRNA loaded FA-PMOXA-PDMS/PMOXA-PABOXA-PDMS based micelles were prepared by aqueous self-assembly. Briefly, the polymers were dissolved in 200 μl ethanol under permanent stirring to give 2.5 % (w/v) solutions. The two polymers were mixed accordingly to the N/P ratio considering that the FA-PMOXA-PDMS/PMOXA-PABOXA-PDMS mixture has a 0.5 mol% FA-PMOXA-PDMS content. The desired amount of this ethanolic solutions were subsequently added to 100 μl of 10 mM PBS buffer containing 40 pmol of siRNA and the mixture was vortexed for 2 min followed by gentle stirring for 1 h at room temperature.

Determination of siRNA loading capacity of pentablock based micelleplexes

The condensing ability of the pentablock based micelles was examined by agarose gel electrophoresis. The siRNA/copolymer polyplexes were self-assembled at different charge ratios (N/P) 0.5, 1, 2, 5, 10, 15, 30 and naked siRNA was used as a control. For electrophoretic separation, samples were loaded on a 1 % agarose gel and visualized with ethidium bromide (0.2 mg/ml). The retardation of siRNA mobility was detected via irradiation with UV light.

Particle size and surface charge

The particle size (diameter, nm) and the zeta potential of the nanoparticles were examined using a zeta sizer (Nano ZS, Malvern Instruments Ltd., Malvern, UK) by dynamic light scattering (DLS) and electrophoretic mobility respectively. All samples were measured in 10 mM PBS buffer pH 7.4 at 25 °C. Data were given as mean ± standard deviation (SD) based on three independent measurements. Transmission electron microscopy (TEM) images have been taken to confirm the size of the complexes.

Transmission electron microscopy

Transmission electron microscopy (TEM) was employed as imaging technique to visualize the size of the pentablock based micelleplexes in aqueous environment as well as to investigate aggregation formation of the micelleplexes and polyethylenimine in serum containing buffer solution. Samples were placed on a copper grid covered with a nitroglycerin film coated with carbon. A staining agent was added (2 % uranyl acetate).

Stability studies

Colloidal stability and agglomeration of the polymer solely and siRNA complexed with the polymer were investigated by incubating the polymer in PBS (pH = 7.4) for 4 days and siRNA-polymer complexes in PBS containing 10 % fetal calf serum (FCS) for 1 h, 6 h and 24 h at 37 ° C, respectively. At each time point, an aliquot of nanoparticle (NP) solutions was collected to measure NP size. All samples were analyzed by dynamic light scattering based on three independent measurements.

Cell culture

HeLa cells expressing green fluorescent protein (GFP) were purchased from Cell Biolabs (catalog number AKR-213) and adapted to grow in Dulbecco's Modified Eagle Medium (D-MEM; Invitrogen catalog number 31870-025). All HeLa cells used in the experiment were cultured in D-MEM containing 10 % FCS, 1 % GlutaMax^{TM}, 1 % NEAA, 1 % P/S and 1% Blasticidin. HEK cells were cultured in D-MEM containing 10 % FCS, 1 % GlutaMax^{TM}, 1 % NEAA and 1 % P/S. Cultures were maintained at a temperature of 37° C in a humified 5 % CO_2 atmosphere.

Transfection procedure

Green fluorescent protein expressing HeLa cells were transfected in 24-well plates (Corning) using either pentablock based micelleplexes or the positive control Lipofectamin^{TM} RNAiMax according to the manufacturers protocol. Cy3-fluorescently labeled anti-GFP siRNA, anti-GFP siRNA and siRNA of random sequence was synthesized by Microsynth (Balgach, Switzerland). The siRNA sequence targeting GFP is 5′-GCA GCA CGA CUU CUU CAA G-3′ (sense) and 5′-CGU CGU GCU GAA GAA GUU C-3′ (antisense).

Polymers were dissolved in Ethanol and gently mixed with 100 µl 10 mM PBS pH 7.4 containing 40 pmol siRNA at the desired charge ratio. The mixtures were further incubated for 1 h at room temperature. Polymer siRNA complexes were used immediately after preparation. Complexes were prepared inside the well after which cells (10'000 cells/well) and medium were added. The cells were washed after 24h with PBS and fresh growth medium was added following incubation at 37 °C for additional 48 h. Quantification of downregulation of target gene expression was analyzed by western blot analysis.

Western blot

HeLa cells were washed twice with ice-cold PBS and scraped into 1 ml cold PBS. The cells were pelleted by centrifugation at 3'000 rpm for 5 min and resuspended using 100 µl RIPA buffer containing protease inhibitors. The pooled cells were lysed mechanically and rotated for 30 min at 4°C. To remove nuclei, the samples were spun down (14,000 rpm, 15 min) and the protein concentration of the supernatant was determined by D_C protein assay (Bio-Rad). Then**,** 30 µg of protein/well were

loaded on 12% gels and separated by SDS-PAGE. Transfer to nitrocellulose membranes was applied at 100 V for 1.5 h, and the blots were blocked for 1 h with 5% milk powder in TBS-Tween. Primary antibodies were applied overnight, secondary antibodies for 1 h. The immunoblots were detected by enhanced chemiluminescence (Pierce) on Kodak BioMax light films (Sigma).

Cellular uptake determination of siRNA

To visualize the cellular uptake of siRNA, the cells were transfected with complexes containing Cy3-labeled siRNA. HeLa and HEK cells were incubated with non-targeted and targeted Cy3-siRNA/polymer, Cy3-siRNA/lipofectamine complexes and naked Cy3-labeled siRNA. Cells were seeded in 24-well plates in medium containing 10 % FCS for 24 h. The naked Cy3-siRNA, Cy3-siRNA/polymer and Cy3-siRNA/lipofectamine complexes were added to each well, and the cells were incubated at 37 °C for 4 h and 24 h, respectively. The culture medium was removed and the cells were washed with PBS (pH = 7.4). The cells were fixed with 4 % Paraformaldehyde (PFA) for 10 min. The cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) solution for 20 min at room temperature. Subsequently, the cells were washed with PBS two times and then mounted with fluorescence mounting medium (Dako, Carpinteria, CA). The cells were visualized using a fluorescence microscope (Olympus BX61 Diana) under a magnification of 20.

Cytotoxicity assay

The cytotoxicity of pentablock based micelleplexes was evaluated with HeLa cells by measuring cell viability using the Resazurin reduction assay. Briefly, cells were seeded in 100 ml media in 96-well microtitre plates at a density of 4000 cells /well. Following overnight incubation, the cells were exposed to a range of different concentrations of pentablock based micelleplexes and grown at 37°C under a 5% CO_2 atmosphere for 24 h and 48 h. Then, 6 ml of 0.02% (w/v) Resazurin (Sigma-Aldrich, R7017) in phosphate buffered saline (PBS) was then added to each well and incubation was continued for an additional 1 h. Finally the fluorescence was read using a spectramax GEMINI XS microplate reader (lexc = 544 nm, lem = 590 nm).

Results and Discussion

Preparation and characterization of nanoparticles

The pentablock based micelleplexes were prepared upon self-assembling in aqueous solution at the desired N/P ratio as shown in Figure 1. Dynamic light scattering (DLS) data showed a hydrodynamic diameter of 21 ± 3 nm (Figure 2). The slightly smaller hydrodynamic diameter observed by TEM analysis can be explained by the fact that the samples are dried during sample preparation, which leads to a decrease in particle size.

To examine the loading capacity of the pentablock based micelleplexes, a gel retardation assay was employed (Figure 2). The quantity of siRNA was set as a fixed value, whereas the polymer concentration was increased accordingly to the N/P ratio. Complexation of siRNA with FA-PMOXA-PDMS/PMOXA-PABOXA-PDMS based micelles completely prevented siRNA migration at an N/P of 5, indicating full neutralization of the negative siRNA charge. Fully complexed siRNA stayed in the gel loading wells due to their large size of the complexes.

The zeta potential of the polyplexes was determined by electrophoretic mobility and reveals that the particle surface of the polyplexes is slightly positive (+ 4 mV) before addition of siRNA, indicating that most of the positive charge is shielded by the outer PMOXA shell. It has been proven that nanoparticles with almost neutral surface charge (zeta potential between – 10 and + 10 mV) are less likely to effect immunological reaction in vivo (20,21). The micelleplexes, prepared at N/P 5, with a moderate positive charge (+ 4 mV) and small size (21 ± 3 nm) were selected for further biological experiments.

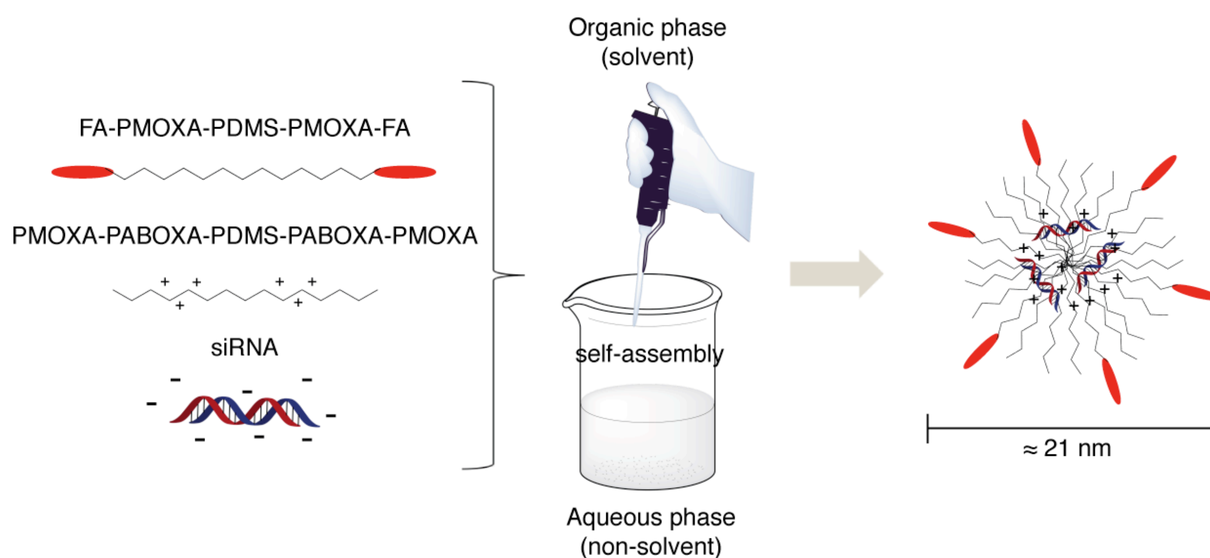


Figure 1. Illustrative representation of siRNA pentablock copolymer formation.

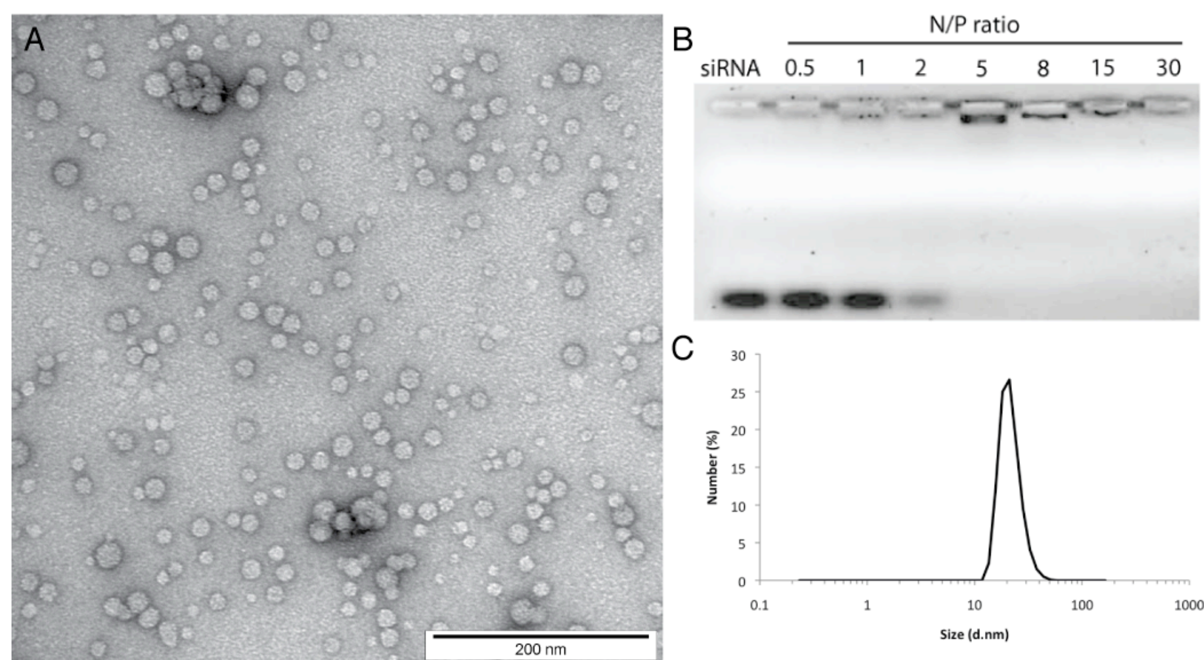


Figure 2. Characterization of the size and siRNA loading capacity of the micelleplexes. (A) Size determination by TEM. (B) Polymer based complexes at different N/P ratios were loaded onto agarose gel and electrophoresis was carried out. (C) Hydrodynamic size of micelleplexes measured by DLS. The FA-PMOXA-PDMS/PMOXA-PABOXA-PDMS micelleplexes have a diameter of 21 ± 3 nm.

Stability studies

Cellular interactions of nanoparticles are well known to be based on physicochemical properties such as size, surface charge, structure and chemical composition of the

particles (22-24). One of the key factors, which directly relates to cellular uptake is the surface charge of NPs (25).

Positive charged NPs are strongly being taken up by the cells due to unspecific electrostatic interactions with negatively charged sugar moieties of monosaccharides and polysaccharides on the cell surface. Furthermore, the use of positive charged NPs in biological systems is often constrained due to the inherent toxicity associated with them (26). In regard to in vivo administration of the NPs, the positive surface charge often lead to binding with biomolecules such as serum proteins in biological fluids (27). Cationic polymers are well known to form aggregates in serum containing medium, which turns them highly unfavorable for further in vivo applications. Therefore, it is important while designing new vectors for siRNA delivery to consider the physicochemical properties to construct optimal nanoformulations for efficient *in vitro* and *in vivo* us.

To evaluate the suitability of the micelleplexes for potential biomedical applications, stability studies regarding long-term storage, degradation and serum interactions were carried out. To evaluate the long-term stability, the NPs were prepared in PBS (pH = 7.4) at 37 ° C for 4 days and the size and zeta potential were measured as shown in Figure 3. During the study there was no significant change in the average diameter of the NPs whereas the surface charge decreased within time, which might be explained by the increased absorption of negative ions from the buffer solution such as phosphate ions.

The stability of the pentablock based micelleplexes and the polymer/siRNA complexes in 10% serum, was investigated by DLS measurements. For the detection of nanoparticle aggregation, we chose to use DLS intensity distribution instead of the more popular number distribution, which is usually being used for nanoparticle characterization, due to the high sensitivity of the detection of larger particles such as aggregates compared to single particles. Based on the Rayleigh scattering approximation, the intensity of the scattered light by a particle is proportional to the sixth power of its diameter. Therefore, larger particles such as aggregates will be easily detected by the intensity distribution even though the total numbers within the whole sample is small. As seen in Figure 4 A**,** measurement of 100% serum shows two peaks at ~ 10 and 50 nm, which are usually observed due to the high concentration of proteins in the serum as described by (28).

Pentablock based micelleplexes and 25 kDa PEI were tested in serum containing buffer solution and analyzed after 1 h, 6 h and 24 h. As seen in Figure 4 B**,** after addition of the micelleplexes to the serum containing buffer solution, no significant change of the intensity spectra for large aggregates could be detected after all measured time-points. The micelleplexes are similar to the size of serum proteins but have in comparison much lower concentrations. On the contrary, the highly cationic polymer polyethylenimine, which is one of the most successful and widely used polymer for drug delivery shows an additional peak at much larger size, indicating aggregate formation. This observation could be assured by TEM images showing aggregate formation as compared to the pentablock based copolymers (Figure 4 D - E).

The high colloidal stability of the micelleplexes can be attributed to their architecture. The PMOXA outer shell masks most of the positive charge of the PABOXA block, therefore reducing unspecific interactions with serum proteins. Former studies have evaluated that nanoparticles with zeta potential in the range between - 10 and + 10 mV are less likely to cause immunological reactions (20,21).

Furthermore, after loading of siRNA onto the micelleplexes a decrease of the zeta potential can be observed compared to the naked pentablock copolymer micelleplexes turning them slightly negative (- 1 mV). These studies demonstrated the excellent stability of pentablock based micelleplexes for potential *in vivo* application.

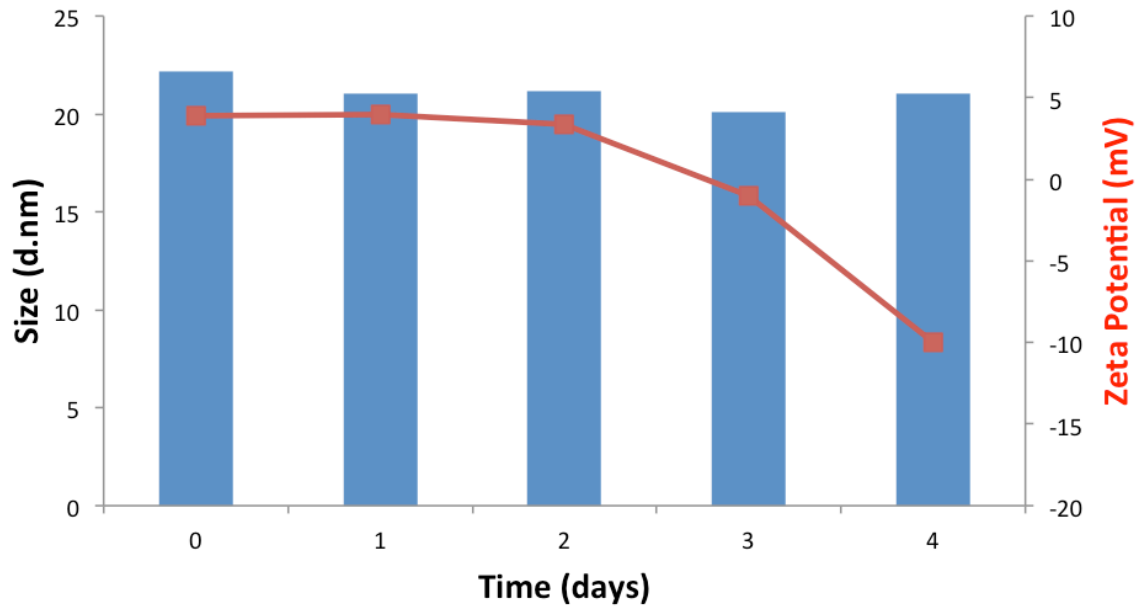


Figure 3. Long-term stability conformation of micelleplexes. Stability conformation over 4 days indicating no significant change in the average diameter (blue), whereas a decrease of the zeta potential (red) can be measured.

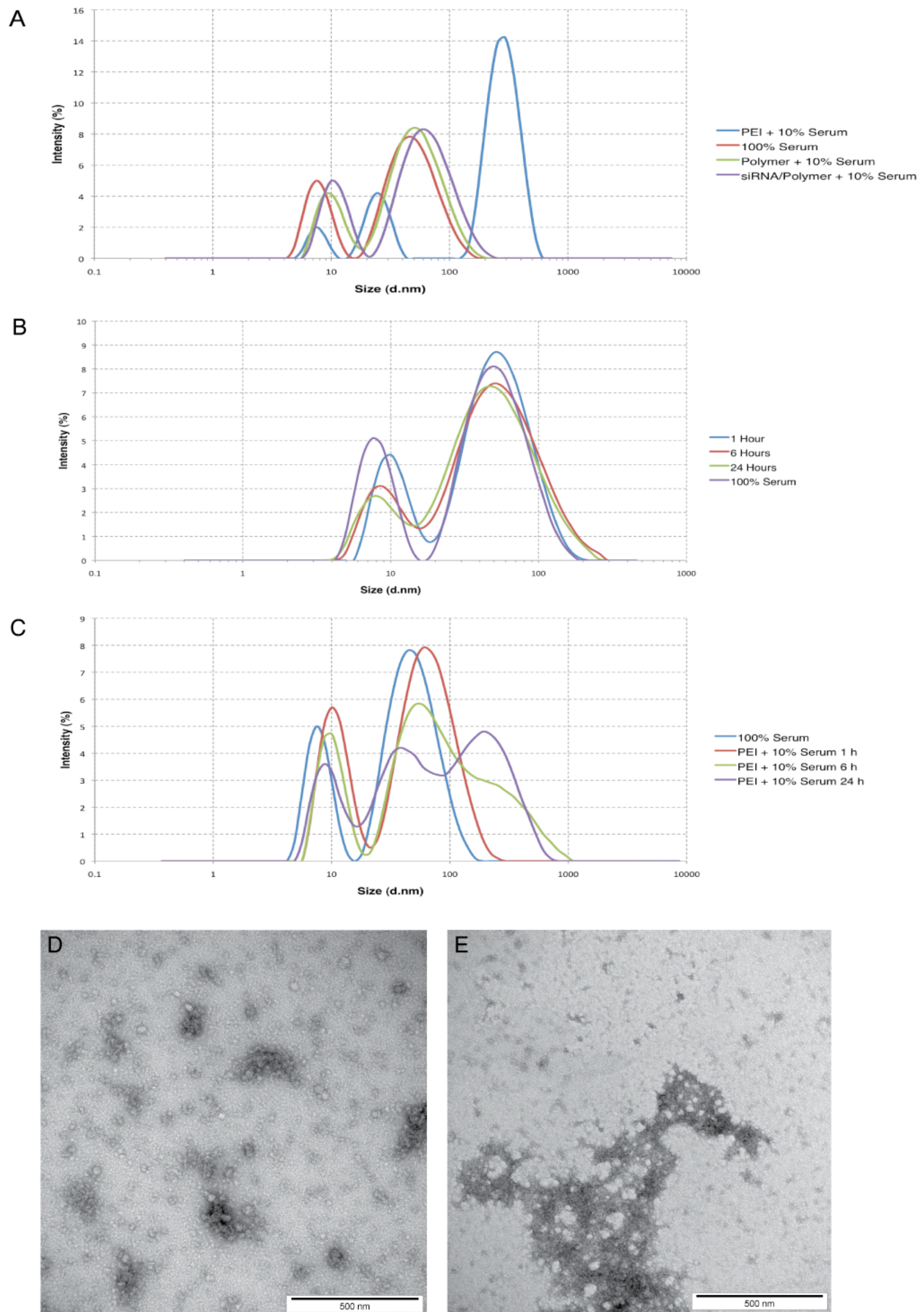


Figure 4. Characterization of the colloidal stability of the micelleplexes. (A) Hydrodynamic size measurements of serum, Polymer + 10% serum, siRNA/Polymer + 10% serum and 25 kDa PEI in 10% serum. As compared with the micelleplexes,

the hydrodynamic size pattern of the highly cationic PEI shows an aggregate peak. (B - C) Hydrodynamic size measurements of micelleplexes and 25 kDa PEI at 1 h, 6 h and 24 h in 10% serum. TEM images of micelleplexes (D) and 25 kDa PEI (E) after 24 h in buffer, containing 10% serum, demonstrating aggregate formation of PEI.

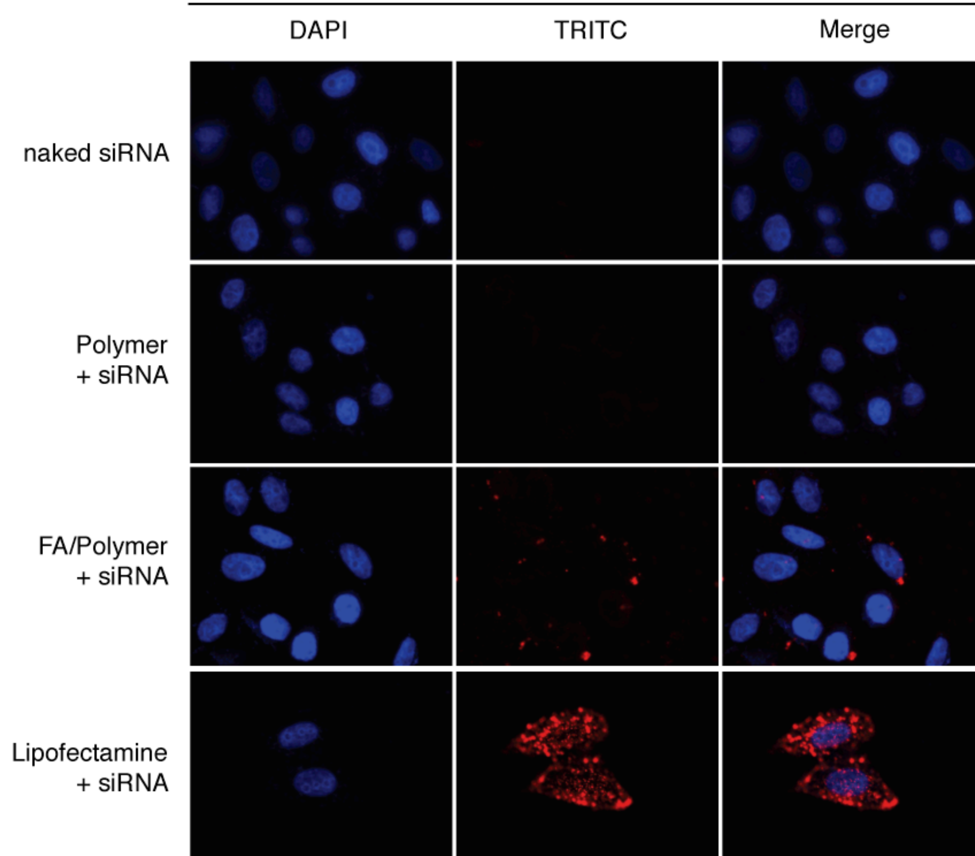
Cellular uptake of siRNA

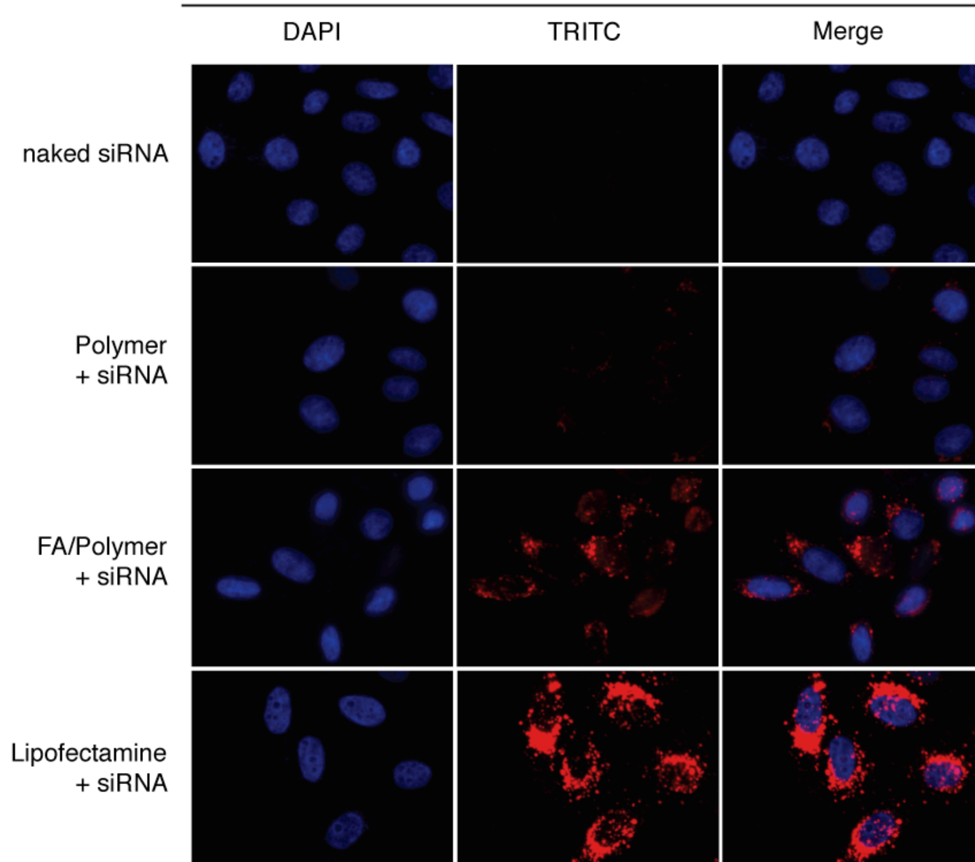
We compared the cellular uptake efficiency of non-targeted and targeted siRNA/pentablock copolymer complexes on cancerous folate receptor (FR) positive (HeLa) or normal FR-negative (HEK293) cells. The cellular uptake of siRNA was observed with Cy3 fluorescently labeled siRNA. Naked Cy3-siRNA was employed as negative control for uptake study. HeLa and HEK cells were incubated with naked Cy3-siRNA, non-targeted and targeted Cy3-siRNA/polymer as well as Lipofectamin/Cy3-siRNA complexes for 4 h and 24 h at 37 °C. As compared to the non-targeted complexes, the red fluorescent Cy3-labeled siRNA were strongly observed in HeLa cells treated with FA targeted complexes after 24 h (Figure 5 A). These results indicate that the cellular uptake of the FA targeted complexes is mainly based on a folate-receptor-mediated endocytosis mechanism.

However a small amount of Cy3-siRNA can be detected in HeLa cells treated with non-targeted complexes, which might be explained by fluid phase endocytosis, whereas particles are spontaneously being taken up through invagination of the cell membrane (29).

The normal FR-negative HEK293 cells showed no significant signal regarding cellular uptake of the Cy3-siRNA, for the targeted and the non-targeted complexes even after 24 h (Figure 5B) as expected, due to the lack of the folic acid receptor expression as proven by western blot analysis (Figure 6B). However, the proper mechanism of cellular uptake still remains a topic of research.

A





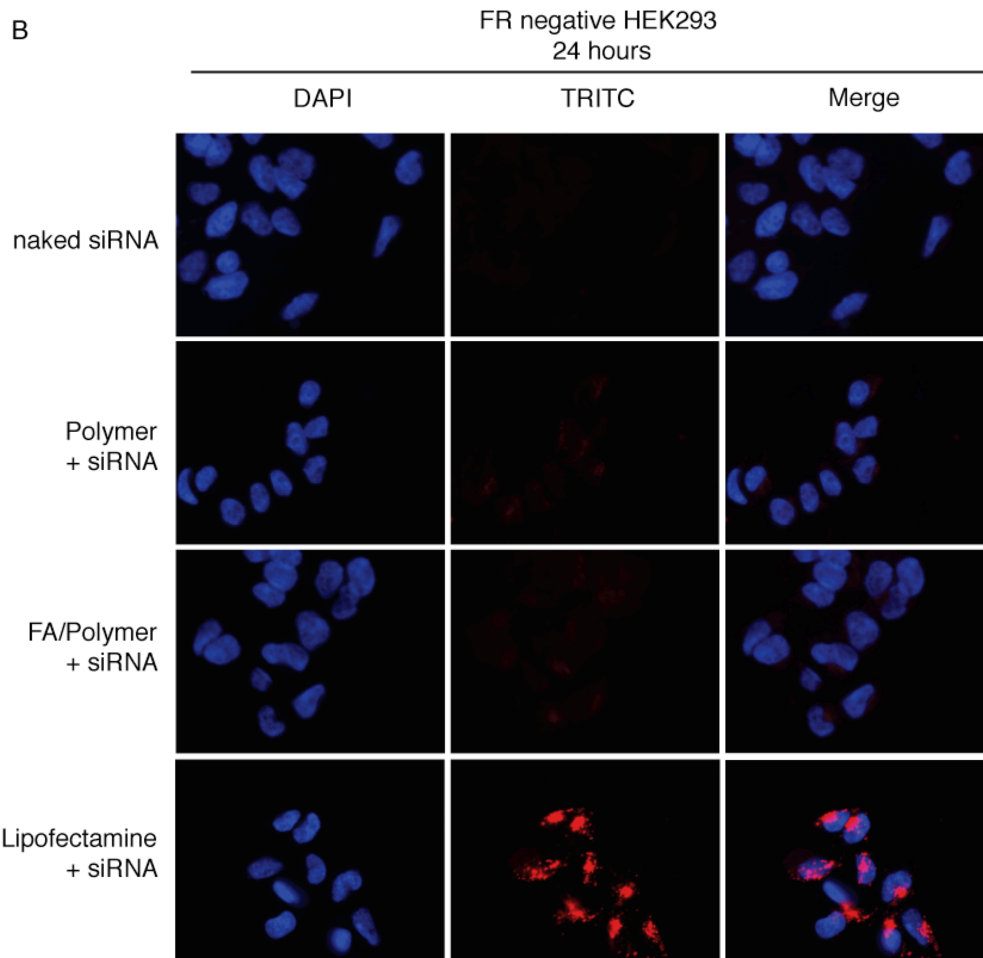


Figure 5. Cellular uptake study of siRNA. Fluorescence microscopy images showing siRNA uptake of FR-positive HeLa (A) and of normal FR-negative HEK293 (B) cells, after 4 h and 24 h, respectively. Lipofectamine shows a high cellular uptake upon unspecific binding to cells, precluding its use in targeted “in vivo” context. DAPI staining, shown in blue, was added to visualize nuclei. Red signals represent Cy3-labeled siRNA molecules (TRITC).

Gene silencing mediated by siRNA loaded pentablock copolymer nanoparticles.

To evaluate the gene knockdown efficiency of the polymer complexes, we used as a model stably green fluorescent protein (GFP) expressing HeLa cells and siRNA targeting GFP. Hela cells highly overexpress folic acid receptors (folate receptor alpha encoded by the FOLR1 gene) as compared to HEK cells (Figure 6 B*).* Quantification and visualization of the gene silencing was done by western blot analysis and fluorescence microscopy. GFP expression reduction was quantified by normalizing with tubulin expression. GFP expression was suppressed to 92 ± 4.2%

and 69 ± 5.5% using non-targeted PMOXA-PABOBA-PDMS polymer and FA-PMOXA-PDMS/PMOXA-PABOBA-PDMS polymer, respectively. In comparison, the common transfection reagent Lipofectamine showed a strong protein suppression to 46 ± 7% (Figure 6 B). Adsorption of serum proteins on the nanoparticle surface may hinder ligand–receptor interaction and therefore preventing cellular uptake.

However, these results demonstrate that introduction of a ligand clearly enhanced siRNA delivery and knockdown. Even though compared to Lipofectamine the decresase is not that high, Lipofectamine itself undergoes a non-specific cellular uptake route and is therefore not suitable for in vivo application in targeted delivery strategies. The gene knockdown efficiency can be qualitatively directly correlated with the uptake efficiency. Whereas lipofectamine shows a high cellular uptake and high level of GFP expression suppression, the targeted and non-targeted polymer/siRNA complexes lead to less cellular uptake and lower levels of protein expression suppression. Furthermore, no silencing effect was confirmed in all complexes using non-targeted siRNA (data not shown). Our results show also that siRNAs were localized to perinuclear regions and this localization was correlated to the silencing efficiency (Figure 6 C). In addition, a slight difference in the fluorescence pattern of the taken up siRNA by Lipofectamine or the micelleplexes can be seen. The micelleplexes show a more even distribution of the siRNA within the cytoplasm, whereas the Lipofectamine samples show concentrated dot like structures, localized around the periphery of the nucleus. This pattern might be explained by the fact that non-targeted micelleplexes are also been taken up by the cells, possibly by fluid phase endocytosis, whereas their intracellular fate might differ from the one of Lipofectamine. These observations possibly correlate, that RNAi activity is associated with siRNA localization to specific compartments in the cytoplasm, such as the perinuclear localization, as already stated by others (30).

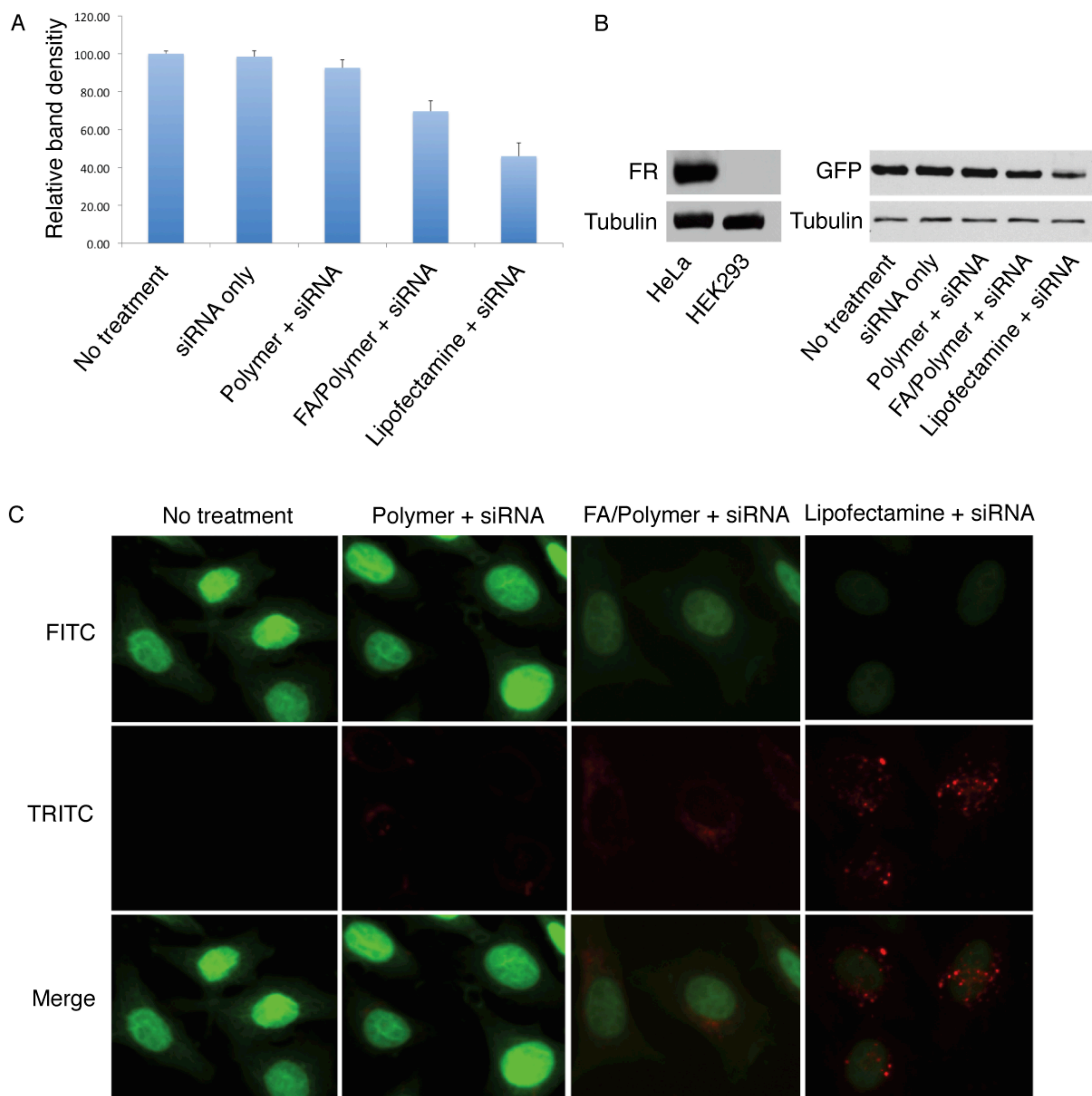


Figure 6. Gene silencing evaluation of siRNA, targeting GFP using non-targeted and targeted siRNA/polymer complexes compared with the classic transfection agent Lipofectamine. (B) Western blot showing protein expression of FR in HeLa and HEK293 cells as well as GFP knockdown in HeLa cells. 30 µg of protein from total lysates were loaded and subjected to WB analysis. (C) Fluorescent microscopy images showing cellular uptake of the siRNA. Green and Red signals represent GFP (FITC) and Cy3-labeled siRNA molecules (TRITC). Data were normalized versus an untreated control. The results are representative of three independent experiments.

In vitro cytotoxicity evaluation

Toxicity is an important consideration in the design of nanoparticles for efficient

nucleic acid delivery. The toxic effect is mostly based on the cationic nature of the carrier system and often combined with high transfection efficiency (31). Therefore, many approaches have been conducted using polyethylenglycol (PEG) to prevent cationic nanoparticles from unwanted interactions and lower cytotoxicity. However, although PEGylation of NPs has been claimed to be safe in particular, recent findings have shown that PEG might lead to complement activation related pseudoallergic reactions and should be considered for design of new delivery systems (7). Cytotoxicity of the pentablock based polyplexes was assessed with HeLa cells by measuring cell viability using the Resazurin reduction assay. As shown in Figure 7 cytotoxicity detection was at much elevated polymer concentrations then the one used in the experiments (5 mm) The low level of cytotoxicity can be explained the architecture of the pentablock copolymer. The positively charged polymer PABOXA block is surrounded by a hydrophilic PMOXA polymer layer, which generates a shielding effect of the nanoparticle from direct charge induced interactions with the cells.

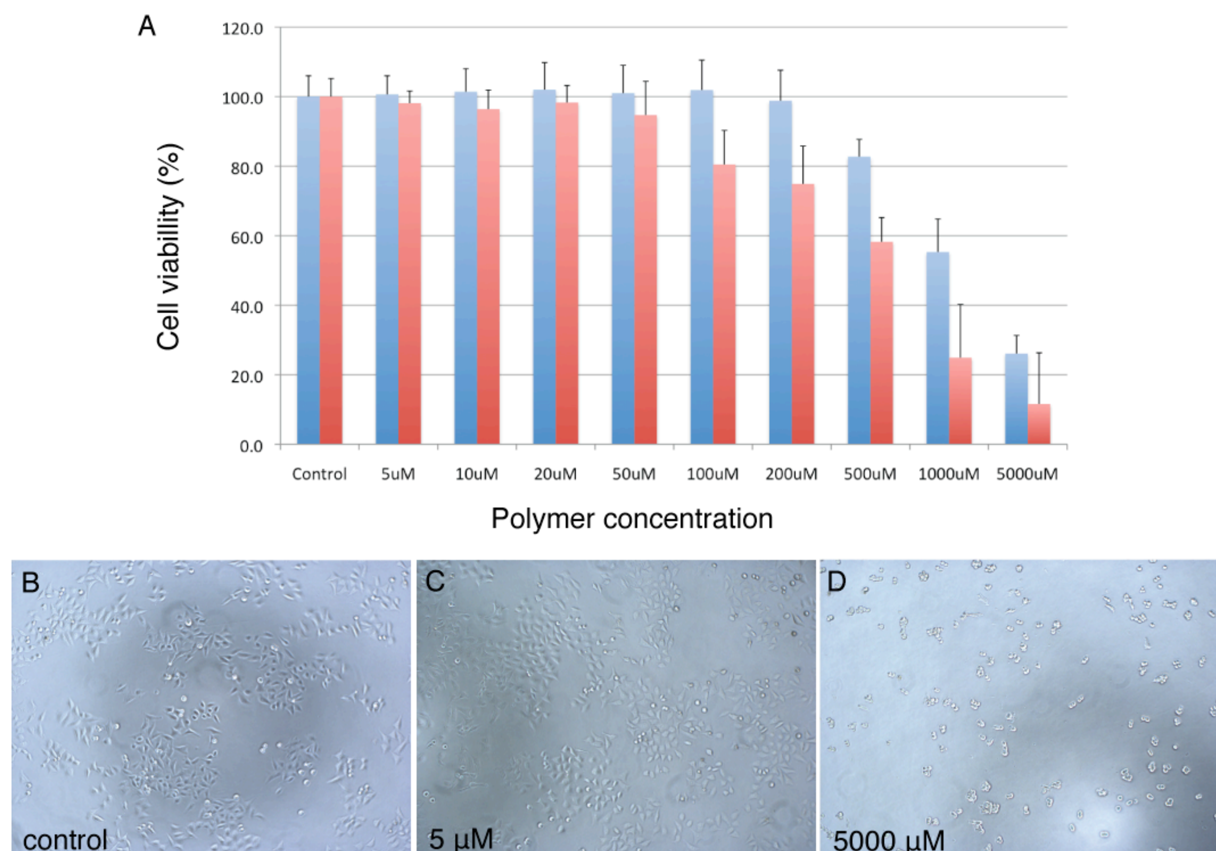


Figure 7. (A) Viability assessment of HeLa cells treated with micelleplexes at various concentrations for 24 h (blue) and 48 h (red). The cell viability is still above 90 % after 48 h at concentrations 10 x higher then the one used in the experiments compared to untreated cells. (B - D) Microscopical images of HeLa cells treated with different polymer concentrations.

Conclusion

In this study, we have developed well defined pentablock-copolymer based micelleplexes of FA-PMOXA-PDMS/PMOXA-PABOXA-PDMS for efficient targeted siRNA delivery in vitro. The currently achieved 31 % knockdown efficiency shows its possible potential regarding gene therapy. The pentablock architecture allows the formation of highly stable micelleplexes with a neutral surface charge, siRNA condensation properties, excellent colloidal stability in serum and good cytocompatibility, due to the absence of considerable cytotoxicity. Furthermore, selective delivery of the siRNA could be proven by the introduction of a ligand linked block copolymer. This pentablock based system design for siRNA delivery might provide a great feasible and potential platform to be applied *in vivo* for cancer gene therapy.

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Chapter 7: Conclusions and outlook

The field of nanomedicine has evolved into an important driver for the finding of new solutions within the field of medicine to improve medical diagnosis and therapy of various diseases. Different nanomaterials have emerged and been tested for biomedical applications whereas drug delivery systems belong to the most successful and versatile examples. As seen by this study, a broad array of different delivery systems exists with the aim to overcome the major barriers such as specific cell targeting, controlled payload release and biocompatibility. But when compared with the number of developed delivery systems, only a few have found their way into the market, demonstrating the still existing difficulties from bench to bedside.
To advance preclinical development and possible clinical application, the design of future delivery systems should comprise stimuli responsiveness and multi-functionality. To progress in this direction, comprehensive and deepened knowledge is required. Light shows highly suitable properties as a stimuli such as sensitivity, biocompatibility and spatial and temporal precision turning light-based therapies into a very promising future application to expand the diagnostic and therapeutic options. Thus, a lot of effort is being put into the usage of NIR linked to two-photon uncaging and upconverting systems, two very interesting approaches with a high impact.

Polymers have received great attention within the last decades due to their multifaceted design properties and their possible application in the biomedical field. Polymers based on block copolymers were developed for the controlled and efficient delivery of siRNA and plasmid DNA. The ability to efficiently transfect plasmid DNA into eukaryotic cells had a major impact on scientific research in the recent years as a potential cure for genetic diseases and the translation to clinical application is ongoing. In a first study we showed that next to the choice of the delivery vector, the topology of the DNA, as well as the control of shape and condensing properties seem to be key factors for efficient transfection. In a second study we synthesized cationic diblock copolymers to understand the influence of primary and tertiary amines in regard to transfection efficiency and DNA condensation. The data observed showed proof of concept and gave insights into polymer/DNA condensation properties by transmission electron and atomic force microscopy.

Small interfering RNA has gained a lot of attraction since its discovery, especially in the field of cancer research, considering a significant potential for personalized cancer treatment. We have explored the potential of novel pentablock-copolymer based micelleplexes for efficient targeted siRNA delivery in vitro with a high potential for future in vivo applications for cancer gene therapy. The design of the micelleplexes incorporated the consideration about future clinical implications such as compliment activation and hypersensitivity, which has been found for polyethylene glycol. Thus, the architecture of the micelleplexes proofed the formation of highly stable nanoparticles with a neutral surface charge and excellent colloidal stability in serum without the usage of polyethylene glycol as a shielding molecule. Furthermore, they show excellent siRNA condensation properties and good biocompatibility due to the absence of considerable cytotoxicity as well as selective delivery of the siRNA by receptor-ligand specific interactions.

Future perspectives

The synthesized pentablock based micelleplexes showed clear receptor mediated cellular uptake and efficient gene knockdown. Future steps would include the *in vivo* application of the micelleplexes to study its distribution pattern within an organism (mouse model) using a fluorescently labeled siRNA. Since the GFP cell line used represents a model system and to evaluate the variety of other possible targets of this delivery system, it would be necessary to switch towards a more clinically relevant application by knocking down specific oncological important targets to inhibit cancer growth.

In addition to properly understand and improve cellular uptake, studies regarding detailed analysis of the cellular uptake mechanism involved such as receptor-mediated endocytosis, normal endocytosis or pinocytosis should be conducted. Intracellular trafficking experiments could give insights into the fate of the micelleplexes, e.g endosomal escape and give further understanding how RNAi activity is associated with siRNA localization to specific compartments in the cytoplasm, such as the perinuclear localization.

Furthermore, targeting is an important topic for the design of a delivery system to increase specific biodistribution and therefore decrease unwanted interactions with healthy tissue. To further increase the specificity and sensitivity regarding clinical application of the system, caging of the siRNA incorporating newest developments such as two-photon uncaging and upconverting systems should be considered.

To improve plasmid DNA transfection first the variation of the polymer architecture of primary amine containing polymers, meaning size and charge ratios between the single blocks (PMOXA vs PABOXA) of the polymers, which plays an important roles in dictating DNA condensation, should be considered. Second, combination of primary and tertiary amine containing polymers should be conducted, since tertiary amine, due to their buffering capacity, can improve endosomal escape and therefore increase transfection efficiency. Furthermore, introduction of a ligand-coupled polymer for specific receptor mediated cellular could be a potential improvement.

Third, the designed primary amine containing polymers show strong aggregate formation. To better understand aggregation formation, comparison studies taking into account salt and pH dependency upon condensation should be executed.

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