Applications for DNA-encapsulated silver clusters in physics, biology and medicine

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CHAPTER 1 – Introduction

In the intricate field of applications in nanoscale science, nucleic acid oligomers have emerged as indispensable instruments as spacers and positioning tools. They owe their reliability to the strength and predictability of Watson-Crick base pairs, making them exemplary components for the construction of experiments on the scale of nanometers.

The introduction of clusters of small numbers of silver ions and atoms yields Ag-DNA constructs, a fusion of few-atom silver clusters and single-stranded DNA (ssDNA). Their optical characteristics are uniquely sensitive to the DNA scaffold, rendering them especially useful in detecting minute changes in their environment. This potential is particularly exciting in experimental biophysics, and theragnostic applications.

In essence, the combination of DNA technology for attachment and spacing, and the optical and chemical properties of Ag-DNA constructs, shows significant potential as a flexible and useful tool in various scientific areas, from fundamental research and cellular studies to ground-breaking therapeutic applications. This thesis will address a number of such applications in its chapters.

1.1 DNA-encapsulated silver clusters

Within the field of nanotechnology, a significant area of research has been silver-DNA constructs, also known as Ag-DNAs. These nanostructures, made by stabilizing silver nanoclusters with DNA oligomers, can be used as versatile fluorescent emitters[1], but also have the potential to open new avenues for applications in chemistry, biology, and materials science.

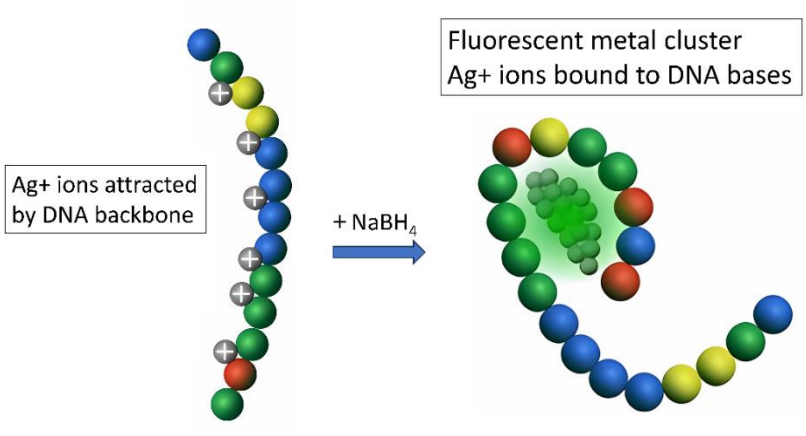

Figure 1.1: Cartoon representing fluorescent Ag-DNA synthesis. Ionic silver is introduced to specific oligonucleotide sequences in solution, which is attracted to the negatively charged DNA phosphate backbone. A chemical reduction is performed using $NaBH_4$ to partially reduce the ionic silver, yielding a partially charged cluster, typically around 10-20 atoms and ions in size. The silver ions bind strongly to DNA bases to produce a stable construct over the course of several hours. The final construct exhibits a wide-ranging fluorescence depending on the size and shape of the metal cluster, and the DNA sequence, length, and conformation.

Fluorescent Ag-DNAs stand out for their exceptionally wide range of emission colors, spanning from blue to infrared[2–5]. These complexes utilize various DNA, or even RNA, oligomers to yield vastly different emission wavelengths, making them tunable fluorophores suitable for multiple applications.

Recent advances in negative ion, high-resolution mass spectrometry have expanded our knowledge surrounding these constructs. It has been found that much of the silver in these complexes exists in a cationic form[6]. The silver nanoclusters in Ag-DNAs are described as neutral, rod-like chains surrounded by a base-bonded Ag^+ frame[7,8]. The size and shape of the metal cluster seems to significantly determine the optical properties of the Ag-DNA emitters.

The constructs are easily compatible with DNA nanotechnology and DNA origami[9,10], with excess DNA not used to stabilize the metal cluster offering an excellent attachment site for the specific addition of targets and recognition elements.

Ag-DNAs have been employed in a wide range of applications, such as biological imaging[11] and sensitive signaling for the detection of objects such as viruses[12]. They can act as sensors for single-base mutations and microRNAs[13]. The dependence of the optical properties to the DNA dynamics makes them highly sensitive to their surrounding chemical environment[14]. They are promising candidates for intracellular bio-sensing applications, competing with existing fluorescent probes.

Finally, silver's inherent antibacterial[15,16] and potential anti-cancer properties can add an additional application to these structures as a therapeutic agent. The combination of optical sensing applications and effective treatment in one several nanometer sized package makes further Ag-DNA development a very exciting prospect.

1.2 A study of DNA hybridization in antisense therapeutics

Modern scientific applications extensively harness the versatile properties of nucleic acid oligomers. Among the array of its utilities, DNA's characteristic Watson-Crick base pairing[17] paves the way for their usage as nanoscale spacers and positioning tools[10,18,19]. Molecular biology has utilized this material in applications such as DNA microarrays[20,21] to monitor significant biological processes like gene expression. Furthermore, the area of therapeutics has seen oligonucleotides emerging as promising candidates. Especially notable is the use of single-stranded antisense oligonucleotides (AONs) in splicing modulation[22–24].

Recent years witnessed two such AONs, eteplirsen and nusinersen, earning FDA approval[25] to combat Duchenne muscular dystrophy and spinal muscular dystrophy, respectively. For these therapies to be effective, it's pivotal to comprehend the hybridization dynamics of AONs with target RNA.

While DNA hybridization energetics are relatively understood[26–29], achieving measurements on the dynamics of these short sequences, on the level of individual base pairs, often necessitates aggressive modifications, such as the incorporation of fluorescent dyes[30]. Such alterations can change DNA conformation and stability[31,32]. Furthermore, most studies have been executed on surface-bound DNA, leaving uncertainties about their behavior inside living cells. For therapeutic AONs, understanding how sequence mismatches can affect binding efficiency is of vital importance. Misaligned binding might inadvertently interfere with sequences that share partial homology, undermining the therapy's effectiveness, and increasing the risk of side effects to the patient.

In Chapter 2 of this thesis, a novel probing approach using fluorescent DNA-stabilized silver clusters (Ag-DNA) is introduced to understand the binding efficiencies of short DNA sequences. The unique fluorescence emitted by Ag-DNA[1], strongly dependent on the DNA sequence and length[4,33,34], was used to develop this method. This study employed a 19-base DNA sequence (19b-Probe), known for its robust fluorescence emission upon silver addition. The 19b-Probe method proves to be a suitable tool to study DNA hybridization without the use of additional fluorescent dyes.

The 19b-Probe was appended to sequences that complement parts of exon 51 in the dystrophin transcript, a crucial segment linked to Duchenne muscular dystrophy. The probe's fluorescence intensity post-stabilization with a silver cluster provides insights into DNA-DNA binding efficiency.

The chapter sheds light on the impacts of nucleotide mismatches on DNA hybridization, which holds great significance for medical applications of DNA. Our work shows that the impact of a base mismatch is not universal but varies based on its location. This is derived from the probe's detailed observations, where mismatches near the DNA strand's center and edges shows a pronounced insensitivity. A simplified theoretical model affirms the experimental observations, suggesting that strands of certain lengths, like the 'AON1' experimental sequence, might inherently be more resistant to mismatches.

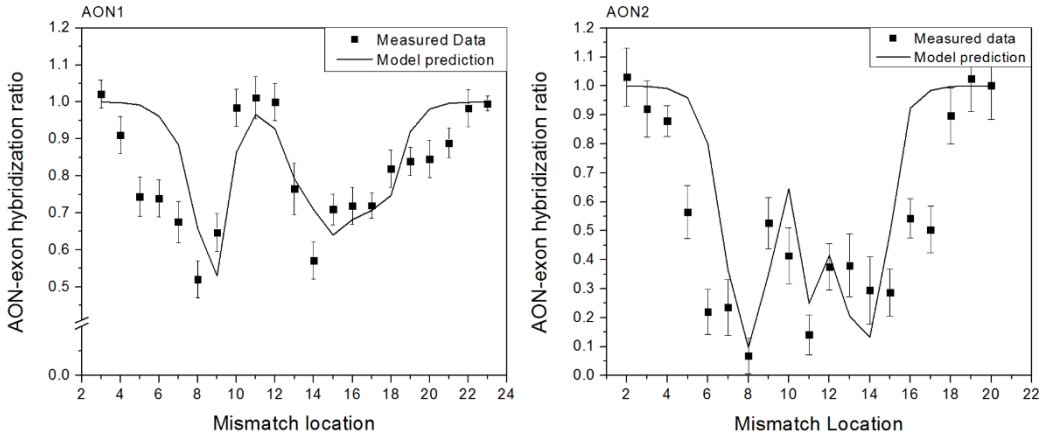

Figure 1.2: Measured DNA-DNA hybridization ratios (black squares), and the calculated behavior from our model (black curve), for the AON1 (left) and AON2 (right) sequences. The AON sequences are chosen to be different in length and sequence, and are relevant test sequences to the treatment of Duchenne muscular dystrophy. Our theoretical model predicts the binding efficiency of two DNA strands with a mismatch positioned in various places along the double helix. Points are experimental results measuring the hybridization, showing agreement with our predicted results.

Although the experiments attempt to closely mimic physiological conditions, there are certain differences to the treatment environment. The primary distinction being the DNA employed in the experiment versus the RNA strands in actual treatments. RNA's distinct molecular and helical structures might introduce unforeseen dynamics in mismatch dependencies[35]. Moreover, intracellular conditions represent strongly varying ionic concentrations which could impact DNA dynamics.

Notably, the 19b-Probe method is widely applicable, promising more profound insights into oligonucleotide binding in diverse conditions. We have proceeded to apply it to detect binding to specific targets relevant to antibiotic resistance in pathogens. Through multiplexing by targeting multiple sections within a gene, a strong signal can be produced even at low target concentrations.

Finally, an alternative method is introduced, which utilizes a hairpin-shaped DNA construct that shows sensitivity to hybridization to a target sequence. The difference in this technique is the use of already fluorescent Ag-DNA constructs to be introduced to the target, unlike the 19b-Probe method, where silver has to be introduced after binding. Although this method shows a weaker specificity and sensitivity, it has potential as an alternative method for use under harsher or live cell conditions.

1.3 A sub-nanometer ruler by Energy Transfer to DNA-stabilized Few-atom Silver Clusters

The precision of observation at the nanometer scale is a cornerstone for various research endeavors, especially when dealing with living biological systems. While optical microscopy offers a significant insight, it encounters inherent limitations when observing moving entities or seeking resolutions in the range of several nanometers. An existing alternative is the utilization of indirect techniques to derive distances, drawing from methods such as optical interactions, Electron Paramagnetic Resonance (EPR), and Nuclear Magnetic Resonance (NMR). Despite their utility, these methodologies are frequently restricted by the incorporation of, for example, unstable spin labels, which often show high sensitivity to chemical reduction and are unsuitable for in vivo environments.

The chemical robustness of fluorescent labels combined with their limited sizes make them crucial in interaction-based optical techniques for measuring proximity, even in live samples. Förster Resonance Energy Transfer (FRET)[36], is one very commonly used interaction, facilitating the detection of two objects within a range of up to around 8 nanometers. This technique typically utilizes a fluorescent dye as a donor, and some form of quencher as an acceptor, which shares its absorption spectrum with the emission spectrum of the donor. Many such combinations exist, including pairs of fluorescent dyes (such as Cy3 and Cy5), leading to flexibility of use in different measurement or microscopy setups, and in experiments with inherent fluorescence such as in labeled cells. Dark quenchers (such as Black Hole Quenchers) that do not exhibit their own fluorescence, and can be used with a wide range of donors, also exist,

which can be beneficial in low intensity experiments that require a low background. These interactions exhibit a distinct $1/r^6$ dependence on distance within a 3-8 nm range, and can be a strong tool for measuring attachment and interactions between objects labeled appropriately.

In order to measure even more subtle distance variations, there's potential in leveraging Nano Surface Energy Transfer (NSET) – an interaction between a fluorescent dye and a metal surface[37]. The underlying principle here lies in the energy transfer from an activated dipole, such as a fluorescent dye, to image dipoles on the metal surface, generated by the metal's free conduction band electrons. This process allows for proximity determination of a fluorescent dye in relation to a metal surface, detected through the measurement of fluorescence quenching. NSET exhibits a high quenching efficiency and range of operation (10-15 nm), and most crucially, a larger potential resolution due to its gentler transition in the distance dependence as compared to FRET. As such it holds significant promise in biological settings[38–40], shedding light on protein interactions or nucleic acid activities.

However, a primary challenge in the path of advancing NSET-based studies has been integrating metal surfaces into experimental settings. Traditional metal nanoparticles, compared to fluorescent dyes, are notably larger than many biomolecules, making their inclusion difficult. DNA technology can provide a suitable tool in these types of experiments. Double-stranded DNA spacers have emerged as a potential vehicle to study NSET processes[41].

In Chapter 3, we utilize the few atom silver clusters in Ag-DNA constructs as a more suitable acceptor in the NSET interaction. Ag-DNA, with its particle minute size typically ranging between 5-20 atoms, operates on the limit between the molecular and particle regimes. The unique properties of these structures allows incorporation of a very small metal surface, that notably is still capable of exhibiting many features of larger metal particles[7,8]. Simultaneously, conventional interactions between molecular fluorophores, such as FRET, have been exhibited using Ag-DNA as well[42].

Ag-DNA can be reliably produced, precisely positioned[9], and can exhibit stability and biocompatibility enabling its use in diverse environments, including living systems[11]. These structures pave the way for the integration of metal surfaces that can potentially participate in NSET, of a size whereby they will not interfere with the biomolecular activity that is being studied.

To explore the interaction further, we perform a study on the size of the metal particle, and its effect on the NSET interaction. Given that the dipole's potential damping is

predominantly a surface component, smaller particles are believed to manifest a more pronounced interaction. Yet, the existing evidence for this remains limited, mainly due to challenges in the synthesis and positioning of nanoscale metal particles. We utilize the potential of Ag-DNA, whereby its nanometer size paired with easy positioning allows for in-depth exploration of the NSET interaction under varying conditions of metal particle sizes, dipoles, and distances.

We exhibit Ag-DNA's potential as an angstrom-scale ruler, based on its competence in quenching fluorescent dyes. By synthesis of few-atom silver clusters in proximity of various organic fluorescent dyes on an attached ssDNA strand, the effect of the energy transfer can be observed. Using double stranded DNA (dsDNA) as a spacer, we can precisely position dyes at variable proximities to the metal cluster, to calibrate the distance dependence. Furthermore, we utilize the quenching interaction to perform studies on particle size and cluster formation, which can be monitored in real time.

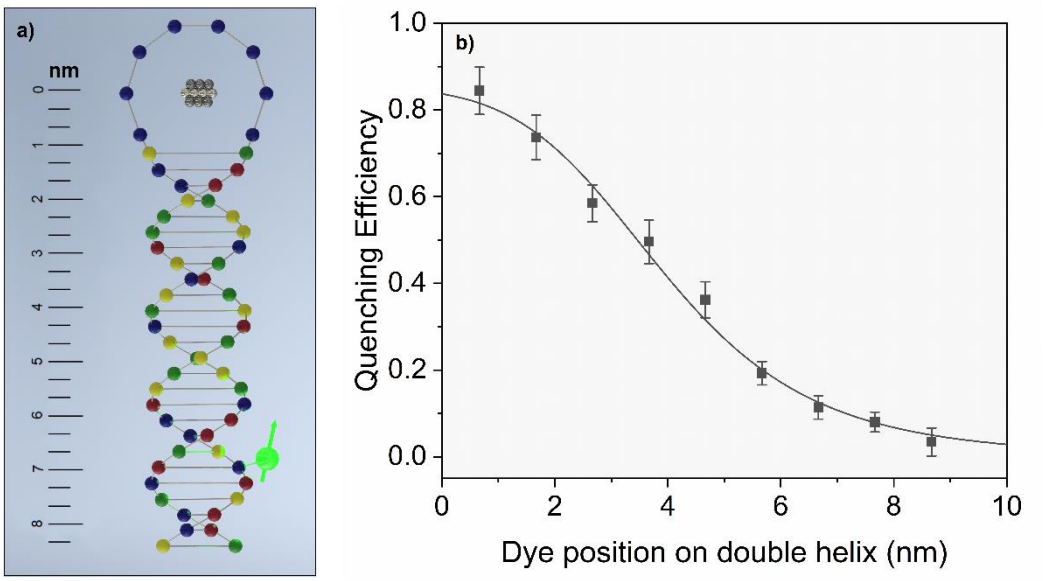

Figure 1.3: Distance-dependent quenching efficiencies of the Ag-DNA cluster on an attached Cy3-dye. As illustrated (a), the silver cluster is contained within a poly-C loop, attached to a 25 base pair double helix that is used to position the dye at various distances. The dye can be placed at any base pair along the double helix. Cy3 is chosen as the fluorescent dye due to it exhibiting the strongest quenching interaction. Clusters were synthesized with a 14.4 Ag/DNA ratio, which produces the strongest quenching as well. The measuring bar is in nanometers. Quenching efficiencies (b) are calculated from the change in bulk fluorescence in solution, measured through fluorimetry. The black line is a fit to the data of the NSET model. The distance is measured from the base of the loop containing the metal cluster.

In summary, Chapter 3 of this thesis exhibits a further notable usability of Ag-DNA, and its significant potential in nanoscale distance measurements, and laying a foundation for future applications for this material in experimental (bio-)physics.

1.4 Ionic sensors to measure real time ionic strengths in live D. discoideum cells and nuclei

The chemical intricacies within biological environments play a pivotal role in our understanding of their functioning, especially in health contexts. Monitoring ionic imbalances, such as those of potassium, sodium, magnesium, or calcium, is crucial as these alterations can reveal pressing health challenges. In particular, the cellular environment's ionic composition, especially within the nucleus, can represent valuable information. Yet, the small and intricate nature of these biological systems, especially individual cells, often hampers precise and detailed measurement. Traditional methods, including conductivity measurements, surface probing techniques like SIMS and MALDI[43], and even advanced FRET-based probes[44], come with their own limitations.

The nucleus of a cell, a central library of genetic information, is something of a mystery zone due to its hard-to-access nature. This secluded environment, guarded by the nuclear membrane, shows very different properties to the surrounding cytoplasm. While ionic channels in the nuclear membrane are known to exist[45,46] and play a part in regulating the charge balance during macromolecular transport, the resultant ionic concentrations within the nucleus largely remain elusive. Any tool seeking to measure this must possess the ability to freely traverse the nuclear membrane and retain its sensing capabilities post-entry.

Crucially, fluorescent Ag-DNA, despite their larger metal particle analogues' plasmonic nature, remain within a scale of a few nanometers[34]. The inherent versatility of DNA allows for tailoring these constructs across a wide spectrum of sizes, shapes, and optical attributes, including covering the entire visible light range. It's the unique interplay between ssDNA's sequence and conformation and the sensitivity of few-atom clusters that gives rise to the variable optical properties observed in Ag-DNA emitters. Due to the DNA strand’s thermodynamics being strongly dependent on the chemical environment[14,47], the optical properties of Ag-DNA respond to shifts in local ionic concentrations. Furthermore, these constructs are small enough to successfully infiltrate the nucleus and maintain compatibility with a range of cell types[11].

Therefore, in Chapter 4 of this thesis, we harness Ag-DNA's potential as an ion-sensitive probe, exhibiting a fluorescence behavior linked to a solution's ionic strength. We calibrate this fluorescence against known ionic concentrations and utilize it to probe

the interior environment of living cells, specifically *D. discoideum*. Utilizing colocalization techniques in fluorescence microscopy, we can separate the nuclear environment from its cellular counterpart, showing a strong, measurable ionic disparity between the two.

Due to the scalability, and economic feasibility of Ag-DNA constructs, we can increase their applicability to home-based health monitoring. As an example we specifically target urine's salt concentration as a diagnostic parameter for kidney health. Utilizing simple detection setups combined with these novel sensors, we show an initial concept for a simple new approach to health monitoring. This has significant potential for early detection, informed self-management, and consequential cost savings in healthcare.

In summary, Chapter 4 exhibits ionic measurements on intracellular and intranuclear ionic environments, and relevant samples for home-based health diagnostics, leveraging the versatile capabilities of fluorescent Ag-DNA constructs.

1.5 A light-activated silver-DNA construct for induced toxicity in cancer cells

Cancer, an ever-present problem in the area of health, remains a persistent challenge in medical science. With a continuously rising global incidence rate, malignant tumors have long been a focus of extensive research efforts, given their significant implications for public health. Current treatments mainly revolve around chemotherapeutic agents, radiation therapy, and surgical interventions. While these approaches have certainly saved countless lives, they are not without drawbacks.

For decades, cancer treatment has been reliant on chemotherapeutic agents[48]. Their advantage lies in their ability to inhibit rapid cell growth, to help treat the disease, often in combination with other treatment methods. Yet, their broad-spectrum action often results in the unintentional damage of healthy cells, which leads to systemic side effects that can severely diminish the patient's quality of life. Immunotherapies[49], a recent addition to the oncological toolkit, have illustrated potential by harnessing the power of the body's own defence mechanisms. However, the inconsistent patient responses and the risk of unintended autoimmune-like symptoms make it a complex approach that is yet to be fully understood and optimized, and its cost is often prohibitive.

Surgical interventions, while often effective in removing localized tumors, have their own set of challenges. Exceptional precision is paramount during these procedures to avoid damage to surrounding healthy tissues, and even the most skilled surgeons face limitations due to the intricate nature of human anatomy, and variability in tumor

structure. Laser-based tumor ablations[50,51], although a promising idea given the precision of light, are currently restricted by their associated costs and the need for specialized expertise, along with the risk of unintended injuries to neighboring tissues.

Additionally, the financial implications of cancer are noteworthy. The costs associated with current treatments pose a significant strain on global healthcare infrastructure. As the demand for more efficient, less invasive, and cost-effective treatments intensifies, there will always remain a need for innovative approaches.

Into this field, we introduce a new approach: the utilization of modified Ag-DNA constructs with a sensitivity to light, named the 9C-PC agent. The structures are designed to be inherently non-toxic, but can be activated into a toxic state by low-intensity light for the precise targeting of cancer cells. Our preliminary research offers evidence for its efficacy, and Chapter 5 of this thesis shows our initial results of this promising technique.

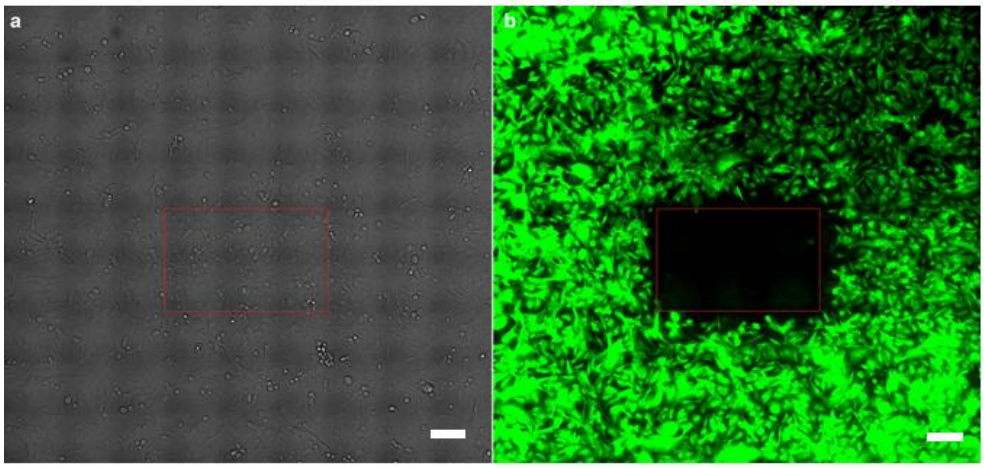

Figure 1.4: Bright field (a) and fluorescence (b) microscopy images of *mda-mb-231* cell culture. The cells were grown densely onto glass intentionally to gain an insight into specific targeting. The samples were exposed to the 9C-PC agent through injection into the medium, followed by 20 minutes incubation. Exposure with the 405 nm laser was performed through the scanning FRAPPA unit within the red box, to induce cell death. Green light indicates live cells, the result of a fluorescein diacetate viability stain.

We are able to target individual cells, and sections of cell culture with single cell precision. Initial results on simple tissue models, in collaboration with our partners at the Leiden University Medical Center, have shown that the technique is also applicable in 3D tissue. This chapter exhibits the potential for Ag-DNA constructs as cancer treatments, not only more precise than existing methods, but also gentler on the patient and more sustainable for our healthcare systems.

1.6 DNA Nanocarriers for Controlled Drug Delivery

The quest for optimum drug delivery has always been of significant interest in research for the healthcare field, because it serves dual critical functions: augmenting therapeutic efficacy and limiting side effects. Historically, this pursuit saw the use of various strategies, each designed to deliver therapeutic agents to the right site, at the right time, and in the right amount. Traditional systems like liposomes, polymeric nanoparticles, and inorganic carriers are currently in use for targeted and controlled release. However, they are not without flaws and limitations.

Liposomes[52,53], for instance, have shown an ability to encapsulate both hydrophilic and hydrophobic drugs, providing improved drug stability and reduction in toxicity. However, their clinical application has been limited by their potential for rapid clearance from the bloodstream, susceptibility to oxidative degradation, and inconsistent release kinetics. Polymeric nanoparticles[54,55], on the other hand, are known for their tunable release profiles and potential to improve drug bioavailability. But concerns regarding their biodegradability, potential toxicity, and limited drug loading capacity often limit their usability and future potential. Other inorganic carriers have shown structural rigidity and multifunctionality, but they, too, show limitations on issues of biocompatibility, long-term accumulation, and potential cytotoxicity.

Against this backdrop of traditional drug delivery methods, we introduce DNA origami structures as an alternative. The unique programmability, impeccable biocompatibility, and robust structural stability of double stranded DNA makes it an exciting tool for constructing the next generation of nanocarriers.

In Chapter 6, we develop several DNA-based nanocarriers, exhibiting their potential as next-gen vehicles for drug delivery. Notably, we are able to produce structures on a scale of less than 10 nanometers. These structures can be customized to house payloads for the purpose of delivering therapeutic agents.

The constructs exhibit two mechanisms of degradation: thermal and nuclease-mediated release. DNA's intrinsic temperature-responsive nature facilitates continuous release dependent on the temperature of the environment. This may open additional potential in oncology, where, for example localized hyperthermia might be used to target cancer cells. Secondly, release occurs through nuclease activity, leading to a slow, prolonged drug release as the result of the DNA-degrading enzymes naturally present in our bodies.

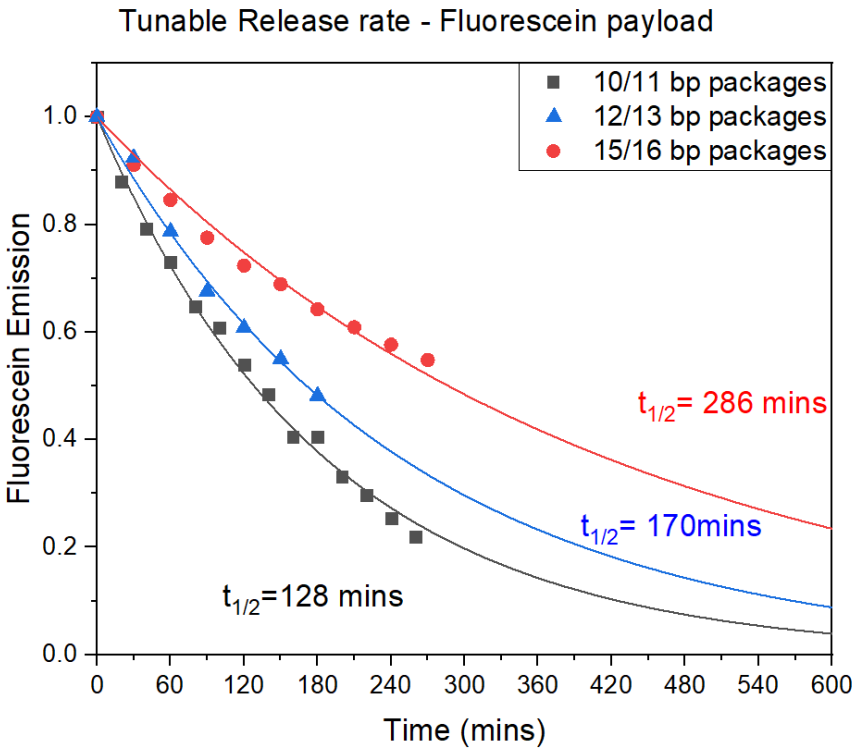

Figure 1.5: Release of fluorescein molecules from three DNA nanocarriers measured over time. Packages are characterized by the length of the various double helices stabilizing the structure, 10/11 base pairs (black) 12/13 base pairs (blue) and 15/16 base pairs (red). The solutions are placed in a dialysis membrane to remove the released payload from the environment continuously. The packages are kept within the TAE buffer, at room temperature. Fluorescein fluorescence intensity is measured using a 488 nm excitation. Exponential fits to the data (lines) show halftimes of the payload concentration of 286 mins, 170 mins, and 128 mins.

We utilize three distinct DNA nanocarriers, encapsulating fluorescein as a model payload. By monitoring the fluorescence, the dynamics of molecular release can be studied in real time. These DNA structures show significant potential to help improve drug delivery techniques.

The modern accessibility of DNA oligonucleotides, and the flexibility of design in these structures, can open a wide range of variants for different applications, from targeted therapies that could redefine cancer treatment, to promising avenues in regenerative medicine.

1.7 Valorization

The quest for innovative cancer treatments has led to significant recent advancements in the area of oncology, particularly in the development of targeted therapies that promise minimal side effects while maximizing efficacy. This thesis concludes with Chapter 7, where we focus on the valorization plans of the anti-cancer agent introduced in Chapter 5, specifically designed for light-activated therapy. The essence of this approach lies in its ability to offer a precise, controlled, and minimally invasive treatment, particularly for skin cancer, which remains one of the most prevalent forms of cancer worldwide.

Skin cancer, due to its high incidence and visibility, is at the forefront of cancers that require innovative treatment methods. Among skin cancers, Basal Cell Carcinoma (BCC) is the most common, characterized by its slow growth but significant potential for disfigurement. Despite its lower metastatic potential compared to melanoma, the prevalence of BCC represents a critical need for effective, targeted treatments.

The introduction of the 9C-PC agent, as detailed in Chapter 5, shows potential to deliver a significant leap in the treatment of cancer. This agent, comprising 2-3 nanometer Ag-DNA constructs, is activated by light to target and destroy cancer cells with single cell precision. The use of a 405 nm laser or a similar light source enables the activation of the agent within the tissue region of the tumor, inducing cell death over a period of 2-24 hours. This method exhibits up to 100% toxicity in targeted cells and tissue models and shows promise in minimizing damage to surrounding healthy tissues, a common drawback of traditional cancer treatments.

Our initial testing in cell and tissue models provide a basis for further validation with the aim of treating various types of cancer accessible with light. BCC, with its high incidence rate and accessibility to light, represents an ideal candidate for initial treatment trials. The increasing global incidence of BCC, driven by factors such as heightened public awareness, improved diagnostic methods, and an aging population, further accentuates the need for such targeted therapies.

The valorization of this light-activated anti-cancer agent involves not only its clinical development but also the establishment of a robust intellectual property framework to facilitate its commercialization. The ongoing patent application by Leiden University aims to protect this innovative technology, as required to attract the necessary investment for its further development. The researchers involved in the writing of this thesis plan to incorporate the license into a spin-off business within the Leiden Bioscience Park, to translate scientific innovation into practical, market-ready solutions.

The transition from laboratory to market for the 9C-PC agent will require a comprehensive validation and regulatory process, anticipated to span the next 5-10 years. This long process underscores the many requirements, between scientific innovation, regulatory compliance, and commercial strategy, all essential for the successful introduction of new medical treatments. In Chapter 7, we will explore the aspects of valorizing the light-activated anti-cancer agent, from scientific development and patent strategies, to commercialization pathways and the potential impact on global health.