

Towards novel cancer therapeutics: Development of programmable, synthetic DNA polymers to inhibit tubulin polymerization

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Abstract

Paclitaxel (Taxol®) is a microtubule stabilizer and an effective chemotherapeutic. Yet, it suffers from limitations, which oligonucleotide therapeutics might be able to address.

Tubulin is a dimeric protein comprising α/β subunits which assemble into microtubules. It is a prominent cancer drug target and essential for cell growth, division and stability, intracellular transport and cell cycle regulation. Paclitaxel's potent mechanism of action leads to hyper-stabilization of microtubules and ultimately, apoptosis. But emerging resistance, undesirable properties as well as its environmentally destructive extraction, scarcity and astronomical price pose severe risks to its therapeutic success and highlight the pressing need for alternatives.

Aptamers are versatile biological polymers made from nucleic acids, which fold into three-dimensional structures and can bind a broad range of targets, with affinity and specificity that rivals antibodies, while offering compelling advantages. Aptamers are versatile 'chemical antibodies' which can be synthesized and modified cost-effectively and rapidly in large quantities, are chemically benign and non-immunogenic. They are typically generated from a random pool of sequences by an iterative enrichment, called in vitro selection (SELEX) and have already found clinical success.

In the motivation to overcome the under-addressed hurdles of chemotherapeutics, aptamers are explored as novel anti-mitotic agents. In this work, a population of previously published tubulin-binding aptamers with affinities in the low micromolar range are evaluated in in vitro drug discovery screening for the first time and a comprehensive analysis framework is developed for the rapid and robust characterization of nucleic acids as drug-like molecules. In vitro selection of more tightly-binding aptamers and a range of post-selection modification strategies are pursued.

This involves development of a rapid prototyping template and a ligand-responsive anti-tubulin chimera. Furthermore, a novel polymeric miniature microtubule target is fabricated to facilitate epitope-specific selection of Paclitaxel-like binders.

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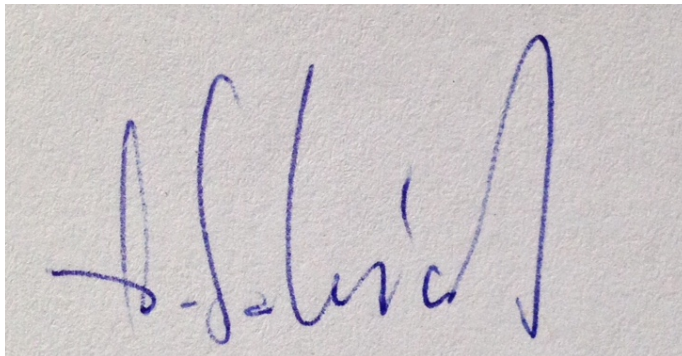
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First and foremost, I thank my friends and loved ones for their support and patience during this time.

Declaration of Originality

I, Elsa Sotiriadis, hereby declare that this thesis and all corresponding work presented herein is the result of my own original research, unless otherwise noted, acknowledged or referenced. I hereby state that I am the sole author of this thesis and that I have written it in my own words.

A handwritten signature in blue ink, appearing to read 'E. Sotiriadis', is shown on a light-colored, textured background.

Elsa Sotiriadis

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Publications and articles

Conference posters

Sotiriadis E., Huang, J.D., Bayer T.S. Engineering synthetic aptamer switches for next-gen cancer therapeutics. Postgraduate Research Day, Imperial College London. April, 2014

Sotiriadis E., Huang, J.D., Bayer T.S. Can we switch off cancer? Rational engineering of aptamers for smart cancer therapeutics. Graduate School Symposium. Imperial College London, July 2014

Sotiriadis E. Identification of tubulin-binding aptamers with small, but significant inhibitory effects on tubulin assembly. Aptamers 2016, St. Hilda College, Oxford, April 2016

Sotiriadis E. The aptamer clock: a rapid prototyping template for post-selection optimization of tubulin-binding aptamers. Aptamers 2016, St. Hilda College, Oxford, April 2016

Sotiriadis E., How synthetic biology startups are building the future at RebelBio. BioCoder (O'Reilly). August 2017

Manuscript in preparation

Sotiriadis E., Huang, J.D., Polizzi, K.M. Rapid prototyping and high-throughput screening of aptamers for drug discovery (in preparation for PNAS or Molecular Therapy)

Sotiriadis E., Polizzi, K.M. A new frontier in medicine: the rise of cognitive machines (target journal to be determined)

List of abbreviations

AMA	Anti-mitotic Agent
APS	Ammonium Persulfate
BSA	Bovine Serum Albumin
CD	Circular Dichroism
CE	Capillary Electrophoresis
DNA	Deoxyribonucleic Acid
Dox	Doxorubicin
dsDNA	Double-stranded DNA
ELONA	Enzyme-Linked Oligonucleotide Assay
FRET	Fluorescence Resonance Energy Transfer
FW	Forward (primer)
GRO	Guanosine-rich oligonucleotide
GmpCPP	Guanosine-5'-[(α,β)-ethyleno]triphosphate
IC ₅₀	Inhibitory Concentration (50%)
K_d	Dissociation Constant
LHC	Light-Harvesting Centre
ML	Machine Learning
MSA	Microtubule-stabilizing agent
MT	Microtubule
NGS	Next-Generation Sequencing
nt	Nucleotides

List of abbreviation

OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RV	Reverse (primer)
RT	Room Temperature
SDS	Sodium Dodecyl Sulfate
SPA	Scintillation Proximity Assay
ssDNA	Single-stranded DNA
TEM	Tunnel Electron Microscopy
Tris	Tris(hydroxymethyl)aminomethane
TX	Paclitaxel (Taxol)
UAV	Unmanned Aerial Vehicle
V-Vis	Ultraviolet-Visible
VC	Vinecristine Sulfate

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The box was a universe, a poem, frozen on the boundaries of human experience.
William Gibson

To my family

1

Literature Review

1.1 Introduction

1.1.1 Medical Innovations are transforming oncology

The promising scientific advances around early detection and treatment of cancer could benefit patients in the coming years.

The ability to grow organoid models of pancreatic cancer from mouse and human cells (Boj *et al.*, 2015), for instance, could be a transformative step towards precision medicine and accelerate drug development in the future, although hurdles remain to be overcome. As the cost of high-throughput, whole-genome sequencing has outpaced Moore's law, tumor genomics and gut or colorectal microbiome data are amassed at great pace to shed new light on underlying disease patterns. Today, algorithms are trained on vast amounts of such information and are drawing from potent computing capacities. This could greatly improve the automated analysis of the complex imaging and structural datasets, such as from mammograms or patterns of drug molecule libraries. With visual pattern recognition, certain types of analyses can be automated, for instance for the rapid classification of mammograms to detect malignancies.

This section is structured in three parts: It starts with an overview on developments in cancer medicine and follows with an overview on the most effective chemodrugs and their unique interaction with the prominent drug target tubulin. Ultimately, a review on aptamers as promising therapeutics and protein inhibitors is given.

1.1.1 Point-of care drug factories and origami-mediated delivery

Recent work demonstrated the use of a portable miniature medical factory to produce drugs on-demand — by adding water (Pardee *et al.*, 2016). It contained freeze-dried drugs and cell-free extract in reaction pellets. This served as cell-free biosynthesis system to which custom DNA can be added (Pardee *et al.*, 2016). The production range of this synthetic biology platform was demonstrated as a proof of concept with antimicrobial peptides, vaccines and combinations of antibody

conjugates and small molecules (Pardee *et al.*, 2016). Other work in point-of-care (POC) synthesis demonstrated near-single dose production of biologics in less than 24 hours. This was achieved by using a strain of genetically engineered *Pichia pastoris* able to produce and secrete two different proteins as biologic drugs in response to external inputs that were controlled with a microfluidic device. The strain contained genetic expression cassettes that could be controlled independently and in combination by the signals methanol and β -estradiol. The group demonstrated that protein production could be rapidly switched from one protein to the other on milliliter-scale, the scale of one dose (Perez-Pinera *et al.*, 2016). Similarly, cell-free microfluidic reactors produced therapeutic proteins at the point-of-care (Timm *et al.*, 2015).

These pioneering projects face multiple hurdles, such as the need for analysis, purification or controlling variability, yet they offer a glimpse into how biologics manufacturing and accessibility to medicines might change in the years ahead. For instance, unmanned aerial vehicles (UAV's, drones) could provide emergency relief, map epidemics and deliver medicines (Floreano and Wood, 2015) or vaccines from local hospitals to patients (Haidari *et al.*, 2016).

Meanwhile, drug delivery is an area of promising developments as well. DNA has emerged as versatile nano-material for this purpose. Self-folding origami, unlike DNA aptamers, are built from long DNA strands into three-dimensional nano-structures. Such origami structures bearing functional logic gates have been used to deliver targeted molecular payloads into cells (Douglas *et al.*, 2012). Implemented in living animals, these origami were able to perform basic computing tasks. In the future, they could potentially serve as molecular processor of disease states and control therapeutic molecules in parallel (Amir *et al.*, 2014). Self-assembled origami were capable of delivering the anticancer drug Doxorubicin (Dox) into cancer cells, as it non-covalently attaches to DNA by intercalation (Jiang *et al.*, 2012). The particles were not actively targeted, yet, in combination with the origami as carrier, the drug reached higher cell internalization efficiency. It showed cytotoxicity to MCF-7 cells (breast adenocarcinoma cancer) and most remarkably, also to doxorubicin-resistant cancer cells, which have shown a reversal of their resistance phenotype. Such smart cancer drug vehicles do not suffer from the shortcomings of nanoparticles, such as a

lack of biocompatibility and show promise in delivery of cytotoxins specifically to tumor cells. However, a hurdle to overcome for origami will be the DNA stability *in vivo*. This lack of specificity features prominently among the drawbacks of current chemotherapeutics (Table 1.2).

An example is the work by Douglas *et al.*, (2012). The researchers have developed a DNA 'nanorobot' capable of delivering molecular payloads into cells in response to cell surface inputs. The purpose was to selectively regulate its efficacy for differential delivery, depending on the cell. The scaffold consisted of a single-stranded DNA multiple kilo-bases long, which was folded into a custom shape with the assistance of shorter 'staple' oligonucleotides, which were later replaced with fully complementary ones. This structure contained AND gates able to sense these molecular inputs and function as a lock. This made it possible to conditionally trigger the nanorobot activation (Figure 1.1).

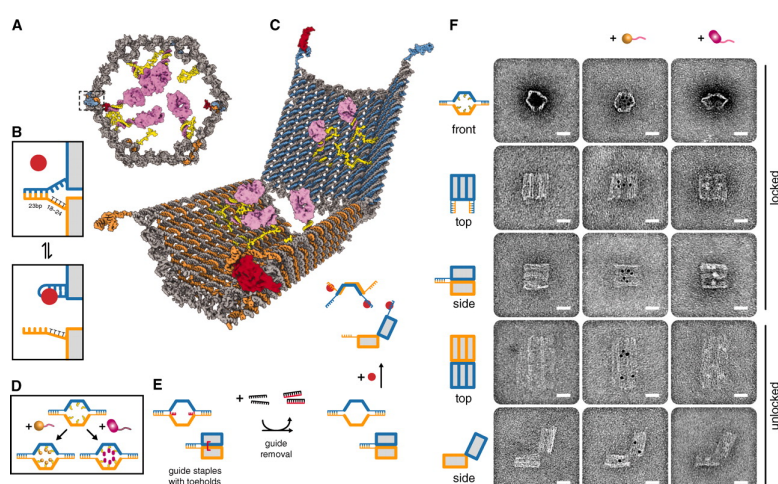


Figure 1.1: Design and TEM analysis of aptamer-gated DNA nanorobot.

(A) Schematic front orthographic view of closed nanorobot loaded with a protein payload. Two DNA-aptamer locks fasten the front of the device on the left (boxed) and right. (B) Aptamer lock mechanism, consisting of a DNA aptamer (blue) and a partially complementary strand (orange). The lock can be stabilized in a dissociated state by its antigen key (red). Unless otherwise noted, the lock duplex length is 24 bp, with an 18-to 24-base thymine spacer in the nonaptamer strand. (C) Perspective view of nanorobot opened by protein displacement of aptamer locks. The two domains (blue and orange) are constrained in the rear by scaffold hinges. (D) Payloads such as gold nanoparticles (gold) and antibody Fab' fragments (magenta) can be loaded inside the nanorobot. (E) Front and side views show guide staples (red) bearing 8-base toeholds aid assembly of nanorobot to 97.5% yield in closed state as assessed by manual counting. After folding, guide staples are removed by addition of fully complementary oligos (black). Nanorobots can be subsequently activated by interaction with antigen keys (red). (F) TEM images of robots in closed and open conformations. Left column, unloaded; center column, robots loaded with 5-nm gold nanoparticles; right column, robots loaded with Fab' fragments. Scale bars, 20 nm. Used with permission of AAAS.

1.1.2 Bioprinting micro-tumor models

Organs-on-a-chip are micro-biomimetic systems from human cells lined inside microfluidic channels (Huh *et al.*, 2013). The work was pioneered by a team at the Wyss Institute (Huh *et al.*, 2013) to create a human kidney on a microfluidic chip. To test efficacy and toxicity of drugs and chemicals, these *in vitro* human disease models could present a lower-cost alternative or complement to animal testing, although a framework of international standards satisfying regulatory bodies remains to be put in place.

Similarly, spherical three-dimensional tissue cultures, so-called spheroids or organoids, are isolated from a patient's tumor and grown on a microfluidic chip, where they serve as a micro-tumor replica representing the diversity of mutations (Ruppen *et al.*, 2015). Alternatively, tumor microenvironment models can be 3D-printed in relatively high-throughput fashion to yield new insights into tumor formation, growth and response to anticancer therapies (Zhao *et al.*, 2014). They further serve to test drug-release kinetics and the biodegradability of novel delivery systems, such as polymer microspheres as drug carriers (Singh *et al.*, 2008). Testing customized formulations in micro-organs and tumors from a patients own cells in this lab-on-a-chip could allow for fine-tuning (adjusting concentrations) of drug cocktails through the rapid assessment of the formulations and present a step-change towards personalized medicine (Ruppen *et al.*, 2015).

1.1.3 Machine learning in drug design and diagnosis

Could an artificially intelligent algorithm design novel drug molecules? Drug design by machine learning has emerged from remote horizon into the realms of the feasible. Sternberg *et al.* pioneered interdisciplinary work in bioinformatics, structure prediction and SAR (structure-activity-relationship) in the 1990s and proposed programming inductive logic for the task (King *et al.*, 1992, King *et al.*, 1996, Agatonovic-Kustrin and Beresford, 2000). Inductive and deductive reasoning are logical methods that describe complex reasoning tasks, such as for medical applications. Unlike deductive reasoning, inductive reasoning allows for the

possibility that the conclusions are false. For example, the model called GOLEM of King *et al.*, (1992) was trained on SAR data of 44 trimethoprim analogues and then able to derive accurate rules (more accurate than linear regression models) to characterize the chemistry from this test data. Aspuru-Gudzik and an international group of colleagues presented a generative model (a branch of unsupervised learning) trained on 250'000 drug-molecules (Gomez-Bombarelli *et al.*, 2016). The deep neural network was able to generate novel structures from a set of operations it performs on molecules. They hope to advance the potency of their model by training it with data of the American Chemical Society's encompassing about 100 million chemical structures towards the automatic design of potent chemical compounds.

Machine learning has already been applied successfully to design antibiotics (Cherkasov *et al.*, 2009). Support vector machine classification, a type of algorithm, which can tackle QSAR (quantitative structure-activity relationships) and pattern recognition problems, has proven effective in designing molecules *in silico* (Burbridge *et al.*, 2001). Deep learning is increasingly applied to medicine (Ramesh *et al.*, 2004). An evolutionary approach for a neural network applied to the diagnosis of breast carcinoma is presented by Abbass, (2002). An artificial neural network was able to accurately diagnose malignancies from gene expression profiles in microarrays, as reviewed by Greer and Khan, (2004). Similar work has demonstrated its potency for analyzing skin lesions and melanoma, reviewed by Dreiseitl *et al.*, (2001). More wide-spread applications of classification algorithms are likely to expand to the diagnostic practice in the coming years.

1.1.4 Developments in cancer theranostics

With more than 7.5 million deaths each year, cancer remains the world's most fatal disease, with breast cancer continuing to be the leading overall cause of death for women (Ferlay *et al.*, 2008). The WHO predicts an increase to 13 million deaths from cancer in 2030 (<http://globocan.iarc.fr>). Despite advances in treatment and diagnosis, still very few patients with metastatic disease are cured, for instance for the aggressive HER2⁺ breast cancer (Artega *et al.*, 2012, Slamon *et al.*, 2001). Major theranostic advances, (unifying therapeutics and diagnostics), are a global and public health imperative — yet cancer therapy remains firmly among the most challenging conundrums of modern medicine.

Regardless of the large investment of public and private funding, oncology drugs in particular remain a challenging and often uneconomical undertaking, in the sense that revenues realized from selling a drug do not cover its development costs (Kamb *et al.*, 2007). The probability for an oncology drug to succeed is over three times lower compared to the other major disease classes (Kola & Landis, 2004 and Chabner *et al.*, 2005). Due to the nature of the disease, if as few as 10% of malignant cells survive treatment, tumors continue to proliferate (Kamb *et al.*, 2007). It requires enormously aggressive approaches, which bring many challenges to strike the balance between patient safety, therapeutic efficacy and permanent remission.

Risk is also reflected in the economics of Research and Development and vice-versa. The average cost to bring a new biologic (protein or monoclonal antibody) to market amounts to \$1.2billion (DiMasi and Grabowski, 2007). Why this high cost? Firstly, pharmaceutical Research and Development remains a slow and expensive process, due to high costs and time required in pre-clinical and clinical trials, which has remained largely unchanged since the 1960s, (Kaitin, 2010). Moreover, revenue from the average drug rarely exceeds the R&D costs to develop it — this occurs in only 3 out of 10 cases on average (Grabovski *et al.*, 2002). At the same time, one highly successful drug on the market can offset such losses.

Rising drug prices are a pressing concern among policy-makers, clinical practitioners and researchers — but first and foremost, patients (Bach, 2009 and, Mailankody and

Prasad, 2015). Paclitaxel treatment typically costs \$80000 per year (Siddiqui and Rajkumar, 2012), monoclonal antibody therapy up to \$9000 monthly, (Malik, 2009). As discussed above, especially oncology drug development poses challenges. Therefore, new approaches to de-risk and accelerate this process are desirable.

Table 1.1: Early stage research shaping tomorrow's cancer medicine

	Key oncology theranostics and drug discovery	Reference
Machine Learning (ML)	ML enables cancer classification from genetic microarray and genomic data, drug design in silico with vector machines and image processing in diagnosis of mammograms and melanoma, or predict recipes personalized drug cocktails	Wang <i>et al.</i> , 2005 Tan and Gilbert, 2003 Guyon <i>et al.</i> 2002 Ledford, 2016
Organs-on-a-chip	This might allow for routine personalized, rapid drug testing with micro-tumors grown from patients cells, and could set a new paradigm for clinical trials	Gao <i>et al.</i> , 2015 Huh <i>et al.</i> , 2012 Boj <i>et al.</i> , 2015 Patel <i>et al.</i> , 2015
Origami, Soft Robotics	DNA origami controllable with logic gates can deliver molecular payloads to cells, origami nanorobots can be used to identify drug targets	Douglas <i>et al.</i> , 2012 Wehner <i>et al.</i> , 2016 Li <i>et al.</i> , 2013 Fu and Yan, 2012
Robotic Surgery	The iKnife to detects tumor tissue with integrated mass-spectrometry capabilities and high-precision robotic surgery has shown improved success rates	Genden <i>et al.</i> , 2009 Iseli <i>et al.</i> , 2009 Camarillo <i>et al.</i> , 2004
Precision Medicine and Genomics	Targeting genetic disease pathways in cancer from computational analysis of mutations from tumor exome-sequencing, portable sequencing for genomics research anywhere, from the arctic to space, novel, non-optical approaches to genome sequencing	Jameson <i>et al.</i> , 2015 Collins and Varmus., 2015 Raphael and Dobson, 2014 Van Allen <i>et al.</i> , 2014 Branton <i>et al.</i> , 2008 Rotheberg <i>et al.</i> , 2011
CRISPR-Cas9 Gene Editing	Logic gates can be used to identify bladder cancer cells as part of screening and progress is being made to apply gene editing to eliminate cancer mutations	Platt <i>et al.</i> , 2014 Shi <i>et al.</i> , 2015 Sanchez-Rivera, 2014 Doudna and Charpentier, 2014
Microbiome	Gut microbiome screening serves as a tool to analyze colorectal cancer and identify patterns in the development of the disease	Zackular <i>et al.</i> , 2014, Ahn <i>et al.</i> , 2012 Schwabe and Jobin, 2013 Gill <i>et al.</i> , 2006

Treatment of cancer typically involves surgical removal, radio- and/or chemotherapy. New directions for the latter include cancer immunotherapy and targeted therapies. For instance, nanodiscs for personal cancer immunotherapy take advantage of each patient's tumor's unique exome sequences (sequences of all the expressed genes) and were recently tested successfully in mice (Kuai *et al.*, 2016), while elsewhere, researchers reported that their cancer vaccine for tumor antigens had successfully triggered immune responses in three people with melanoma (Carreno *et al.*, 2015). However, translating early research success into clinical success is not trivial.

Targeted therapies, on the other hand, are established (like Trastuzumab, Atezolizumab and Bevacizumab) and consist of cytotoxic molecules linked to proteins, usually antibodies as biologic-cytotoxic conjugates (Sawyers, 2004). A large number of targeted antibody-therapies have been approved by the FDA and are in the clinic for different types of cancer, from leukemia, brain, bladder and cervical cancer to other types. This is promising, as a wide range of targets is accessible to biologics (Fischbach *et al.*, 2013). Antibodies have been applied as antibody-drug conjugates (Casi and Neri, 2012), targeted antibodies (Schrama *et al.*, 2006) and therapeutic antibodies (Elvin *et al.*, 2013). Examples of RNA therapeutics include RNA-interference for therapeutics (Bora *et al.*, 2012), RNAi for drug discovery (Iorns *et al.*, 2007) and micro-RNA Mir-43 (Misso *et al.*, 2014) and small molecule cancer drug discovery (Hoelder *et al.*, 2012) and are covered by Bumcrot *et al.*, (2006).

While targeting strategies address the lack of specificity of general chemotherapy, they still rely on the cytotoxic agents associated with severe adverse effects and emerging resistance. Historically and presently, apoptosis promotion remains the prominent therapeutic oncology strategy (Fesik, 2005).

1.2 Anti-mitotics: effective microtubule disruptors which induce apoptosis

As apoptosis-inducing agents, anti-mitotics are highly effective as anti-proliferative and are therefore the most widely used chemotherapeutics (Fesik, 2005). They are natural and complex biomolecules isolated from plant sources and belonging to the class of either vinca alkaloids or taxanes. Their unparalleled potency to modify the tubulin, a key cellular and cytoskeletal protein central to the cell cycle, stems from their mechanism of action.

AMAs disrupt microtubule dynamics and cause mitotic arrest, which endows them with high cytotoxicity relative to drugs which target non-essential functions.

Microtubules are cylindrical polymers of α/β -tubulin heterodimers (Downing, 2000). They form an integral part of the cytoskeleton of all eukaryotic cells and play a key role in cell motility, development and shape, as well as in intracellular transport, cell signaling, cell division and mitosis (Jordan, 2004). Dynamic instability is a central property of microtubules. It describes the fact that the polarized microtubule (+) and (-) ends constantly fluctuate between assembly and disassembly of dimers, prompting continuous, rapid shrinking and growing of the polymer (Mitchinson and Kirschner, 1984). While it is a dynamic process, which does not reach an equilibrium, it can reach a steady-state, where shrinkage and growth are balanced. The resulting net growth and net shortening of this dynamic process at each end is termed treadmilling (Mitchison and Kirschner, 1984).

This quick (millisecond scale), precisely timed switch between elongation and dissociation of kinetochore microtubules is a critical transition point for successful mitosis (Hayden *et al.*, 1990, Skibbes *et al.*, 1993 Saxton *et al.*, 1984) and has been studied widely, for instance in cultured cells expressing GFP- α -tubulin (Rusan *et al.*, 2001). As the central dynamic regulator of cell cycle control and mitosis, microtubules are an appealing drug target (Millinedo and Gajate, 2003).

Paclitaxel (Taxol ®) is considered the most effective and commercially successful cancer drug both historically and currently, owing to its unique mechanism of action on microtubules: it capitalizes precisely on this sensitive dynamic interplay (Jordan and Wilson, 2004). Taxol is a natural cyclic diterpenoid, originally isolated from the bark of the Pacific Yew *Taxus brevifolia* (Wani *et al.*, 1971).

It has shown significant anti-tumor activity in clinical studies with metastatic ovarian, lung and breast carcinoma, including lung, head, neck, bladder and cervix melanoma as well as AIDS-related Kaposi's sarcoma (Woo *et al.*, 1994). Taxanes, such as taxol and its close structural relative Taxotere (Docetaxel®), belong to the class of microtubule-stabilizing agents (MSAs), together with Epothilone A and B, Discodermolide and Eleutherobin. MSAs bind to polymerized tubulin and prevent their disassembly from the microtubule (Kumar, 1981, Altman *et al.*, 2001).

The other class, microtubule-destabilizing agents, includes vinca alkaloids (Vinblastine, Vinflunine and Vincristine), Podophyllotoxin, Colchicine and Nocodazole. In contrast to the former, they inhibit microtubule assembly by preventing attachment of the microtubules to the kinetochore in the cell (Jordan, 2002).

Interaction kinetics with tubulin are well-studied for both compounds classes, for destabilizer colchicine (Banerjee and Luduena, 1992, Banerjee and Luduena, 1991, Wilson and Farrell, 1986), and stabilizer Paclitaxel: (Kumar, 1981, Derry *et al.*, 1997, Banerjee and Kasmala, 1998, Kowalski *et al.*, 1997) and, epothilones, which share the same mechanism of action with the latter (Bollag *et al.*, 1995).

1.2.1 Paclitaxel's unique mechanism of action

Paclitaxel acts as an inhibitor of microtubule depolymerization by interlocking the polymerized tubulin dimers onto the microtubule. More precisely, it binds to the taxane site on the β -subunit and prevents its dissociation from α -tubulin. This prompts the hyper-stabilization of the microtubule by lateral polymerization (Schiff *et al.*, 1979, Yvon *et al.*, 1999), thereby blocking progression of mitosis (Yvon *et al.*, 1994, Manfredi *et al.*, 1981, Jordan *et al.*, 1993). The disruption of spindle microtubule dynamics results in aberrant mitosis, dysfunctional chromatid separation and fragmentation, causing cell cycle arrest at the G2/M transition point between metaphase and anaphase (Jordan, 2004 and Jordan *et al.*, 1993). This forced arrest sets the cell cycle back from metaphase to anaphase, which eventually leads to cell death (Fan, 1999, Torres and Horwitz, 1998).

X-ray crystallography studies have revealed the taxane binding site on the β -tubulin subunit of the heterodimer (Nogales *et al.*, 1995), where it binds polymerized tubulin on the luminal side of the microtubule (Parness and Horwitz, 1981). Nogales *et al.* also determined that these binding interactions are formed via hydrogen bonds and hydrophobic association, over a T-shaped Taxol structure, which interacts with part of beta-tubulin over functional similarity (Snyder *et al.*, 2001).

Residues 217-233 of β -tubulin have been identified by extensive and detailed photo-affinity labeling studies by the Horwitz group to constitute the taxane binding pocket (Rao *et al.*, 1992, 1995). The detailed molecular mode of action, especially the transition from the cytosolic side to the luminal side of the microtubule, remains to be determined. However, a study revealed an intermediate binding site of taxol on microtubule pores (Freedman *et al.*, 2009).

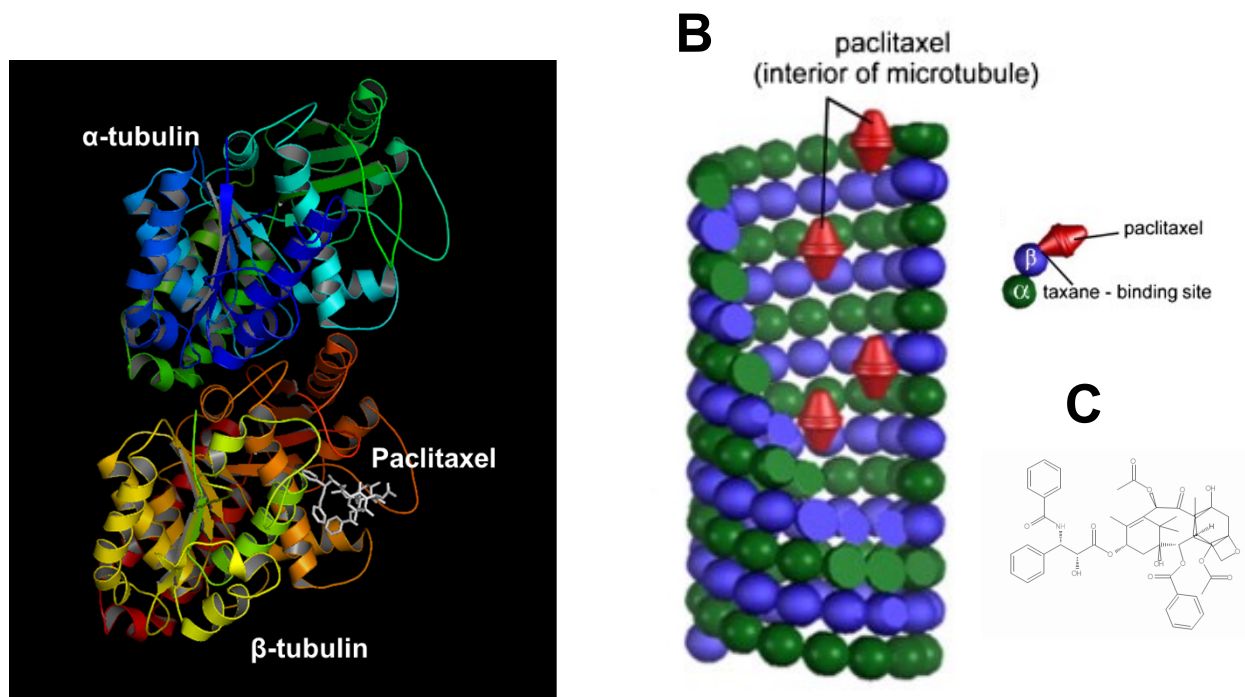


Figure 1.2: Interaction of Taxol on polymerized β -tubulin.

A) Taxol binds to the pharmacophore of β -tubulin. The figure was generated with PyMOL (Schroedinger) under university license and with sequences from doi:10.2210/pdb1jff/pdb. B) Lateral stabilization at the tubulin-dimers in the lumen of the microtubule by paclitaxel. Figure B is used with permission from Elsevier. C) Chemical structure of Paclitaxel (Taxol). Used with permission of www.emolecules.com under public domain license. Binding sites and SAR of Taxol and tubulin are presented in detail by Nogales *et al.*, (1995) and Snyder *et al.*, (2001).

1.2.2 *In vitro* cancer drug discovery screening

As illustrated, Taxol is a unique, highly clinically effective anti-mitotic agent and extensively studied, ever since it was first discovered in the Pacific Yew by the National Cancer Institute (Jordan and Wilson, 2004). But how are drugs discovered in the first place?

The process to identify potential cancer drugs typically starts with drug discovery screens of natural or synthetic compound libraries and monitors their potential interference with microtubule dynamics in this case or other targets — a promising first step, by which Paclitaxel was discovered (Cragg, 1998). This is carried out in high-throughput fashion (around 10'000 compounds per day) with *in vitro* tubulin

polymerization assays, a sensitive and robust method for the parallel screening of larges 'files' (compound libraries).

Interference with microtubule dynamics *in vitro* can be a promising hint for later anti-neoplastic effects in cell lines. To assess compounds, their interference with microtubule assembly is analyzed. Turbidity assays, where optical density is measured at 340nm, offer a rapid way to monitor the progression of tubulin polymer formation (Hall and Minton, 2005), as the polymer formation increases the OD in the solution.

For instance, researchers succeeded in synthesizing a novel triazole-based compound through rational drug design (Zhang *et al.*, 2007), which suppressed tubulin polymerization dynamics in the turbidity assay. These results were investigated further with tumor cell lines, where the cellular toxicity of the lead compound was confirmed in low nano-molar range, both to tumor- and multi-drug resistant cell lines. Interestingly, the compound did not affect healthy cells. The group also identified the compound's binding site through inhibition assays with colchicine (Arora *et al.*, 2009).

In another study, *in vitro* screening revealed interference with tubulin polymerization of a new molecule and cytotoxicity in low micro-molar range in various human cancer cell lines was confirmed. It caused the characteristic G2/M phase arrest, as well as down-regulation of cyclins and other checkpoint proteins. The compound was found to inhibit NSLC carcinoma in nude mouse xenografts (Chen *et al.*, 2006).

Similarly, a new synthetic compound called I-387, (short for (3-(1H-indol-2-yl)phenyl) (3,4,5-trimethoxyphenyl)methanone) was screened *in vitro*, where it induced microtubule depolymerization by binding on the colchicine site (a follow-up experiment). It was found to inhibit the growth of cancer cell lines with IC₅₀ in low nano-molar range and also induced the characteristic G2/M-arrest in a concentration-dependent manner (Ahn *et al.*, 2010, Kuo *et al.*, 2004 and Tuma *et al.*, 2010). An example of the work on a synthetic compound tested with turbidity assay and in cell lines is also given below (Zhang *et al.*, 2006).

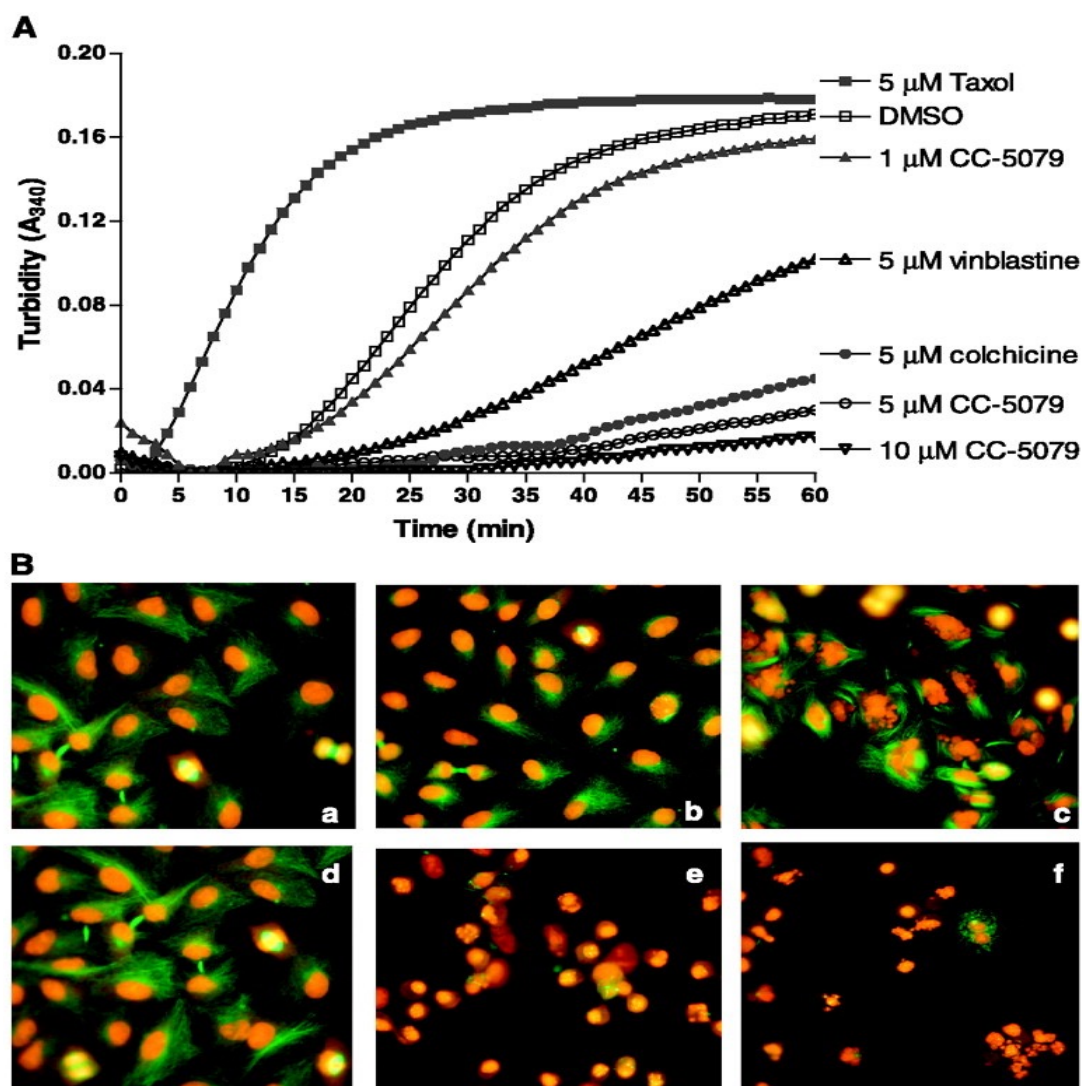


Figure 1.3: Effect of CC-5079 on microtubules.

A) Tubulin polymerization *in vitro*. The ability of CC-5079 at indicated concentrations to inhibit polymerization of purified tubulin was assessed by turbidity change using spectrophotometry. DMSO was used as vehicle control. Taxol, Vinblastine, and colchicine were used as control for positive tubulin-targeting agents. **B)** microtubule polymerization in cancer cells. A549 cells were grown in medium containing 0.1% DMSO (a) or 40 nmol/L Colchicine (b), 40 nmol/L Taxol (c), 8 nmol/L CC-5079 (d), 40 nmol/L CC-5079 (e), 200 nmol/L CC-5079 (f) for 24 hours. After treatment, cells were fixed. Microtubules were stained green with FITC-conjugated anti- β -tubulin antibody, and chromosomal DNA was stained red with PI. C, effect on tubulin binding of colchicine, vincristine, and Taxol. Tubulin binding was tested with a SPA-based competition assay. Representative of three independent experiments. Used with permission of AACR (American Association for Cancer Research).

As effective as Paclitaxel might be, it suffers from severe drawbacks typical to many clinically active compounds from natural sources, such as low solubility, fluctuating supply and difficult synthesis (Table 1.2). New classes of drugs, such as biologics, could offer an alternative.

Table 1.2: Limitations of Paclitaxel

Limitation	Explanation	Reference
Development of Resistance	Acquired resistance to paclitaxel treatment poses limitations to therapeutic success. Includes slow-growing microtubules and overexpression of p-glycoproteins, (drug efflux pumps) and MDR cell types, among other types of resistance.	Gonçalves <i>et al.</i> , 2001, Horwitz <i>et al.</i> , 1993, Dumontet and Branimir, 1999
Lack of Specificity	Traditional chemotherapy is highly aggressive and destructive to all cells, including healthy tissue. Tubulin as target protein is essential in all cells, therefore the drug does not discriminate between tumor and healthy cells.	Ross <i>et al.</i> , 2004 Brannon-Peppas and Blanchette, 2012
Scarcity	The destructive extraction from the slow-growing Western Yew tree is an environmentally unsustainable method (~0.5g per tree). The compound is scarce, supply fluctuates and the cost for research and therapy is high.	Oliver, 1993; Si-Shen and Guofeng, 2001
Problematic Synthesis	As complex molecules with many stereo-centres, Paclitaxel is not amenable to synthetic modification and synthesis involves many steps. Semi-synthesis of precursor Baccatin III has been achieved from plant cell culture and paclitaxel was synthesized from fungal endophyte cultures, but this is not economically viable.	Pyo <i>et al.</i> , 2006, Li and Tao, 2009, Frense, 2007 Witherup <i>et al.</i> , 1990 Nicolaou <i>et al.</i> , 1994; Holton <i>et al.</i> , 1995;
Water Insolubility and Toxicity	Paclitaxel is insoluble in water. It requires formulation with toxic Polyoxyethylene pastor oil (Cremophor EL) as vehicle and dehydrated ethanol, which often causes hypersensitivity reactions. Poor solubility, metabolic instability and permeability contribute to the high attrition rate in development of many chemodrugs. Paclitaxel can cause peripheral neuropathy, neurotoxicity, cardiac and other toxicity effects.	Panchagnula, 1998, Di and Kerns, 2003 Singla <i>et al.</i> , 2002 Rowinsky <i>et al.</i> , 1995

1.3 Aptamers are emerging therapeutic agents

Nucleic acids are essential molecules for storage, transport, processing and expression of genetic information as well as catalyzing reactions similar to enzymes. Aptamers have emerged as promising class of biologics made from nucleic acids. Currently, there are 11 aptamers in the clinic for treatment of various disease areas including macular degeneration, oncology and inflammation (Zhou and Rossi, 2017). They are composed of short, single-stranded sequences of non-coding RNA or DNA of a typical length of 15-100 nucleotides. By folding into secondary and tertiary structures, they are able to bind a broad range of targets with high affinity and selectivity. Secondary structures are formed between bases over hydrogen bonds and tertiary structures are formed between regions of the aptamer in the three-dimensional space. While they can cover the same therapeutic spectrum as monoclonal antibodies (mAbs), they offer a number of compelling advantages over antibodies (Table 1.3).

Aptamers and *in vitro* selection were introduced by Ellington and Stozak (1990), Tuerk and Gold (1990) and Robertson and Joyce (1990). These are considered the founding works of the field. Demonstrating remarkable affinities from pM to nM against protein targets, aptamers compete with monoclonal antibodies (mAbs) in analytical and medical applications (Luzi *et al.*, 2003, Tombelli *et al.*, 2005). Aptamers possess selectivity similar to mAbs (Bell *et al.*, 1998, Cox and Ellington 2001). The aptamers were found to distinguish even between closely related targets, differing as little as one chemical group (Zimmermann *et al.*, 1997, Sazani *et al.*, 2004) or between the native and denatured conformation of a protein (Bianchini *et al.*, 2001). Another example is the distinction between closely related types of human influenza viruses (Gopinath *et al.*, 2006).

As an illustration of their broad spectrum, targets include a wide range of different molecules, from small molecules (Mannironi *et al.*, 1997, Sassanifar and Szostak 1993, Geiger *et al.*, 1996) to peptides (Nieuwlandt *et al.*, 1995) and proteins (Famulok and Meyer 1999, Brody and Gold 2000). This makes them a valuable asset in versatile applications in diagnostics (Osborne *et al.*, 1997, Famulok *et al.*, 2007), biosensors (Navani and Li, 2006) and anticancer therapeutics (Lee *et al.*, 2015).

As they overcome major limitations of mAbs, such as costs and limits to *in vivo* production, they are increasingly adopted as therapeutics and affinity agents, for instance in sensors or as diagnostic agents (discussed further in section 1.3.2). To begin with, the cost to synthesize DNA and thereby, aptamers, has decreased significantly over the last years, due to advancements in the productivity of DNA synthesis. An example is the large-scale gene synthesis of DNA constructs by relatively new companies in the field, such as Twist Bioscience or Gen9 (recently acquired by Ginko Bioworks). This stands in contrast to the high production costs of antibodies.

The decreasing price to synthesize and sequence DNA (Cyranoski, 2016) could contribute to the appeal of aptamer therapeutics. This is because *in vitro* synthesis can be achieved with rapid turnaround times and at low cost even at larger scale. *In vitro* synthesis also secures supply stability without the batch-to-batch variability associated with antibody production. As aptamer enrichment does not rely on *in vivo* production, aptamers can be selected against toxic targets and cover an extended spectrum. Reduced shelf life is a concern for antibody biologics, while aptamers can be stored practically infinitely and refolded after denaturation anytime and with great ease, (Keefe *et al.*, 2010, Jayasena, 1999).

Furthermore, their much smaller size results in low or non-immunogenicity (Eyetechnology Study Group, 2002 and Eyetechnology Study Group, 2003) and allows for better penetration of tumor tissue (Keefe *et al.*, 2010).

After selection, nucleic acid ligands can be modified to improve their *in vivo* stability and bioavailability by a number of modifications, such as 2' fluoro-modifications on ribose (Jellinek *et al.*, 1995), O'-methylation on hydroxyl groups and 3'-deoxythymidine capping (Beigelman *et al.*, 1998). PEGylation and other modifications increase plasma circulation and counteract the rapid renal clearance due to their small size, (Tucker *et al.*, 1999). They can also be converted post-selection into the nuclease-resistant spiegelmers, which contain enantiomeric L-nucleotides, (Keefe *et al.*, 2010). Furthermore, they can be conjugated to other molecules, which makes them immensely versatile. Linkers for conjugation, immobilization as well as dyes and detection labels can be inserted at various sites, (Nimjee *et al.*, 2005).

Table 1.3: Comparison of aptamers and antibodies.

Aptamers	Antibodies
Binding affinity in low nanomolar to picomolar range	Binding affinity in low nanomolar to picomolar range
Entire selection is a chemical process carried out in vitro and can therefore target any molecule	Selection requires a biological system, therefore difficult to raise antibodies to toxins (not tolerated by animal) or non-immunogenic targets
Can select for ligands under a variety of conditions for in vitro diagnostics	Limited to non-toxic conditions for optimizing antibodies for diagnostics
Iterative rounds against known target limits screening processes	Screening monoclonal antibodies time consuming and expensive
Uniform activity regardless of batch synthesis	Activity of antibodies vary from batch to batch
PK parameters can be optimized	Difficult to modify PK parameters
Wide variety of chemical modifications to molecule for diverse functions	Limited modifications of molecule
Return to original conformation after temperature insult	Temperature sensitive and undergo irreversible denaturation
Unlimited shelf-life	Limited shelf-life
No evidence of immunogenicity	Significant immunogenicity
Cross-reactive compounds can be isolated utilizing toggle strategy to facilitate pre-clinical studies	No method for isolating cross-reactive compound
Aptamer-specific antidote can be developed to reverse the inhibitory activity of the drug	No rational method to reverse molecules
Production process not prone to contamination (bacterial or viral)	Production process highly susceptible to contaminations
Small size enhances access to biological compartments	Large size reduces bioavailability and access to biological compartments
Conjugation chemistries are orthogonal: can be readily introduced during synthesis, usually do not interfere with activity	Conjugation chemistries are stochastic: leads to product mixtures and reduces activity

Adapted from Jayasena 1999 and Nimjee *et al.*, 2005, Keefe *et al.*, 2010.

1.3.1 *In vitro* selection

In single-stranded form, the polar, negatively charged phosphate backbone of RNA and DNA form secondary structures which can participate in a variety of non-covalent interactions, such as van der Waals forces based on electrostatic interactions.

For instance, the secondary structure of the DNA double-helix is formed over hydrogen bonds between the bases (Watson-Crick base-pairing), as well as Pi stacking of electron orbitals and Dipole interactions. As example, ions from salts in solution will affect the folding of an ssDNA strand.

The precise stacking of flat moieties enables precise molecular recognition, alongside specific hydrogen binding and molecular shape complementation, which determines the binding specificity (Hermann and Patel 2000). Binding specificity is among the basic principles for *in vitro* selection, as well as counter selection.

As demonstrated by Tuerk and Gold (1990) and Ellington and Szostak (1990), target-binding aptamers can be selected *in vitro* from a library of random nucleotide sequences through a cyclic procedure commonly referred to as SELEX (Systematic Evolution of Ligands by eXponential Enrichment). It is also termed *in vitro* evolution, as it involves iterative steps of binding, partition and amplification to select the best binders and eliminate non-binders.

A chemically synthesized DNA library typically consists of 10^{12} - 10^{15} unique sequences (10^{15} unique sequences per 100mmol in the case of this thesis). A sequence is typically between 50-100 nucleotides long and bears constant PCR primer-binding regions of around 18nt (nucleotides) on each end and a random region of several tens of nucleotides long. Either DNA or RNA selection can be pursued, as the DNA library can be converted into an RNA library through transcription by T7 RNA Polymerase and insertion of a T7 promoter region on either end. The library is then incubated with the target of choice and the bound and unbound sequences are partitioned by either filtration, affinity chromatography, gel shift or gel electrophoresis. Subsequently, the bound sequences are eluted from the complex with the target, are PCR-amplified and separated from the dsDNA back into ssDNA to generate the pool for the next round. This may include a reverse transcription step of RNA to cDNA (and T7 transcription of DNA to RNA). Depending

on the method, the cycle continues for 10-35 rounds until the pool reaches a high level of convergence. This is observed by the occurrence of repeat sequences and clusters in sequencing results after cloning. In late rounds, the diversity of the pool will increasingly diminish and ultimately reveal the most effective, evolved binders.

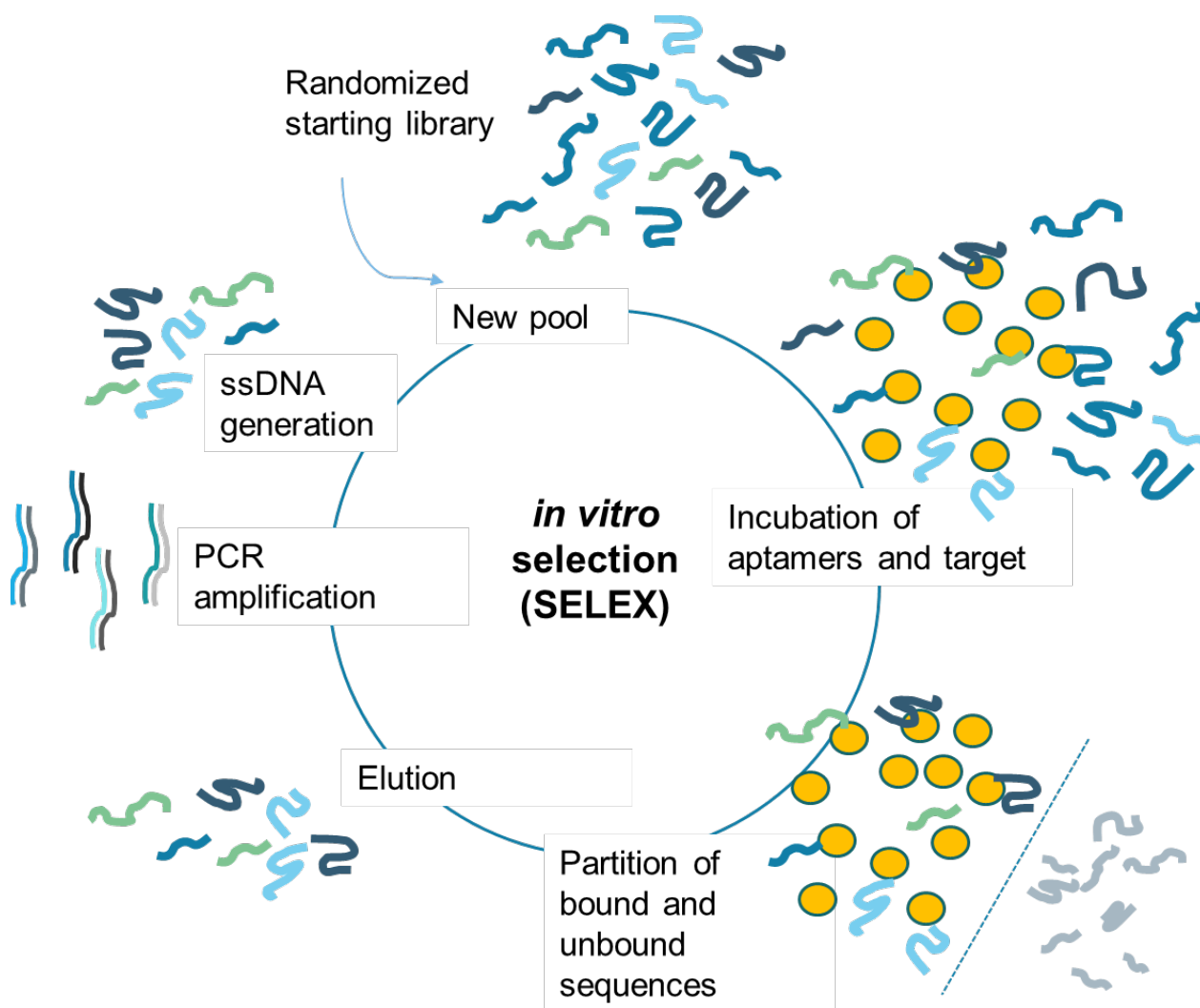


Figure 1.4: In vitro selection of DNA aptamers.

Incubation of the random oligonucleotide library with the target molecule is followed by a partition step to separate bound from unbound sequences. The bound sequences are then eluted from the target-aptamer complex, purified and amplified with PCR and finally separated into ssDNA to yield the pool for the next round. Ultimately, over the course of the selection, tight binders will become more dominant, known as enrichment. Pools are then cloned into bacteria and sequences to probe the convergence and enrichment. These consensus sequences are then tested for their binding ability with analytical methods like SPR and gel shift assays.

1.3.2 Oncomers: New directions to develop aptamers for oncology

Since their first introduction in 1990, possibilities for protein-binding aptamers have expanded. Cell-SELEX (and tumor-cell SELEX) allows aptamer selection against extracellular targets, for instance with the aim of targeting cancer cells through membrane receptors (Shangguan *et al.*, 2006) and Tenascin C (linked to oncogenesis), as demonstrated by Gold and colleagues (Daniels *et al.*, 2003).

SELEX has great potential for automation (Cox *et al.*, 2002, Cox and Ellington, 2001). Microfluidics is a versatile platform technology and has been applied for selection-on-a chip (Lin *et al.*, 2014), where selections can be automated (Huang *et al.*, 2010). Recently, researchers have developed an automated microfluidics chip utilizing magnetic beads, creating a device which enables high-throughput screening of various cell lines for competitive selection of aptamers. The goal is selection of aptamers which bind histologically different ovarian cancer cells for early detection of the cancer (Hung *et al.*, 2014). Quantitative Parallel Aptamer Selection System (QPASS) combines microfluidic selection with NGS and *in situ* synthesized aptamer arrays to accelerate the generation of high-affinity aptamers (Cho *et al.*, 2010). This microarray technique has made it possible to simultaneously screen 15000 aptamers for a given target. Next-generation sequencing (NGS) is discussed further in chapter 5. Using DNA microarrays, aptamers have been identified in a single round (Martin *et al.*, 2015).

SOMAmers are short DNA sequence, which are used in a protein analysis scan called SOMAScan. Bearing slow off-rate modifications (modified bases that contain 'protein-like' side chains and a 5' linker for greater affinity) endows them with greater sensitivity than antibodies, while functional side chains enable binding to specific proteins or conformational epitopes (discussed in chapter 5). New proteomic multiplex technology has been used for biomarker discovery for early lung cancer diagnosis (Ostroff *et al.*, 2010), malignant mesothelioma, (an aggressive pulmonary cancer related to asbestos) (Ostroff *et al.*, 2012), as well as Alzheimers disease with the SOMA scan (Sattlecker *et al.*, 2014), or aptamer array (Baird *et al.*, 2012). Here termed oncomers, aptamers for oncology are at the core of this thesis' motivation. These new directions above are promising improvements for coming years, which precisely address the aims of the work which were so far pursued with traditional methods.

1.3.3 From *in vitro* selection to the clinic: Aptamers as protein inhibitors

As proteins fulfill essential functions in disease, they are an integral part of therapeutic strategies. A rising number of aptamers have proceeded to late stage clinical trials and the first were released to the market, as discussed thoroughly by Nimjee *et al.*, (2005) and Keefe *et al.*, (2010), Sundarama *et al.*, (2013).

The success story of the first FDA-approved aptamer drug Macugen (Eyetech Pharmaceuticals/Pfizer Pegaptanib) has paved the way for oligonucleotide therapeutics. The vascular endothelial growth factor (VEGF) is a protein involved angiogenesis and mediation of vascular permeability. Initially intended as an anticancer agent, the RNA aptamer was successfully selected for this target. It was found to act as an effective anti-VEGF factor, which inhibits certain VEGF-mediated cellular responses, including age-related macular degeneration (AMD), a serendipitous discovery, (Green *et al.*, 1995).

Another milestone to modulate protein activity with aptamers was demonstrated by the precise, reversible control of a blood-clotting factor with an aptamer and its antisense antidote for use during surgery (explored in chapter 7), (Rusconi *et al.*, 2004). The thrombin-binding aptamer (TBA) is among the most widely studied anti-protein aptamers — a high-affinity binder and inhibitor of human thrombin with anticoagulant activity (Bock *et al.*, 1992). As such, it has found uses in theranostics: from immobilization for ELONA assays (Baldrich *et al.*, 2004) or immobilized on the gold surface of an electrochemical biosensor via thiolation (Lu *et al.*, 2008). The TBA also served as FRET probe (Nagatoishi *et al.*, 2006). Another example is a modification to respond to light-activation with the aim to control its activity (Heckel *et al.*, 2015). Incorporation of unlocked nucleic acid monomers improved its binding affinity (Pasternack *et al.*, 2010), while incorporation of G-LNA improved structural variability and biostability (Virno *et al.*, 2007). This represents the versatile applications of aptamers.

1.3.4 Aptamers in oncology theranostics

Targeted delivery strategies involving mAbs and cytotoxic agents hold promise to improve therapies, as mentioned previously. Trastuzumab (Herceptin® by Roche/Genetech) is a targeting antibody-Paclitaxel conjugate for drug delivery specifically to HER2-positive breast cancer cells — the most aggressive type of breast carcinoma — and has been met with great success (Collins and Workman, 2006).

But aptamers, as novel biologics, were also used to deliver cytotoxic drugs into HER2⁺ breast cancer cells (Liu *et al.*, 2012) and to deliver chemo-sensitizing siRNAs to tumor cells (Thiel *et al.*, 2012). Furthermore, aptamers were used to coat targeted supra-magnetic nanoparticles (Pala *et al.*, 2013) and were conjugated to silica nanoparticles carrying Doxorubicin (Dox). This enabled a novel targeted chemo-photo-thermal combination therapy (Wang *et al.*, 2015). Cell-SELEX with HER2⁺ cells has yielded more aptamers which specifically recognize the tumor cell surface (Dastjerdi *et al.*, 2011). A trimeric version of a newly selected HER2-aptamer inhibited tumorigenic growth of gastric cancer cells and mice xenografts. Remarkably, the cytotoxic effect was more than twice as strong as with the antibody version Herceptin (Mahlknecht *et al.*, 2013). Receptors which are greatly overexpressed on cancer cells offer an opportunity for discriminatory modes of delivery.

Similarly, nucleolin is overexpressed on the surface of multiple types of cancer cells. Anti-nucleolin aptamer AS1411, (previously called AGRO100) by Antisoma has entered stage II of clinical trials for cancer therapy (Que-Gewirth *et al.*, 2007). Inside the cell, it interferes with Bcl-2 mRNA and induces apoptosis by preventing the successful replication of DNA and cell cycle progression, which is mediated by the NF- κ B signaling pathway (Girvan *et al.*, 2006).

Tenascin C, an ECM protein that is highly expressed in the microenvironment of various solid tumors and is believed to play a role in tumor initiation (Hicke *et al.*, 2001) — aptamer TTA1 binds this target. The combination of TTA1 with an imaging agent for computer tomography allowed to visualize 3D structures of breast carcinoma and murine models of glioblastoma (Hicke *et al.*, 2006). Cy5-fluorescently

labelled aptamer, meanwhile, enabled imaging tumors on xenografts *in vivo* for cancer diagnosis (Shi *et al.*, 2011 and 2013).

Oncomers were also generated successfully against other receptors, including Tyrosine Kinase RETC634Y (Cerchia *et al.*, 2005) and Mucin1 (a tumor marker) for prevention of tumorigenesis (Ferreira *et al.*, 2006). The latter was subsequently developed into an ELISA assay to detect endothelial tumors (Ferreira *et al.*, 2007). Others inhibited Tyrosine phosphatase (Bell *et al.*, 1998) and interfered with $\alpha v \beta 3$ integrin for inhibition of oncogenesis (Mi *et al.*, 2005).

Two oncomers (A10 and A9) specifically target human prostate cancer cells via the extracellular portion of the prostate-specific membrane antigen (PSMA) (Lupold *et al.*, 2002), another prominent target next to HER2. Anti-receptor aptamers like these open possibilities for targeted aptamer-drug conjugates specific to prostate or other cancer cells, as discussed above. As aptamers can be modified with versatile conjugation chemistries, they can be applied in combination with gold nanorods, for instance for therapeutics and diagnostics of cancers (Gallina *et al.*, 2016).

But could aptamers themselves serve as drug-like agent? Platelet-derived growth factor (PDGF) is linked to malignant tumor growth and expressed and secreted by many tumor cell lines (Heldin, 1992). Gold and Janjic were among the first to generate aptamers as PDGF ligands as (Janjic *et al.*, 1997). Administered to rats, these aptamers were found to reduce proliferating mesangial cells by 95% (Floege *et al.*, 1999). Further work revealed that administering the PDGF-B aptamer to tumors reduced interstitial fluid pressure in the tumor tissue and thereby enhanced chemopharmaceutical uptake (Pietras *et al.*, 2001). This, the team hypothesized, could be explored as a novel treatment strategy to increase chemotherapeutic efficacy. More research followed for a combination of an anti-PDGF aptamer mixed with a cytotoxic agent (United States Patent 6699843, Pietras *et al.*, 2004). Aptamers in clinical trials for cancer targets are summarized recently and thoroughly elsewhere (Le *et al.*, 2015).

This demonstrates the multifaceted ways that nucleic acid ligands against protein targets offer potential to improve upon existing therapies and pave the way towards aptamer-based oncology drugs in the coming years.

To summarize, cytotoxic chemotherapeutics interfere with tubulin dynamics and block cell cycle progression. Treatment success of Paclitaxel, the most potent among them, is impeded by fluctuating supplies, low-yield harvesting methods, resistance and the need for toxic formulations due to poor water solubility. Aptamers rival antibodies as new affinity agents, while possess great qualities, like robust, fast and relatively cheap *in vitro* synthesis and good amenability to a range of post-selection modifications. These can address some of the limitations of aptamers, like low *in vivo* stability or fast renal clearance (therefore low bioavailability). Another attractive property is that aptamers can be targeted with conjugations and linkers. They are proven as versatile diagnostic and therapeutic agents both in oncology and as protein inhibitors.

The motivation of this thesis was to develop oncomers — aptamers as drug-like tubulin inhibitors in a step towards better, more affordable cancer therapeutics, developed from chemically benign biological building blocks. The following chapters are aimed at evaluating existing tubulin-binding aptamers for their anti-mitotic effects *in vitro*, establish thorough nucleic-acid based analysis tools for drug discovery screening and generate more tightly binding aptamers with *in vitro* selection and various post-selection optimization strategies. Further, a polymeric target was explored to facilitate selection of Paclitaxel-like binders in the future and tubulin purification is attempted to gather bulk quantities of the protein for screening and selection.

1.4 Aims and scope of the thesis

The overall aim of this research was to explore aptamers as anti-mitotic agents (AMAs) towards therapeutic application. The aims are detailed as follows:

To further refine an established drug discovery assay for the evaluation of potential tubulin-interfering aptamers. This involved developing a sensitive, robust and comprehensive analysis framework specifically for characterizing nucleic acid agents.

To screen and assess a panel of tubulin-binding aptamers (Fukusaki *et al.*, 2001) as potential tubulin inhibitors for the first time.

Fabricating a novel miniature microtubule, assembled in presence of GmPcPP (GPC), to explore the feasibility for its routine use as polymeric *in vitro* selection target.

Setting up and optimizing *in vitro* selection protocols and library design and investigating the amenability of tubulin, a relatively unproven, but essential target, to selection with nitrocellulose filter membranes and lambda exonuclease for ssDNA generation.

Pursuing different post-selection modification strategies to improve aptamer activity, including an aptamer clock as rapid prototyping template.

2

Materials and Methods

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

All chemicals were supplied either by Affymetrix or Sigma Aldrich and were of molecular biology grade (nuclease-free) unless otherwise indicated. Water used was from Milipore or double-deionized (ddH₂O), 0.2µm filtered and/or autoclaved. Selection buffers were 0.2µm -filtered. Reagents for SDS Page were supplied by BioRad, enzymes were purchased from NEB (New England Biolabs). Purified tubulin protein was purchased from Cytoskeleton Inc. and chemodrugs as controls were purchased from Sigma Aldrich and Cytoskeleton Inc.

Nucleotides

Nucleotides and DNA libraries were supplied by IDT, Singapore. GPC was purchased from Jena Bioscience, Germany.

2.1.2 Consumables and instruments

Sterile micro-centrifuge tubes and PCR tubes were purchased from Eppendorf or Affymetrix.

Falcon tubes and pipette tips were supplied by Corning Inc.

96-wells assay plates were supplied by Cytoskeleton, Inc.

Micro-volume syringe was purchased from Hamilton

Filters and nitrocellulose membranes were supplied by Milipore

Absorbance and purity measurements were performed with NanoDrop ND-1000 and NanoDrop ND-2000 spectrophotometer.

SDS-page were performed with a BioRad apparatus

HTS assays were performed in a plate reader instrument from Omega

High-speed- and ultracentrifuges used were from Hitachi

2.1.3 Buffers and Solutions

Tubulin purification buffer BRB80

80mM PIPES

1mM EGTA

1mM MgCl₂

pH 6.8

Made 5x stock stored at 4°C

PEM (General Tubulin Buffer)

80mM PIPES

2 mM MgCl₂

0.5 mM EGTA

pH 6.9

TPB (Tubulin Polymerization Buffer)

PEM w. 30% Glycerol (v/v) and 1mM GTP

Glycerol content was 30% and 10% for polymerization buffer and compound dilution buffer, respectively. With 750µL PEM + 250µL Glycerol and 15µL GTP (100mM).

For facilitating assembly, percentages were adjusted to 25%, 40% and 50% Glycerol.

SBB (Selection Binding Buffer)

Per 200mL:

Tris-HCl pH 7.4	20mM	(2.6mL stock of 1.5M)
NaCl	100mM	1.1682g
MgCl	2mM	0.0393g
KCl	5mM	0.0763g
CaCl	1mM	20mL stock with 177.4mL H ₂ O

EB (Elution Buffer)

For 300mL: 8M Urea, 200mM NaCl, 25mM EDTA

3M Sodium Acetate solution

For 100mL: 40.83g, pH adj. with 50% HCl

0.5M KOH solution

For 200mL: 5.61 g KOH pellets

5M NH₄OAc solution

For 100mL: 38.54g NH₄OAc, adjusted pH with HCl

TBE

54g Tris Base

27.5g Boric Acid

20mL 0.5M EDTA (pH 8.0)

For 1L 5X stock solution

SDS Running Buffer

For 1L (10x)

30.0g Tris Base (0.025 M)

144.1g Glycine (0.192M)

10.0g SDS (1%)

3X Laemmli Buffer

For 100mL:

18.8mL 1M Tris (pH 6.8), 6g SDS (2%), 15 μ L β -mercaptoethanol,

30mL Glycerol, with 1mL bromophenol blue

Coomassie Blue Staining Solution

1g of Coomassie Brilliant Blue R-250 (Bio-Rad) was dissolved in 1L of 50% (v/v) methanol, 10% (v/v) glacial acetic acid and 40% H₂O. The solution was stirred for a few hours and filtered through Whatman filter paper, stored at RT and protected from light.

Destaining Solution

For 1L:

400mL MeOH, 500mL H₂O, mixed by stirring. 100mL glacial acetic acid was added under stirring and stored at RT.

GTP

Snap-frozen GTP 11μl of 100mM: 10μL of this solution was added to 1mL PEM buffer (1mM final concentration) for polymerization buffers. GTP was weighted in powder form for addition to the HMPB (High-Molarity Polymerization Buffer) for protein purification.

ATP

Powder was weighted and added to tubulin purification buffers.

Paclitaxel

Dissolved in pure DMSO and stored in small aliquots at -20°C until use.

Vinecristine Sulfate

Dissolved in pure DMSO and stored in small aliquots at -20°C until use.

2.2. Methods

2.2.1 Protein technology

Microtubule fabrication

GmPcPP (GPC) and Paclitaxel-stabilized microtubules

20µg tubulin (10% X-Rhodamine labelled) was polymerized in 10% GPEM-10 buffer (100mM Pipes 1mM MgCl₂, 1mM EGTA, 1mM GTP) containing 10% glycerol (v/v), for 10mins at 37°C. Tubulin polymerization was performed in reaction tubes in 200µL buffer at 37°C in reaction mix: 160µL GPEM30+80µL of 1mM Taxol. Polymerization buffers were G-PEM10 (1mL PEM10+10µL GTP at 100mM) and G-PEM30 (1mL PEM30+10µL GTP at 100mM). GPEM30 was prepared with either 1mM Taxol for Taxol-assembled MTs or 1mM GPC and 5mM NaCl (NaCl facilitates polymerization) for GPC-assembled MTs was added to the buffers.

Seed fabrication

Seeds and polymerization mix was prepared from the manufacturers' protocol (Cytoskeleton Inc.) as above, but instead of Paclitaxel, 1µL GPC (Jena Bioscience) was added (10mM final concentration) and the mix was not polymerized, but kept at RT in G-PEM10 buffer.

Polymerization on glass slide

A 5µL aliquot of polymerization mix (above) was added onto a glass slide and imaged at regular intervals with an LSM 780 confocal/wide-field microscope (Zeiss) with heated (37°C) stage and imaged in regular intervals to monitor growth (avoiding photobleaching and potential interference from antifade solutions).

Shearing of MTs for miniature MT fabrication

Microtubule seeds (prepared as described above) were polymerized for 30mins (as above) and were sheared by carefully passed them 3x through the needle of a 50-microliter 4S gauge Hamilton syringe to generate short seeds as previously

described (Caplow *et al.*, 1987). The syringe was cleaned and maintained as suggested by the manufacturers (Hamilton).

Imaging microtubules

Fluorescently labelled tubulin from Cytoskeleton, Inc was used to image microtubules and samples were loaded in 5 μ L aliquots onto a glass slide (Omano OMSK-50PL), covered with a cover slip and imaged with a fluorescent microscope (Zeiss LSM780) at according excitation and emission wavelengths for Rhodamine (Ex. 530-550nm, Em. 580-600nm) or TAMRA (Ex. 350-370nm, Em. 430-450 nm) fluorescent labels. Imaging data was analyzed with Metamorph software.

Length assay with standard fluorescence microscope

TAMRA fluorescently labelled tubulin was polymerized according to the manufacturer's instructions (Cytoskeleton Inc.) and aliquots at various time-points were gathered from a polymerization reaction tube (results in appendix). Tubes were covered in aluminum foil to prevent photobleaching. The reaction was stopped by placing the aliquots at RT to both prevents further polymerization and disassembly. Samples were imaged and MT length was compared qualitatively with a fluorescent microscope (Olympus) with 20x objective. Images analyzed with SPOT software.

2.2.2 HTS and statistical methods

Aptamer screening *in vitro* with tubulin polymerization assays

***In vitro* polymerization assays**

HTS assays were carried out with >99% pure tubulin supplied by Cytoskeleton Inc and prepared according to the manufacturers instruction (<http://www.cytoskeleton.com/tubulins/kits>).

Prior to each assay, fresh aliquots of aptamers were denatured at 95°C for 5 minutes in boiling water and refolded at RT for 30mins. Experiments were performed in triplicates in three independent experimental experiments, unless otherwise stated.

***In vitro* disassembly assays**

The disassembly reaction was performed as additional measurement directly after the assembly reaction by decreasing temperature to RT (~20°C) for 60mins at an estimated rate of ~2 degrees Celsius per minute. Experiments were performed in triplicates and averaged over three independent experimental runs as above.

Statistical analysis

Normalization

Raw O.D. values were normalized with the integrated baseline-correction function in Prism 6.0 (Graphpad). For some assays, polymerization levels were expressed as percentage to ensure the most accurate and meaningful inter-assay comparison. In addition to baseline-subtraction, OD curves were normalized relative to upper and lower levels (the enhancer and inhibitor) as it is conventionally expressed (Inglese *et al.*, 2007).

Outlier elimination

Outliers caused by spikes and irregularities in readings can cause artifacts in statistically sensitive analysis methods. Therefore, standard error elimination was performed. Outliers were identified by automatic outlier detection from linear or non-

linear regression analysis by Prism (Graphpad), with the recommended, conservative setting of Q= 1% (only the most likely outliers are eliminated).

V_{max}

To estimate kinetic parameters, linear regression analysis was performed either with Prism 6.0 (GraphPad) or manually on 12-18 data points in a section of steady growth (or disassembly). The microtubule growth in this phase is linear, hence this method was chosen as the most precise in accordance to the recommendation of the assay provider (Cytoskeleton Inc). $1/V_{max}$ can be determined from the best-fit value of the y-intercept of the slope, but is not an ideal method (best done from dose-response slopes). Alternatively, V_{max} was determined manually from the incline [mOD/min] over 18-20 data points of linear growth from as advised in conversation with representatives from the manufacturer.

MT polymer mass and stoichiometry

0.1 OD unit at 340nm corresponds to approx. 0.1 $\mu\text{g}/\mu\text{L}$ polymer (www.cytoskeleton.com/pdf-storage/datasheets/bk006p.pdf). With this conversion factor, the total polymer mass formed was determined from absorbance readings and relative to the tubulin control to assess the inhibitory potency of the aptamers at 10 μM .

Student-t-tests

Unpaired, two-tailed student-t tests were performed for determining significance of statistical results (Pearson values), either compared to the tubulin control or the original aptamer, as appropriate depending on the assay. Two-tailed P-values of less than 0.05 were considered significant. P values were represented as * for $P < 0.05$ and for ** $P < 0.01$, but significance of results was also considered in the wider context beyond the statistical significance alone. For instance, a result may be statistically significant, but differences between certain samples may still be small and hence, this was considered as well).

Nuclei size distribution

Nuclei size were estimated as: $A(t; A_{\infty}) = A_{\infty} f[t/t_0(A_{\infty})]$ across reaction times as previously described (Oosawa and Kasai, 1962, Bonfils *et al.*, 2007, Flyvbjerg, 1996)

2.2.3 Post-selection optimization

Randomers

Randomers of identical length and base composition of the sample average per each assay series were generated by scrambling one of the oligos. Each experiment therefore was run with its own representative randomer controls to pursue to highest degree of accuracy possible.

Minimization

To minimize aptamers, 28 nucleotides on each side were removed (a total of 56 from 115 or 116nt sequences), including the 18nt primer-binding regions on both sides.

Switchable chimera

The switch was designed to include two modules, a ligand-binding domain and a therapeutic, target-binding domain. For the former, an Arginine-binding DNA aptamer was used (Harada and Frankel, 1995) with apparent K_d of 2.5mM (as measured by CD titration) and, the Tuda 1 aptamer for the latter (Fukusaki *et al.*, 2001). These domains were combined in different versions of the hybridizing regions, which varied in length of the hybridizing bases (stem length) and thereby would represent self-complexing features of varying strength. The aptamers were refolded by denaturing at 95°C for 5 minutes and cooling down at RT. Then, Arginine was added at 100 μ M final concentration (10 μ L of 1mM stock), samples were mixed and left to incubate 10 minutes. The Arginine-binding aptamer has demonstrated strong affinity. It was hypothesized therefore, that this timeframe should provide sufficient time for the binding kinetics.

‘Aptamer Clock’ prototyping truncates

The predicted secondary structure of Tuda 11, the parental sequence which had shown promise in a previous assay series, was represented with the mfold web server (<http://unafold.rna.albany.edu>). The predicted central stem-loop was utilized to investigate the ‘clock’ concept. Single nucleobases were deleted from this parental sequence to generate a population of deletion mutants with the same sequence, but altered (predicted) structural features. In certain cases, deletions resulted in

completely altered structures *in silico*, other times, the motifs underwent a shift 'around the clock' template, restructuring, or stem-loops were constrained, expanded or novel ones appeared. A number of derivatives deemed worthy of further investigation were selected and tested.

Antidotes

Complementary antisense antidotes were designed as hybridization probe for one of the *in silico* predicted secondary structure features (<http://unafold.rna.albany.edu>). For this, the aptamer sequence of the motif and of the entire oligo were converted into their antisense complementary sequence to yield partial or complete 'antidotes' to control aptamer activity.

Both oligonucleotides were denatured at 95°C for 3mins, mixed and left at RT to refold for 30mins, the general refolding time that seemed sufficient for nucleic acid screening. This allowed for the strands to be fully hybridized at t=0 of the reaction, even though folding kinetics and time to hybridize were not elucidated further.

Secondary Structure Prediction

In silico secondary structure predictions were performed with the mfold web server (<http://unafold.rna.albany.edu/?q=mfold>) which applies a folding energy minimization algorithm (Zuker, 2003). Ion concentrations were estimated from tubulin folding buffer and 20°C folding temperature (RT) and for folding, the option for natural angles were selected.

2.2.4 DNA Technology

Polymerase Chain Reaction

All PCR reactions were performed using a PTC-200 Gradient Cycler (BioRad).

Standard PCR

The standard reaction mix according to the manufacturer's instructions (NEB) was prepared, unless otherwise stated. In a 100 μ L reaction, 10-100ng DNA template was amplified (unknown for selection pools) in 1 $\times$ reaction buffer (Promega), 25 mM magnesium chloride, 0.25mM dNTPs, 15 μ M forward (FW) primer and 15 μ M reverse (RV) primer (or 7 μ M RV primer for asymmetric PCR) and 0.1U/ μ L *Taq* DNA polymerase (NEB), unless otherwise noted. For asymmetric PCR, double the amount of FW primer was used. An initial denaturation step of 95°C for 2mins was followed by 15-20 cycles with a denaturing step at 95°C for 30sec, an elongation step at 56°C for 30sec and an extension step at 72°C for 30sec. A final extension step followed at 72°C for 2mins. Selection 1 was performed in 3x100 μ L PCR reaction, selection 2-4 in 100 μ L volumes, whereas after each PCR, 5 μ L aliquot was stored at -20°C as backup.

PCR optimization

Annealing temperature: Same as above, but the annealing temperature was applied as a gradient across a PCR block of 12 tube holders across the range 45-60 °C in 50 μ L reaction volume.

Cycle number: Same as above, but aliquots were collected in intervals after completion of the respective number of amplification cycles.

Specificity testing: Same as above, but the DNA template was added across a concentration gradient.

Determination of nucleic acids on gels**Agarose Gel Electrophoresis**

3% agarose gels were prepared for dsDNA with less than 100bp and ssDNA nucleotides for best resolution. Gels were prepared with 1× TAE buffer and 0.5µg/ml Ethidium Bromide (EtBr) and were run with a current of (8V/cm²) at 100V for approximately 1hour. The gels were visualized under ultraviolet (UV) light. Surface plots and band intensity diagrams were generated with the automated feature of the gel analyzer software GelDoc (BioRad).

Ethidium Bromide (EtBr) Staining

Agarose gels were stained by homogeneously mixing EtBr solution stock solution (10mg/mL) with liquid agarose in an Erlenmeyer flask (0.5µg/mL final concentration) prior to casting the gel. Gels were then visualized on an ultraviolet transilluminator and GelDoc instrument (BioRad) at 302nm in either case.

Purification of nucleic acids**Ethanol precipitation**

DNA was precipitated by addition of 1/10th volume of 3M sodium acetate pH 5.2 and 2.5x volumes of 100% ice-cold ethanol in presence of 20µg glycogen to facilitate precipitation and pellet visibility. This mix was dipped into liquid nitrogen for a few seconds or alternatively stored in the -80°C freezer for one to a few hours. Afterwards, DNA was centrifuged at 4°C for 30-40 minutes at 17'000rpm in a Heraeus Biofuge. The supernatant was carefully aspirated with a pipette, the pellet resuspended in 600µL of 70% ethanol and spun again for 10mins at 4°C at 17'000rpm. The supernatant was removed, the pellet was washed again with 250µL ice-cold 70% Ethanol and spun down again for 5-10mins. The pellet was then dried and resuspended in 10-20µL sterile miliQ or TE by gentle flicking and used for PCR and stored as 1-2µL backup in advanced rounds of *in vitro* selection.

QiaEX II kit

The kit was used according to the manufacturer's (Qiagen) protocol for desalting to purify nucleic acids after PCR and after lambda exonuclease digest.

Quantitation of DNA with spectrophotometry

The concentration of DNA was obtained from absorbance values at 260nm using a tabletop spectrophotometer (Nanodrop), calibrated against TE or sterile miliQ water. The purity of DNA samples was estimated by absorbance ratio 260/280nm. Ratios of ~ 1.8 were considered optically pure and ratios of up to ~2.0 were considered acceptable to use. The measurement served to verify that all protein had been removed.

Lambda Exonuclease digest for ssDNA generation

Standard reaction

The reaction mix was prepared according to the manufacturer's protocol (NEB). The amount of enzyme was adjusted to the amount of DNA present in pure samples according to nanodrop spectrophotometry (the accuracy for ssDNA is not ideal) for selection 1. For selection 2-4, the amount was kept constant according to the protocol. The antisense strand was 5'-phosphorylated with PCR.

Time-course

For a test reaction, 4.0µg purified dsDNA, 10U and 20U were added in a total volume of 100µl in 1× λ exonuclease buffer. The mixture was incubated at 37°C and aliquots of 10µl were collected across time points at 0, 10, 20, 30, 40, 50 mins and the reaction was terminated by heat-inactivation at 80°C for 1min (results in appendix).

2.2.5 *In vitro* selection

Library and primers

A random DNA oligonucleotide library was synthesized at 250nmole scale (IDT, Singapore) and contained 10^{15} distinct sequences (0.21mg oligos total)) with equimolar base incorporation. The library was diluted to 0.1nmol/ml in 10mM Tris (pH 8) and stored at -20°C until use. This library contained a random region of 54 nucleotides incorporated at equimolar proportions, flanked by 18nt primer regions on each side:

5'-ggattgagggtcgcac-54N-gatggctctaactctcctct-3'

Primers were supplied by Integrated DNA Technologies, Singapore. The reverse primer was phosphorylated on the 5' end and HPLC-purified to remove undesirable un-phosphorylated primer.

Library design: nucleobase composition and length variants

GC% content and sequence length were varied by generating randomized nucleotides across a gradient of either length or composition, while the other variable remained fixed. These oligonucleotides were tested with HTS assays to determine whether there were non-specific effects due to basic properties (in statistically limited manner).

Filter alkaline treatment

Nitrocellulose membrane filters (nitrocellulose/cellulose acetate-mixed matrix, 0.45µm pore size from Millipore Corp.) were equilibrated by soaking the disks in 0.5M KOH at RT for 20mins and then again at 37°C for 10mins. The disks were subsequently washed for 45mins in binding buffer/tubulin buffer on a shaking platform and stored in new buffer at 4°C, as described previously (McEntee *et al.*, 1980).

Filter retention assay

An aliquot of library pool was incubated on a filter disk and eluted under identical conditions as in the selection. The fractions of the flow-through and the background (non-specific filter binders) from the membranes were loaded on a gel for visualization and measured on a nanodrop spectrophotometer.

Preparation of target

Purified tubulin (Cytoskeleton Inc.) was reconstituted from 10 μ M aliquots of 2 μ L to different concentrations.

***In vitro* selection**

The library (at 50 μ M for round 1) was dissolved in 1mL selection binding buffer (SBB), denatured at 95°C for 3mins and immediately placed at 4°C for 30mins for refolding. For the first round, 70pmol ssDNA were used. Then, the aptamer pool was measured by nanodrop (albeit suffering from inaccuracy) and molar ratios of aptamer to target of 10:1 were kept for all rounds of selection 1 and slightly modified for the others. Tubulin was prepared from thawed aliquots (as described above) and added to the library for incubation in a sterile Eppendorf tube at 37°C for 30mins (carried out in a warm room).

During incubation, filters were placed on filter holders (MilliPore) with sterile tweezers. The filter disks were pre-wetted with sterile miliQ water and silicone O-rings (13mm, MilliPore) were placed on top of it. This ensured air-tight application of the top part of the filter holder to prevent leakage.

After incubation, the aptamer-protein mixture was loaded onto a pre-wetted membrane disk prepared as described above. For partition, a 1mL syringe was applied to the filter holder tightly. Then, the solution was passed through the membrane by gently pumping through airflow. The flow-through fraction collected in the tube contained the unbound sequences, while tubulin-binders were retained on the filter. Non-binding oligonucleotides were removed by additional wash steps with warm SBB. Bound sequences were eluted from the disk by boiling at 95°C for 30mins in denaturing 7M urea. Denaturing causes the aptamers to unfold and dissociate from the aptamer-target complex. The eluted ssDNA was precipitated (see section 2.3.4) to remove residual protein. Then, the pool was PCR amplified, λ exonuclease digested and purified to generate a new protein-free pool for the next round of selection (described above). A counter-selection step against the filter matrix was performed by filtering the aptamer pool in the absence of target protein (for all selections). For different selections, some factors were slightly varied, like

incubation times (between 20mins to 1 hour), as well as target concentration and volume of washing steps (no washing to x-4x volume).

After selection (no enrichment observed), the backup aliquots of pools were PCR-amplified to compare their relative intensities on 3% agarose gels.

3

**Assay and analysis development
for drug discovery screening with
tubulin-binding aptamers**

3.1 Introduction

Aptamers have demonstrated their versatility as probes in functional screening and analytical applications in drug discovery (Dausse *et al.*, 2009). A rapidly increasing number of aptamer-based microarrays for proteomics is published, including functional screening of protein-binding RNA aptamers (Collet *et al.*, 2005), an aptamer-based cocaine sensor (Stojanovic *et al.*, 2001) and a fluorescent displacement screen with protein-bound aptamers (Hafner *et al.*, 2008). Furthermore, they have found application as probes against bovine thrombin (Liu *et al.*, 2004). Anti-thrombin aptamers have also been screened using an algorithm that mimics evolution (Ikebukuro *et al.*, 2005). Firstly, ten short 15-mer oligonucleotides were randomly designed and tested for their thrombin-binding activity. These oligonucleotides were presumed to form G-quartets. Then, the best sequence was selected (showing the greatest thrombin inhibition) and reshuffled *in silico* to generate more versions, then they were tested again. After repeating the cycle a few rounds, aptamers with greater affinity were generated. (Ikebukuro *et al.*, 2005)

In the HTS context they have proven useful in displacement screens with small molecules (Green *et al.*, 2001), as drug discovery aptazymes (Burgstaller *et al.*, 2002), for target validation (Blank and Blind, 2005) as well as target prioritization and lead identification (Burgstaller *et al.*, 2002). Within the field of aptamers as active therapeutic molecule, however, a similar number of readily available screening methods is not available yet to build upon, nor much reported in current reviews. This reflects how much progress remains to be achieved in the area (Mayer and Jenne, 2004).

Aptamers exhibit a host of compelling intrinsic properties: excellent water solubility, cell permeability, low or no immunogenicity and amenability to post-selection modifications to optimize their pharmacokinetics and improve biostability (please see chapter 1 for a review). The good water solubility for aptamers means, the oligonucleotides are soluble in high concentration in aqueous solution and do not require solvents. This places them in an advantageous position within the ADME space (Absorption, Distribution, Metabolism, and Excretion) and increases the likelihood to overcome the high attrition rates associated with poor solubility in drug development pipelines. However, clinically successful aptamers have not yet been delivered at significant scale.

Aptamers as new functional inhibitors achieve their effect by binding a protein's small cavity or groove (Bell *et al.*, 1998) and often mimic natural interactions (Long *et al.*, 2008). The fact that they furthermore represent pure, synthetic compounds, contrary to the mixture isolated from natural products but then purified or synthesized and their aforementioned solubility across varied buffer systems makes them ideal candidates for existing drug discovery screens. Drug discovery, performed as HTS in the pharmaceutical industry, is the first step to refueling the drug development pipelines on the pre-clinical end and is based on rapid, sensitive, and robust identification of lead chemical matter from compound libraries for further development. Potential anti-neoplastic agents benefit from accessibility to this technology, since tubulin as drug target is readily applicable for screening *in vitro*.

The turbidity assay (discussed in context of drug discovery in chapter 1) lies at the core of the method to identify anti-mitotic lead molecules. It allows monitoring interference with the dynamics of microtubule assembly and analysis of polymerization kinetics (Gaskin *et al.*, 1974, Hall and Minton, 2005, Bonfils *et al.*, 2007). Kinetic methods can serve to elucidate the mechanism by quantitatively measuring either the incorporation of monomer into the growing microtubule (by absorbance) or by observing the formation of specific polymer structures (Frieden, 1985).

In vitro tubulin polymerization assays provide an excellent platform for the former and for varied further analysis like V_{max} , IC_{50} estimations, monitoring progression of key steps and nucleus size with high efficacy and reproducibility. But kinetic methods are not without their pitfalls, since there is considerable heterogeneity in the microtubules, a system of much greater complexity than for instance actin polymerization and it is not understood in detail (Frieden, 1985). A re-examination of the turbidity assay discusses potential and limitations thoroughly, (Hall and Minton, 2005). However, for the purpose of a first evaluation of aptamers, the assay serves well to shed light into the functional-kinetic interface between proteins like tubulin and aptamers in this manner for the first time. As the field begins to forge ahead to decipher protein-nucleic acid interactions, it is desirable for the larger community to devise efficient, reproducible methods that scale.

Therefore, 16 discrete sequences of tubulin-binding aptamers, which were named Tuda 1-16 (Fukusaki *et al.*, 2001) were evaluated to potentially identify a subset of positives binding controls for future assays, like dose-response curves to confirm (or disprove) the effect. From the reported weak binding affinities (45 μ M and 19.4 μ M for T1 and T2, respectively) only small effects are expected, but could serve as invaluable proof of concept.

At the same time, various analysis methods were applied to assess the HTS system for its compatibility with aptamer screening in general (such as kinetic parameters, time-point analysis of polymer mass and others) and the future use for larger scale libraries, which could be applied to many drug targets in HTS screening.

3.2 Results and discussion

3.2.1 Overall reaction

Here, a general overview on the range of effects of the panel is provided by the reaction curves of the entire Tuda population. Figure 4.1 illustrates the extent and nature (inhibitory or enhancing) of the effects on the polymerization (left) and depolymerization (right) of the target, tubulin. Figure 4.2 serves the same purpose with a more in-depth look at each single aptamer to identify the most promising candidates. This commonly used method allows to evaluate anti-mitotic agents (AMAs) *in vitro* and compares their potency, as described in chapter 1 (Hall and Minton, 2005).

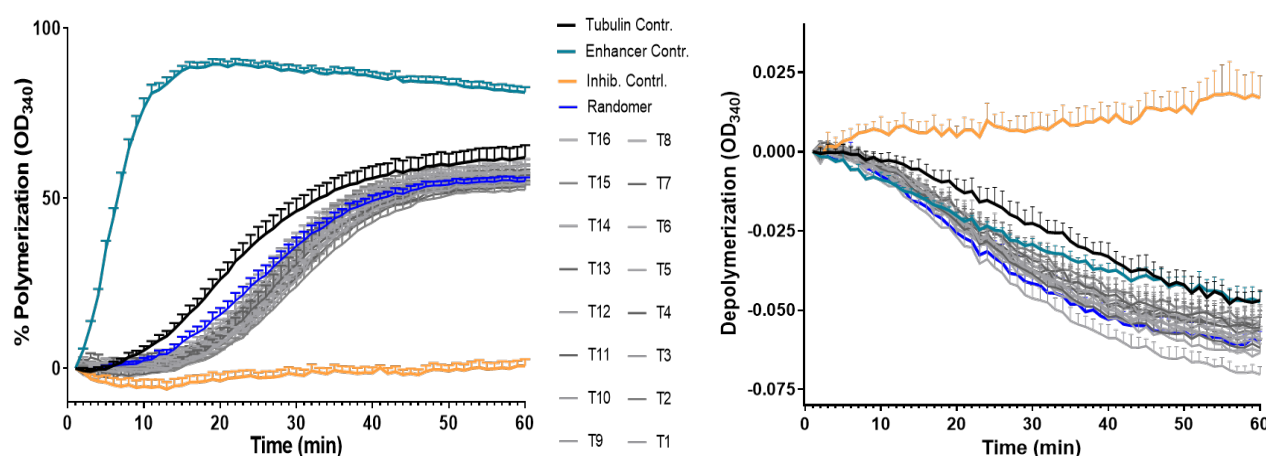


Figure 3.1: General *in vitro* assembly and disassembly curves for Tuda aptamers.

Reaction slopes for assembly (left) and disassembly (right) were determined with *in vitro* absorbance measurements (O.D. 340nm) of tubulin polymerization at 37°C (left) and by cooling down the plate (right). Curves represent TX enhancer (orange), VC inhibitor (orange), Tubulin (black), randomer (blue), Tuda aptamers (grey). Values represent averages over experiments repeated three times in triplicate. Error bars express SEM. Assembly is expressed as normalized %O.D. and disassembly as O.D. values.

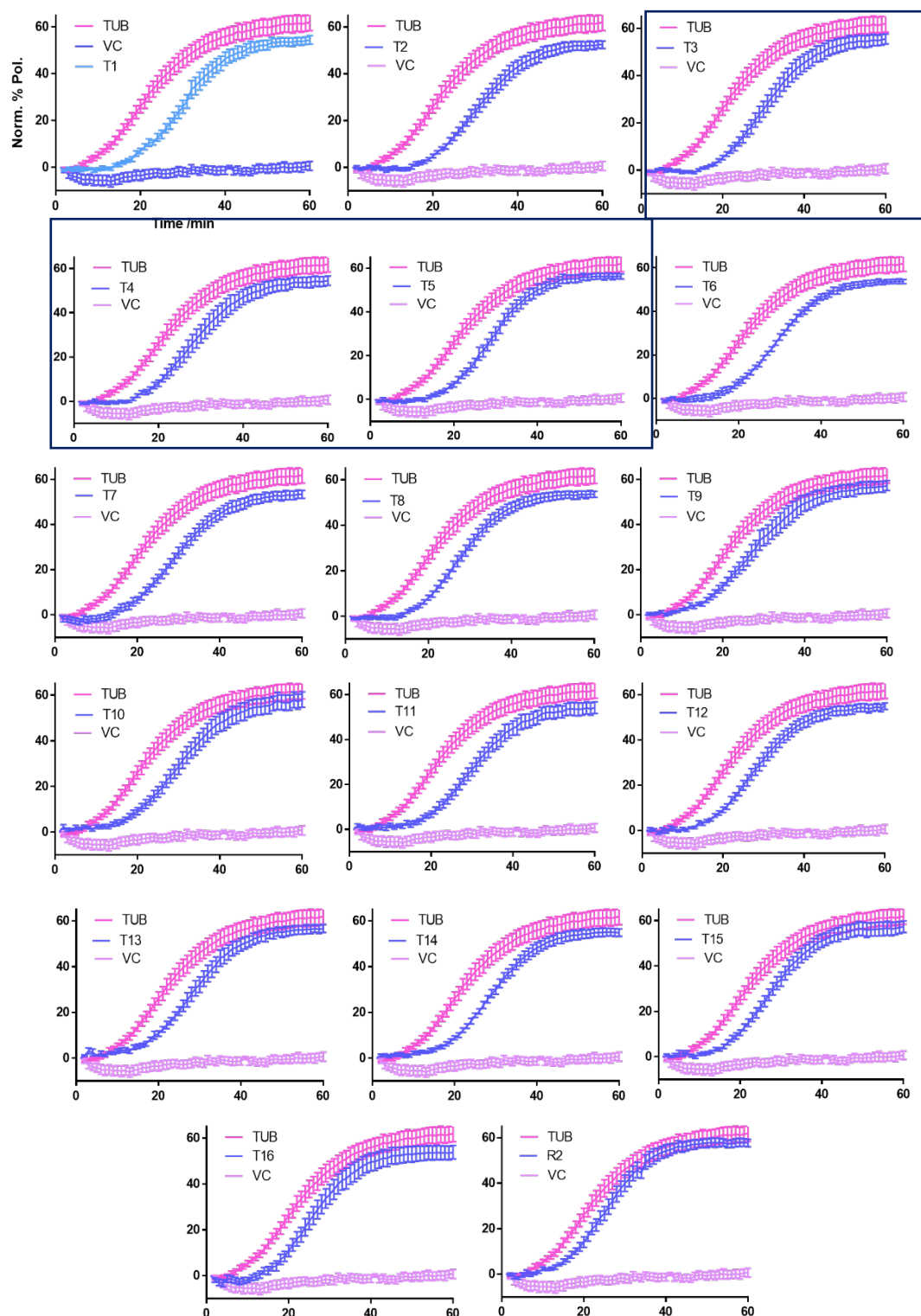


Figure 3.2: *In vitro* assembly curves per Tuda aptamer.

Curves represent the average of three independent experiments performed in triplicate and error bars represent SEM. Reaction slopes for assembly were determined with *in vitro* absorbance measurements (O.D. 340nm) of tubulin polymerization at 37°C. Values represent averages over experiments repeated three times in triplicate. Error bars express SEM. Assembly is expressed as normalized %O.D.

In figure 3.1, the overall reaction profile of the Tuda panel shows very small inhibitory effects in the beginning of the reaction that mostly remain of the range of the randomer control (same base composition and length as aptamers). This control was designed to assess non-specific effects of oligonucleotides, as many nucleic acids weakly bind proteins non-specifically.

The most interesting section is phase I and II within the first 30minutes, nucleation and growth phase. Compared to the randomer, slightly stronger, but clearly visible phase I inhibition is observed until around 20mins, but the difference is small. This effect is not sustained until the end of the reaction and appears to be reversible. MT mass at the end of the reaction approaches very similar levels to the tubulin control and the randomer. This may indicate that the initial inhibitory effects are reversible and/or that binding is not strong enough to impact the reaction overall like for instance the clinical inhibitor (VC) does. The disassembly curves (right) all fall within the range between the levels of tubulin and randomer controls, therefore no strong effects manifest here.

Figure 3.2 depicts the overall screen for each of the Tuda aptamers. The panel exhibits some variation in activity. The aptamers, T9, T13, T14, T15 and T16 do not show significant effects at any stage, while others, T4, T5, T10 and T12, appear to slightly perturb the nucleation phase (0-15mins): The onset of polymer growth (the sharp absorbance increase) is visibly delayed beyond randomer effects. But polymer levels reach the range of tubulin at endpoint.

The group, T1-T3, T7, T8 and T11 also seem to delay phase I. But in addition, they decrease MT mass at the end, even though difference is very small.

Overall, these results show that a few of the Tuda aptamers have small inhibitory effects by perturbing initial stages of the reaction, some more than others. Several slightly decrease endpoint MT mass. In summary, the subpopulation T1-3 (T-rich), T7 and T8 (G/T-rich) and T11 (G-rich) emerged as the most promising of the panel and slightly inhibit the kinetics, yet effects remain within a small range.

3.2.2 Phase I: Nucleation and molar stoichiometry

Phase I analysis serves to evaluate inhibitors in nucleation: Analysis was developed to determine aspects ranging from nuclei size to nucleation progression, the half-time, tenth-time and a O.D.-to-polymer mass conversion to gain quantitative understanding and a comparative tool to assess the specific effects (please see methods section 2.2.2 for calculations).

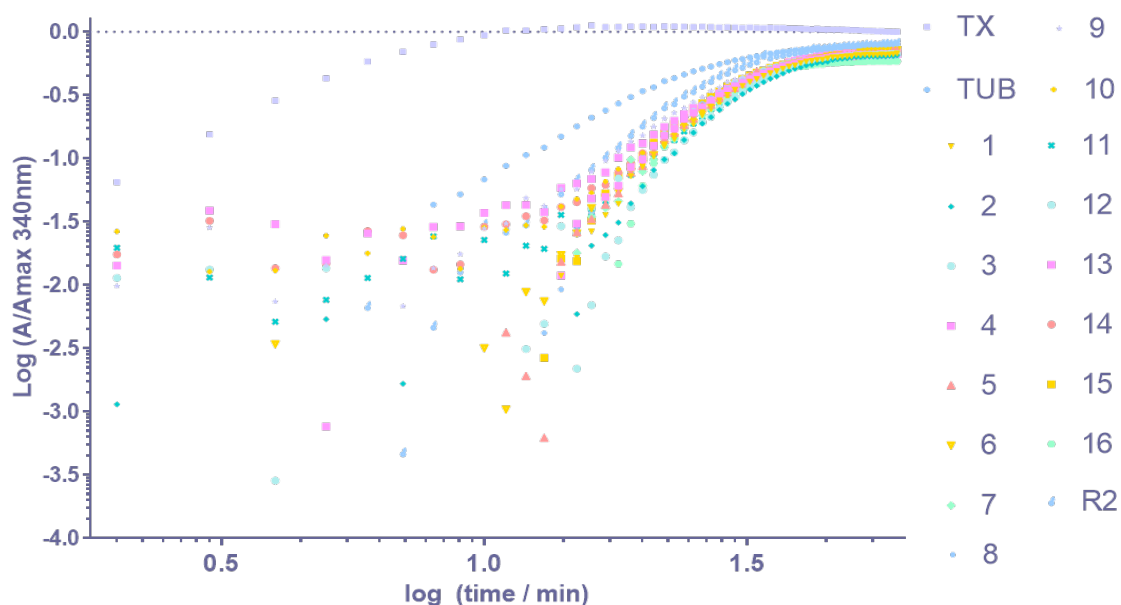


Figure 3.3: *In silico* estimates of nuclei size distribution.

Data points represent the size of the nuclei of averaged samples from three independent assays performed in triplicate. Nucleus size estimation across reaction by parameter p ($\log(t) / \log(\Delta A)$). Numbers represent the Tuda aptamers.

Nucleus sizes in figure 3.3 are estimated *in silico* (see methods 2.2.2) from the optical density across time-points. The figure shows the general nucleation phase dynamics of the panel with *in silico* predicted nuclei sizes based on absorbance values, another way of revealing perturbations in the reaction. As expected, the figure shows how microtubules across all samples grow from small nuclei into longer polymerized MTs in general. For the enhancer Paclitaxel (TX), the nucleus size grows much larger than tubulin control earlier, which corresponds with its ability to hyper-stabilize microtubules and 'supercharge' assembly. The inhibitor VC is omitted, as it is not amenable to this analysis (nuclei growth is inhibited overall). Average nuclei size for Tuda aptamers are smaller than for the tubulin control, which represents an inhibition of progression into the growth phase.

Table 3.1: Key kinetic parameters of nucleation phase (mins)

*

Sample	T 1/10	T1/2	Sample	T1/10	T1/2
TUB	11	22	TX	2	5
R	16	27	VC	n.a	n.a
1	19	30	9	16	28
2	20	31	10	17	29
3	21	31	11	18	30
4	18	29	12	18	28
5	19	30	13	16	29
6	19	30	14	17	29
7	18	29	15	18	27
8	18	28	16	20	27

Sample numbers denote the Tuda aptamers and R stands for randomer, TX for Paclitaxel (enhancer) and VC for Vincristine (inhibitor). T1/10 is tenth time and T1/2 is halftime, the time it takes to polymerize a tenth or half the total microtubules, respectively.

Values were determined optically from *in vitro* polymerization curves. Values represent averaged results from experiments repeated three times in triplicates. Boxed aptamers represent the most effective ones.

Table 3.1 summarizes the tenth time, time required to reach 1/10th of total MT mass. It is delayed between ~45-75% for the aptamers, some aptamers almost double the tenth time. This is a common for inhibitors, but clinical inhibitors (VC) have much more potent effects at 10µM concentration, (therefore not amenable to this analysis).

Here (Table 3.2), the MT polymer mass is calculated from absorbance values across phase I/II and steady-state, which offers another means to compare AMA's in-depth in a novel way. Inhibitors and their concentrations can affect the way nuclei form, from numerous small nuclei, or very few, large nuclei (Jordan 2002).

Table 3.2: Inhibition across time-points expressed as polymer mass.

#	Nucl/ Elong	Equ.	Diff.
TX	192.11 ± 21.9	45.73 ± 22.0	146.37
VC	-70.54 ± 5.6	-182.1 ± 6.8	111.56
R	-34.23 ± 2.6	2.57 ± 32.1	36.84

#	Nucleation/Elong. (18 min)	Equilibrium (min 60)	Difference	#	Nucleation/Elong. (18 min)	Equilibrium (60 min)	Difference
T1	-53.23 ± 3.7	-30.52 ± 7.3	27.71	T9	-36.76 ± 11.0	-24.82 ± 16.1	11.94
T2	-56.17 ± 1.8	-30.71 ± 9.0	27.48	T10	-44.18 ± 10.1	-26.64 ± 20.2	17.54
T3	-58.19 ± 2.5	-30.71 ± 10.2	27.48	T11	-49.38 ± 12.0	-26.64 ± 16.8	17.54
T4	-49.22 ± 9.4	-31.93 ± 11.6	17.28	T12	-47.16 ± 6.2	-34.59 ± 11.6	12.57
T5	-51.14 ± 7.0	-22.93 ± 5.3	28.21	T13	-39.75 ± 1.6	-25.61 ± 9.4	14.14
T6	-50.68 ± 11.6	-19.41 ± 20.0	31.27	T14	-45.34 ±	29.52 ± 7.0	15.82
T7	-52.75 ± 4.2	-33.58 ± 5.6	19.17	T15	-46.43 ±	-23.37 ± 18.3	23.06
T8	-49.7 ± 3.7	-33.39 ± 6.6	16.34	T16	-60.03 ± 43.3	-51.52 ± 43.2	8.51

Blue box highlights the most effective aptamers of the tested panel. Values were calculated from averages of three independent experiments performed in triplicates and according to commonly used conversion of absorbance values to estimated MT mass. Errors represent SEM. This series was not tested for significance.

TX= enhancer, VC = inhibitor, R= randomer

Table 3.3 Stoichiometric relationship of 10 μ M aptamer to impact MT polymer formation compared to clinical controls.

	Tub. Poly mer	Enhanc.	Inhib.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	R
Compound mass (μ g)	0.19	0.85	0.92	35.54	33.56	35.79	35.00	35.98	36.26	35.87	35.87	34.85	36.14	35.41	36.05	36.09	35.87	35.98	33.95	35.0

An estimate of clinical efficacy relative to clinical inhibitor (VC) that does not rely on dose-response IC₅₀ measurements. Values were calculated based on the average over three independent assays done in triplicate and represents the average of 9 samples.

Table 3.2 demonstrates that the Tuda aptamers vary in their effects. All of them slightly decrease the polymer mass formed, most in the range of the non-specific randomer and a few beyond it. During nucleation/growth phase, this inhibition again is greater than at equilibrium (endpoint). This is also observed visually, as phase I is impacted the most, while the suppression declines towards the end. This is revealed though the change in polymer mass difference between nucleation and steady-state. T1-3, T5 and T6 seem the most promising aptamers under this lens, again within a small range of effects. At equilibrium, the aptamers suppress MT formation, beyond the randomer (which has no effect). It appears reasonable to assume that more tightly binding aptamers would impact MT formation more effectively.

Table 3.3 compares the estimated molar stoichiometry between the aptamers and units of dimer affected (this was used as indirect, alternate expression of IC₅₀, which requires dose-response data) – in other words, to what extent does 10 μ M aptamer inhibited the assembly of tubulin dimers (in μ g)? These results show that aptamers require several orders of magnitude greater concentration to achieve effects comparable to the clinical inhibitor. This is consistent with the moderate-to-poor binding affinities (reported for the ‘top aptamers’ T1 and T2, therefore likely less for the rest)

3.2.2 Microtubule mass for assembly and disassembly

Following phase I analysis, a snapshot of the next critical parameters is provided by contrasting polymer mass at mid-and endpoint (phase II, III) of assembly (Figure 4.4A) and disassembly (Figure 4.4B) and the kinetics of both reactions expressed as V_{max} (endpoint is the bar on the right, left is the growing / shrinking phase).

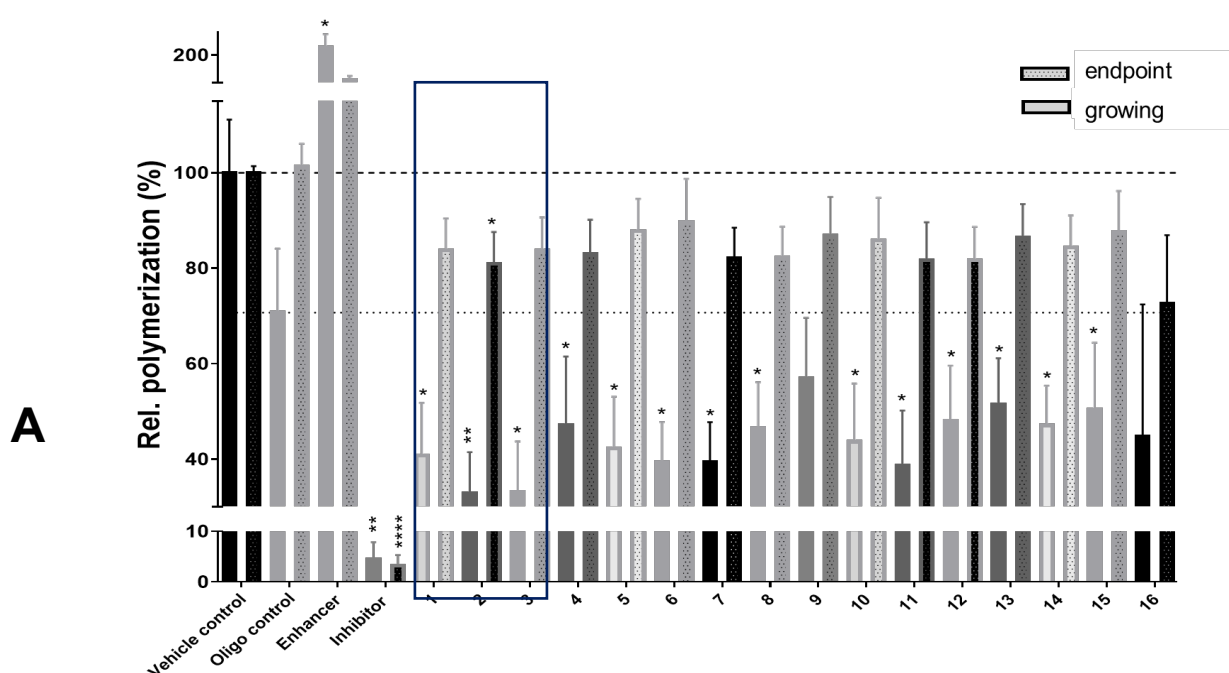


Figure 3.4A: Relative microtubule levels of polymerization.

A) Assembly reaction in growth phase at 25mins. Values were taken from the average of three independent experiments performed in triplicate. Error bars represent SEM. Pvalues were calculated as one-tailed student-t-tests between samples and tubulin (non-parametric). Pvalues: *=0.05 ** = 0.01 ***= 0.005 ****=0.0001

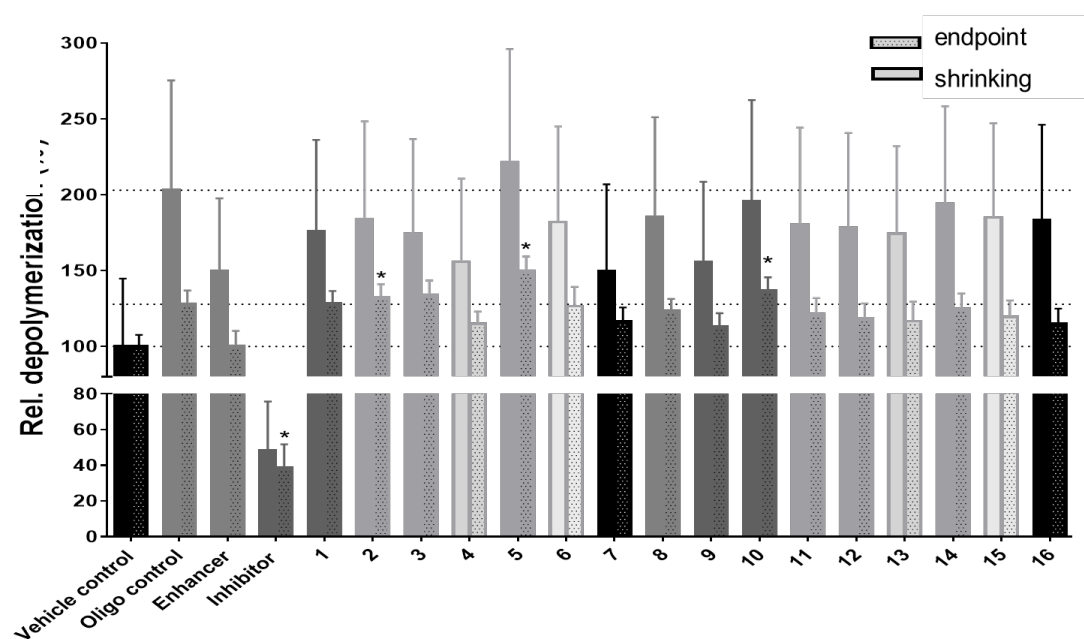
B

Figure 3.4B: Relative microtubule levels of depolymerization.

B) Disassembly phase at time-point=25mins. Values were taken from the average of three independent experiments performed in triplicate. Error bars represent SEM. Pvalues were calculated as one-tailed student-t-tests between samples and tubulin (non-parametric). Pvalues: *=0.05 ** = 0.01 ***= 0.005 ****=0.0001

Here, growth phase kinetics of polymerization allow for V_{max} comparison across the panel. For easy comparison, values are also represented as $\%V_{max}$. To yield additional insights V_{max} of depolymerization was included as well (goes beyond analysis typically performed, see methods 2.2.2). V_{max} values were calculated from the reaction slopes.

Table 3.4: Relative V_{max} and $\%V_{max}$ of assembly.

#	vmax	%vmax
TUB	5.06 ± 0.018	100
TX	20.6 ± 0.025	407.11
VC	0.417 ± 0.010	8.24
R	5.17 ± 0.003	102.17

#	Vmax [mOD/min]	% vmax	#	Vmax [mOD/min]	% vmax
T1	4.05 ± 0.015	80.03	T9	4.34 ± 0.013	85.77
T2	3.80 ± 0.018	75.1	T10	4.06 ± 0.018	80.24
T3	4.09 ± 0.020	80.83	T11	3.85 ± 0.020	76.09
T4	4.30 ± 0.018	84.98	T12	4.41 ± 0.011	87.15
T5	4.36 ± 0.016	86.17	T13	4.24 ± 0.012	83.79
T6	4.30 ± 0.011	86.17	T14	4.23 ± 0.013	83.6
T7	4.25 ± 0.011	83.99	T15	4.58 ± 0.018	90.51
T8	4.41 ± 0.011	87.15	T16	4.73 ± 0.050	93.48

Blue boxes denote the most effective aptamers for impacting this parameter and error represents SEM. $\%V_{max}$ is given relative to tubulin. V_{max} was determined with linear regression over 12-18 time-points of the steadily increasing growth phase.

Table 3.5: Relative V_{max} and % V_{max} of disassembly.

	#	vmax	%vmax
+ faster	TUB	-0.0097 ± 0.0047	100 ± 48.09
	TX -	-0.0065 ± 0.0025	67.2 ± 25.46
	R -	-0.0089 ± 0.004	91.26 ± 41.03

#	Vmax [mOD/min]	% vmax	#	Vmax [mOD/min]	% vmax
T1 +	-0.0103 ± 0.0024	105.2 ± 24.16	T9 -	-0.0097 ± 0.0044	99.62 ± 45.59
T2 +	-0.0104 ± 0.0034	106.4 ± 34.7	T10 +	-0.0107 ± 0.0042	110.0 ± 43.26
T3 +	-0.0107 ± 0.0027	109.5 ± 27.88	T11 -	-0.0086 ± 0.0049	88.45 ± 50.62
T4 -	-0.0091 ± 0.0034	93.31 ± 34.38	T12 -	-0.0088 ± 0.0046	90.27 ± 47.39
T5 +	-0.01 ± 0.0023	103.0 ± 23.80	T13 -	-0.0092 ± 0.0052	94.30 ± 53.28
T6 -	-0.0096 ± 0.0042	98.71 ± 43.36	T14 -	-0.0087 ± 0.0043	89.36 ± 43.62
T7 -	-0.0087 ± 0.0042	89.21 ± 42.98	T15 -	-0.0088 ± 0.0044	90.35 ± 45.50
T8 -	-0.0077 ± 0.0037	79.11 ± 37.95	T16 -	-0.0084 ± 0.0033	86.63 ± 33.52

Green (+) signs denote accelerated velocity compared to tubulin and red (-) signs signify decreased velocity. Values were taken from the average curve of three independent experiments performed in triplicates. Error represents SEM. V_{max} was calculated with linear regression in the growth phase across 12-18 time-points and mathematically represents the incline of the curve over this linear section.

Figure 3.4 A and B provide an overview of the panel and polymer mass differences. A shows that most Tuda aptamers reduce growth-phase polymer mass statistically significantly compared to tubulin, in consistence with the other results. But most of them do not sustain the suppression throughout to the endpoint, which would be typical of a

strong inhibitor (VC), possibly because the aptamers have only modest affinities. The exception is T2 (T-rich), which suppressed MT formation statistically significantly also at the equilibrium. Part B shows that most aptamers of the panel do not have considerable effects on depolymerization. Most exhibit only non-specific, weak effects falling within the range of the randomer. Exceptions are T2, T5 and T10, which appear to promote disassembly statistically significantly.

Table 3.4 presents V_{max} of assembly, the velocity at which the microtubules assemble. It is generally clearly decreased for the aptamers compared to tubulin (100%) and the randomer (102%) and reaches between 75.1-93.5% of V_{max} (for 10 μ M final concentration of aptamer each). The most promising group with the lowest values are the T-rich T1-3 and the G-rich T10, T11 sequences: V_{max} here is decreased to 75.1%-80.8%, inhibition reaching beyond the randomer, which has no effect (102.2% V_{max}). This may indicate that the panel does specifically interact with tubulin in this phase, but effects are mostly very small (consistent with modest affinities).

Table 3.5 shows V_{max} of disassembly, the velocity at which the microtubules disassemble. Promotion of depolymerization would be consistent with inhibitors (as opposed to Paclitaxel, which promotes stability). Overall, most aptamers curb these dynamics slightly, but remain at the randomer baseline. This pinpoints the general nucleic acid effects. A few, however, T1-3 (T-rich), T5 (G/T-rich) and T10 (G-rich) appear to slightly promote disassembly indeed with increased V_{max} , within a very small range of 103-110% but to the contrary effect of the rest of the panel. This may suggest a specific, but weak interaction, but the difference is too small to draw conclusions. Due to the large variation of the disassembly velocity, this part of the reaction seems less suitable for conclusive analysis. Regardless, V_{max} serves as good initial comparison and overview of general features and can reveal basic trends, even as follow-up investigations with dose-response data would further inform the present data.

3.2.3 T-rich ‘hit’ aptamer candidates

From the general discovery screening and evaluation of the Tuda aptamer panel, the three T-rich aptamers (T1-3) have emerged consistently as the most effective inhibitors across different parameters. Here, they are examined in more detail (Figure 3.5).

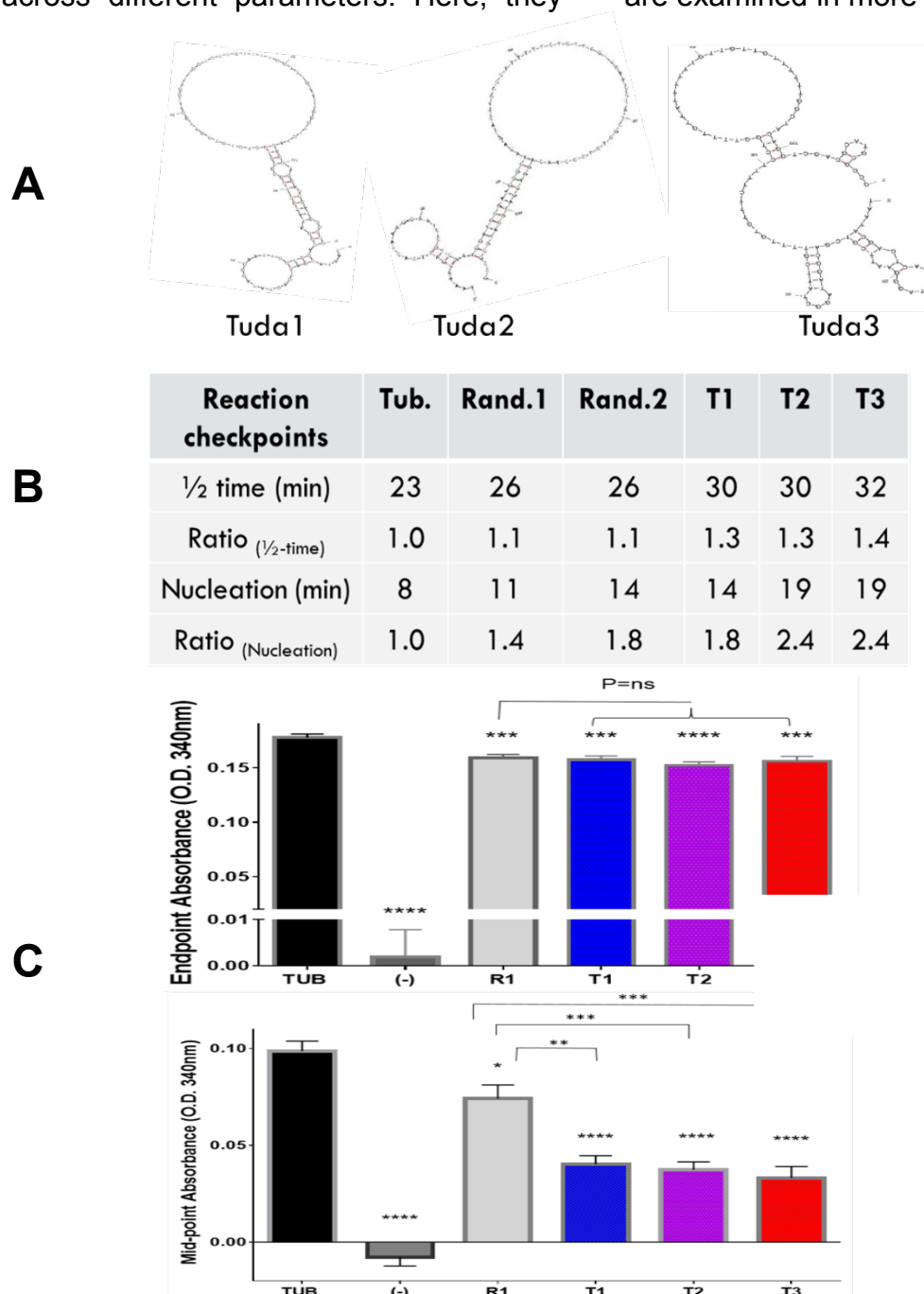


Figure 3.5: Analysis of identified ‘hit’ aptamers: phase I

A) Predicted secondary structures (mfold) under assay folding conditions **B)** Critical nucleation phase parameters half-time and overall duration (mins) **C)** MT polymer levels at mid-and endpoint of the assembly reaction. Values were averaged over three independent experiments performed in triplicate and error bars represent SEM. Pvalues were calculated as one-tailed student-t-tests between samples and tubulin (non-parametric). Pvalues: *=0.05 ** = 0.01 ***= 0.005 ****=0.0001

In Figure 3.5, A represents the predicted secondary structures by the mfold prediction tool, which is based on energy minimization in folding. T1 and T2 bear a highly conserved large stem loop, a long stem, a small interior loop and a small loop, while T3 is composed of a large multi-branch loop and multiple short stems and loops.

Represented in panel B, half-time is the time it takes to polymerize half of the maximal amount of tubulin subunits (to reach 50% maximum absorbance). Here it becomes apparent that T1-3 suppress nucleation and half-time to a greater extent than the randomers. T1 and T2 increase half-time from 23mins to 30mins compared to tubulin alone (26mins for the randomers). Half-time of T3 takes 32mins, 9mins longer than tubulin and 6mins longer than randomers. T2 and T3 more than double nucleation time from 8mins to 19mins compared to tubulin (2.4fold increase) and beyond randomers R1 and R2 (11mins and 14mins).

Panel C visualizes how T1-3 slightly, but statistically significantly decrease the microtubule mass at endpoint. However, the perturbation remains in the same range of the randomer control, therefore this is likely a general feature of nucleic acids (when expressed as relative polymerization in figure 4.1, the difference was not significant except for T2). The randomer control is used to specify the range of these non-specific interactions - by contrast this provides a sense of scale of the actual, aptamer-specific effects.

At midpoint, T1-3 visibly suppress absorbance levels, both compared to tubulin and the randomer and statistically significantly in both cases. This is interesting, because this likely reveals the aptamer-specific effects of binding. The results show that even moderate binders like T1 and T2 with affinities of 45 μ M and 19.4 μ M, respectively (Fukusaki *et al.*, 2001) have the potential to visibly curb reaction kinetics during polymerization. To affect the reaction in a more significant, persistent way, much stronger binders would be better suited for the task.

After a closer look at phase I, key kinetics of phase II and III are evaluated here (Figure 3.6).

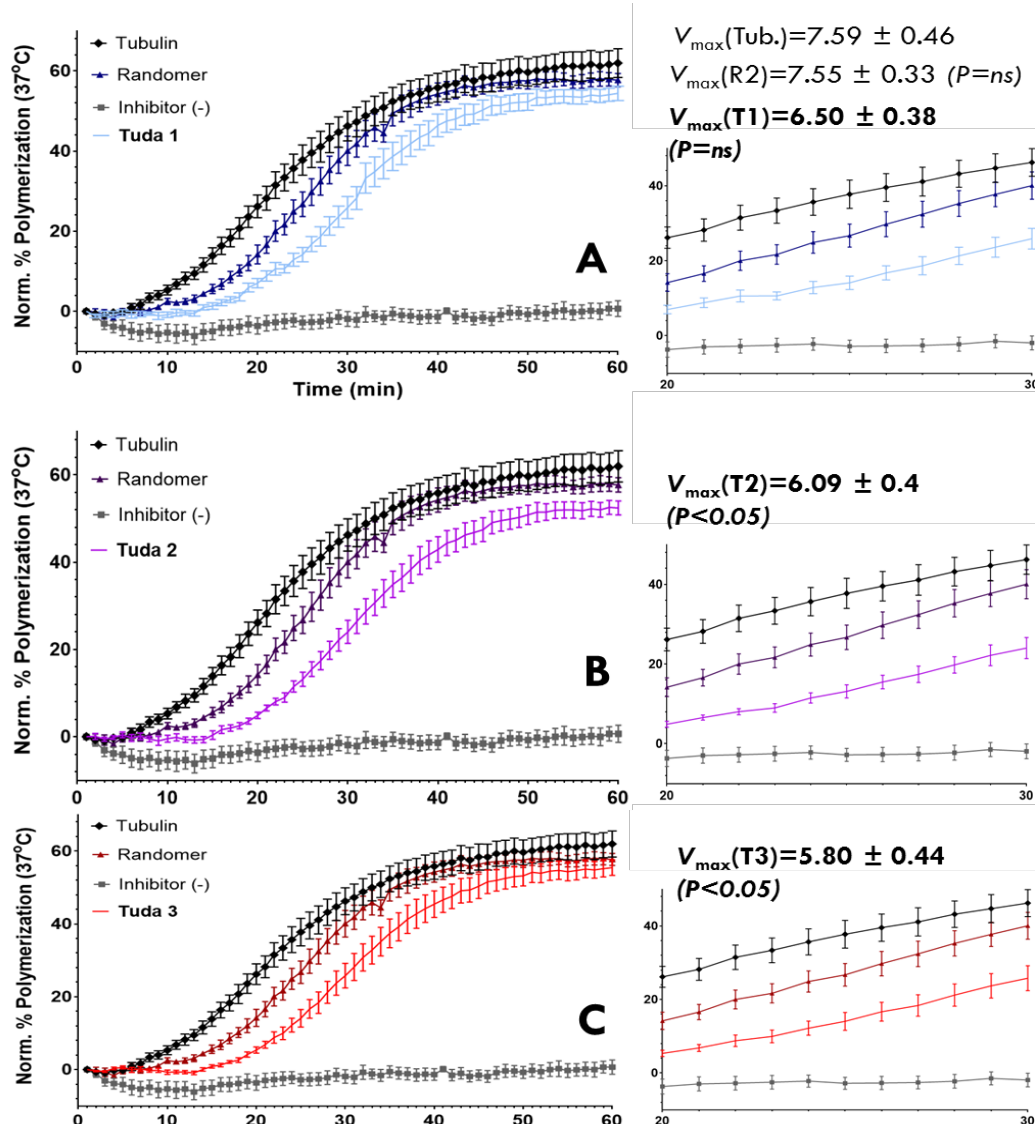


Figure 3.6: Analysis of identified 'hit' aptamers: phase I and III.

A) Tuda 1 ($K_D = 45\mu\text{M}$) **B)** Tuda 2 ($K_D = 19.4\mu\text{M}$) **C)** Tuda 3 (K_D unknown)

Overall *in vitro* assembly curves of tubulin (left), growth phase intersection (right) and V_{max} . Curves represent the average of three independent experiments performed in triplicate and error bars represent SEM. V_{max} was calculated with linear regression in the linear growth phase across 12-18 time-points and given in units of [mOD/min].

In Figure 3.6, polymerization slopes of T1-3 appear very similar: the reaction starts off with a noticeable small inhibition in phase I (nucleation) from 0-15 minutes and progresses with continued suppression in phase II (growth) from 15-35mins. Finally, the aptamers reach polymer levels similar to tubulin in phase III from 35-60mins. T2 inhibition is slightly greater than that of the other two. For all three aptamers, effects beyond general nucleic acid-protein interaction (randomer) are achieved.

V_{max} is statistically significantly decreased for T2 and T3. It is lowest for T3 (5.8mOD/min), followed by T2 (6.09mOD/min) and T1 (6.5mOD/min) compared to tubulin (7.59mOD/min) and randomer, which has no effect (7.55 mOD/min). This suggests that in general, nucleic acids do not impact tubulin formation, as expected, but tubulin-binding aptamers of modest affinity can interfere slightly.

Overall reaction curves show that effects extend beyond the randomer control and therefore are most likely a result specific to interaction with the target. T3 emerges as the most effective of the three T-rich aptamers, given its overall modest, but consistent inhibitor profile across the analyzed parameters. Another promising aptamer is T2 from the overall results.

3.3 Discussion

In vitro polymerization screens are a widely established, efficient and robust means to evaluate potential anti-mitotic compounds. The assay was set up and analysis method were developed for the first time to accurately evaluate a population of tubulin-binding aptamers across different parameters in great depth.

This served to concurrently examine nucleic acid agents which have not previously been characterized as well as to explore the limitations, scope and possibilities of the assay for drug discovery with aptamers. Specifically, the experiment aimed at gaining unprecedented insight into high-throughput oligo drug discovery by exploring non-specific protein-nucleic acid interaction baselines and novel kinetic parameters in addition to the limited set typically discussed.

Since the two repeat sequences from the selection, T1 and T2 possess rather weak reported binding affinities of 45 μ M and 19.4 μ M by SPR, (Fukusaki *et al.*, 2001) only small effects were expected.

Overall results show that the aptamers generally appear to inhibit *in vitro* microtubule formation to a small, but statistically significant extent. Some of the aptamer effects clearly reach beyond the randomer control. This is consistent with the knowledge that target-binding was confirmed (for T1, T2) and effects resulting from specific interaction would be expected. The modest range of effects also corresponds well to the binding affinities (where reported) and are slightly stronger for T2 than T1, but the difference is small. The aptamers seem to suppress progression of nucleation phase (1-10mins) and half-time to varying degrees. Most aptamers suppressed reaction kinetics of the polymerization more strongly than the randomer, but the V_{max} decrease remained in the magnitude of about 15-25%.

In agreement with general observations about tubulin inhibitors, five of the aptamers seemed to slightly promote disassembly and increases the velocity. This is in a very small effect, but it lies in stark contrast to the rest of the panel and randomer, which do not appear to affect the disassembly reaction.

Form these results, T1 T2, T3, (T-rich) T5, T6 (G/T-rich) and T11 (G-rich) seem the most promising. Considering consistent effects across several parameters, three 'hit'

candidate aptamers were identified for further analysis. T1-3. T1 and T2 appear to assume very similar secondary structures based on mfold predictions, which may or may not be accurate, while T3 might contain more complex features. The effects of T3 appear the strongest among these three aptamers across nucleation and half-time analysis, kinetics and polymer levels at characteristic time-points.

This may be a tantalizing hint to investigate this relationship further and most importantly, offers a strong rationale to develop more tightly—binding anti-tubulin aptamers. The results are a reassuring proof of concept within small, but significant inhibitory effects within the range of expectations. The randomer control has proven invaluable to discriminate between non-specific and specific interactions in a first instance. The limitations of the assay to reveal conclusive further information serves as motivation for follow-up experiments on structure, potential G-quartets, protein-aptamer interaction and kinetics in the future.

3.4 Conclusions and outlook

Overall, this experiment showed that oligonucleotides are in general amenable to classical drug discovery screening in HTS format. It also demonstrated that by including random oligo controls, in addition to clinical compounds, it becomes possible to distinguish the aptamer interaction from non-specific effects. This is a desirable feature unique to running this assay with nucleic acids. It revealed that the assay has its limitations when the effects are small, as with any experimental techniques. Inter-assay variations (CV) have to be considered as well (however, a low 12% CV was achieved, which is typically ~16% for this assay).

Furthermore, to unlock more information and utilize resources to their full capacity, the assay was performed both as the traditional polymerization assay and as depolymerization assay.

Another goal of this chapter was to provide experimental guidelines and an in-depth, robust analysis framework to comparatively evaluate aptamers in the most accurate way, both within the context of literature, but also in relation to one another.

In summary, Tuda aptamers seem to have small, in some cases statistically significant inhibitory effects on polymerization and some also in promoting depolymerization, as it would be expected for inhibitors. This effect manifested as changes V_{max} of both reactions, decreased mid-and endpoint polymer mass prolonged nucleation phase and half-time. The most interesting aptamer inhibitors are T1-3. T1 and T2, interestingly, also are the only repeat aptamers from sequencing results after the selection and have confirmed binding affinities by SPR. Other noteworthy aptamers were T5, T6 and T11, which are either G/T-rich or G-rich sequences. This draws attention to the possibility that these oligos could bear non-canonical secondary structure motifs. This would be interesting to explore further in the future.

However, the results also show limitations of the assay. For inhibitors with only small effects, inter-assay variation can potentially obfuscate results, as mentioned above. Hence, the assay appears ideal for compounds that have a stronger effects. Not all aspects of the analysis have been equally useful, and some remain of rather theoretical value.

For instance, overall comparison of polymerization slopes across the reaction has revealed useful information about the inhibitory potential. This revealed that the ‘hit’ aptamers suppressed nucleation and growth phase, but that the effect did not last into the steady-state and was an asset for easy visual comparison to the clinical and randomer control.

Comparison of endpoint/midpoint polymer levels was a good way to create a snapshot-like comparison across the population and across time-points. Representations should be scaled with clinical controls as to give a representative overview and for the best sense of magnitude, the full slopes are best to compare.

While V_{max} may give a useful first indication of overall patterns and can indeed be routinely estimated from slopes (Cytoskeleton, Inc), values would be more accurate when derived from dose-response curves (this would allow for K_D estimate as well) and would be more informative for compounds with stronger effects.

Other analysis, like stoichiometric potential and nuclei size estimations are rather theoretical and serve best as relative comparison within the assay series. Morphology of microtubules and nuclei could be investigated with electron microscopy imaging (which would be a more complex experiment) and for stoichiometric estimates, dose-response curves would be well suited to estimate IC_{50} values instead.

For future assays, it is desirable to rapidly explore the most relevant parameters per assay, depending on the assay rationale and limit in-depth analysis to the potential positives of the assay series. Full-scale analysis does not appear necessary or desirable to perform each time and the purpose of the work was to allow for robust and meaningful screening of aptamers and to enable more detailed analysis, where appropriate (applied in chapter 5).

In conclusion, oligo-specific analysis was developed across multiple reaction parameters to reveal critical dynamic and kinetic insights from nucleation phase, growth-phase kinetics and steady-state, as well as polymer mass differences and stoichiometric estimates. A population of tubulin-binding aptamers was characterized for the first time with a drug discovery screen adapted to nucleic acids, and novel analysis methods. These efforts yielded a set of positives and attractive ‘hit’ candidate aptamers

for follow-up studies. The emerging key hypothesis based on these results is that greater binding affinity is likely a prerequisite for achieving greater tubulin inhibition.

For future work, an interesting assay would be to measure IC_{50} values of a select few sequences and observe the effects at higher concentrations. To this effect, dose-response measurements could provide more accurate V_{max} and yield IC_{50} and K_D estimates with dose-response assays over a range of concentration gradients or cell culture studies. Simply using higher concentration (here: $10\mu M$) of the few select ‘hit’ candidate aptamers could potentially demonstrate much greater inhibition across the entire reaction. It would be exciting to investigate, whether this achieves more irreversible, permanent suppression or if it would still manifests predominantly during growth only.

Competitive binding studies with other clinical inhibitors, such as Colchicine or Vincristine could reveal the binding site of the aptamers (vinca or colchicine site) and allow simple models of the mechanism of action based on this data.

Structural studies with UV/VIS spectra, circular dichroism (CD) and more in-depth experiments with NMR could reveal valuable information for binding motifs and structural information like binding conformation and presence of non-canonical secondary structures. Since the most active aptamers of the population are either T- or G-rich (or both), this would be worth investigating.

This information could be used to pursue an *in vitro* selection with a partially structured library to facilitate selection of tightly-binding aptamers with greater inhibitory effects.

As next step therefore, a new *in vitro* selection for more tightly-binding aptamers was attempted (see chapter 4), while another assay series explored the potential of post-selection optimization of the aptamers (see chapter 7).

4

Fabrication of miniature microtubules

4.1 Introduction

Microtubules are highly dynamic biopolymers and possess the ability to control cell cycle through their characteristic dynamic instability. Paclitaxel (Taxol ®, Bristol-Myers Squibb) belongs to the class of microtubule-stabilizing agents (MSAs) with a potent mode of action. Paclitaxel (TX) binds the β -tubulin subunit of the polymeric α/β -heterodimers in the microtubules' lumen. There, it interlocks them on the microtubule and hyper-stabilizes them by preventing their dissociation - thus inducing apoptosis, (Horwitz, 1993, Kumar, 1981). This is reviewed in more detail in chapter 1.

A number of a few other promising MSAs have been identified (Table 5.1) with analogue mode of action and similar or improved potency. Strikingly, the majority of them binds this same site (Byron, *et al.*, 1998, Kowalski *et al.*, 1997).

Table 4.1: Binding sites of microtubule-stabilizing agents

Microtubule-stabilizing agent	Binding site	Reference
Taxanes (Paclitaxel, Docetaxel)	Taxoid site	Hirao <i>et al.</i> , 2000
Epothilones	Taxoid site	Bollag <i>et al.</i> , 1995
Discodermolide	Taxoid site	Kowalski <i>et al.</i> , 1997
Sarcodictyin	Taxoid site	Hamel <i>et al.</i> , 1999
Dictyostatin	Taxoid site	Madiraju <i>et al.</i> , 2005
Eleutherobin	Taxoid site	Lindel <i>et al.</i> , 1997
Cyclostreptin	Taxoid site, pores	Buey <i>et al.</i> , 2007
Laulimalide	<i>unknown</i>	Pryor <i>et al.</i> , 2002
Peloruside A	<i>unknown</i>	Gaitanos <i>et al.</i> , 2004
Estramustine	EM site	Panda <i>et al.</i> , 1997
FR182877	not yet investigated	Sato <i>et al.</i> , 2000
Zampanolide	not yet investigated	Field <i>et al.</i> , 2009

For instance, epothilones are a new class of taxol-like compounds with favorable properties, which also bind the taxoid site (Bollag *et al.*, 1995) and so do their derivatives (Lee *et al.*, 2001). Similarly, Discodermolide (Kowalski *et al.*, 1997),

Eleutherobin (Lindel *et al.*, 1997), Dictyostatin (Madiraju *et al.*, 2005) and Sarcodictyins (Hamel *et al.*, 1999), all bind to the same site and hyper-stabilize tubulin.

A small number of stabilizers have been found, however, which do not bind the taxoid site, such as Peloruside A (Gaitanos *et al.*, 2004) and Laulimalide (Pryor *et al.*, 2002) or interact with a newly discovered site, termed Estramustine site (Panda, 1997). In other cases, it remains to be investigated, such as for Zampanolide (Field *et al.*, 2009) and a substance called FR182877 (Sato *et al.*, 2000).

Interestingly, diverse organisms ranging from Pacific yew tree *Taxus brevifolia* and its endophytic fungus (Stierle, 1993), marine corals and sponges have evolved to biosynthesize these structurally diverse molecules, which nonetheless act as analogues and most remarkably - bind the same site of β -tubulin on the microtubule. The convergence of these vigorously potent chemistries has stimulated the search to further dissect the interactions between this pharmacophore and taxol-like MSA's in excellent precision (Buey *et al.*, 2004) in the quest for better drugs that share this mechanism. This could facilitate rational drug design (Giannakakou *et al.*, 2000), while understanding binding site thermodynamics helps to predict an MSAs' cytotoxicity (Buey *et al.*, 2005). A derivative of the estradiol metabolite, 2-methoxyestradiol, which has modest anti-tumor activity, shares the mechanism of action with colchicine and interacts with the respective binding site. Acetylation of certain hydroxyl groups and introduction of a ketone group, however, completely abolished this interaction and instead, the compound assumed a Paclitaxel-like mechanism of action (Verdier-Pinard *et al.*, 2000). This suggests that interaction with a particular binding site prompts a specific mechanisms of action.

But the taxane site is not always accessible, as it has a curious property: It only emerges in microtubule polymers with a substructure of well-defined protofilaments (Parness and Horwitz, 1981; Takoudju *et al.*, 1988), but not so in the dimers.

Potentially as a result of this, the vast majority of anti-mitotic agents (AMA's), albeit a chemically diverse group, bind the dimeric subunit form, most commonly via the

colchicine or vinca site and function as inhibitors. Vinca alkaloids like Vincristine and vinorelbine bind the vinca site. Compounds binding the colchicine site on α/β -heterodimers have shown generally weak effects, whereas colchicine itself is not used clinically due to its low potency. Many newly discovered inhibitors strongly compete with Colchicine, indicating they bind the same pharmacophore.

Within the context of existing evidence to date and as is generally inferred, it appears plausible that the β -tubulin binding sites directly governs the mode of action, either resulting in destabilization or stabilization, whereas compounds binding the latter tend to be orders of magnitude more cytotoxic. For an *in vitro* selection target, in consequence, the abundance of three major pharmacophores – two easily accessible, one conditional - presents both a chance to select diverse binders, but also additional impetus to generate aptamers of taxol-like potency.

In consideration of this, the work of this chapter aspired to fabricate a novel polymeric MT target presenting the taxane site to recruit polymer-binding aptamers. It aimed at exploring the feasibility and microtubule plasticity for the subsequent selection (chapter 6) and a more advanced selection with CE and NGS (covered in the future outlook). Design thinking guided the specifications to construct the ideal target:

- Firstly, the microtubule has to be stabilized, or otherwise disassembles quickly (due to rapid GTP hydrolysis). This is commonly achieved with Paclitaxel. However, this agent is not at disposal, because it would occupy this very pharmacophore and defy the experimental logic. The imperative alternative was found in GmPcPP (GPC), a slowly hydrolyzing analogue of the GTP nucleotide (Hyman *et al.*, 1992).
- Secondly, TX binds on the luminal side of MTs (Nogales *et al.*, 1999). TX can access the lumen by binding to a lower-affinity site on the outer wall first and translocate to the high-affinity site, as proposed by Snyder, 2007. But scenarios of inaccessibility of the binding sites exist as well and is not yet fully understood (Diaz *et al.*, 2003). To facilitate luminal access in practice by simple means, the geometric ratio of lumen to external surface (can be imagined as a long, hollow cylinder) was optimized by shortening MTs. Hence,

minimizing probability that binders would attach and remain at the outer wall, in theory.

- Thirdly, obtaining pure polymeric target requires elimination of the off-target structures, such as aggregates, soluble dimers and denatured subunits. This was attempted by ultracentrifugation with use of a sucrose cushion to preserve the structure.

In summary, work in this chapter aimed at facilitating in vitro selection of taxane-site binders with a target of rationally designed architecture by 1) constructing GPC-stabilized microtubules, 2) their miniaturization for luminal accessibility and 3) micro-ultracentrifugation for purity

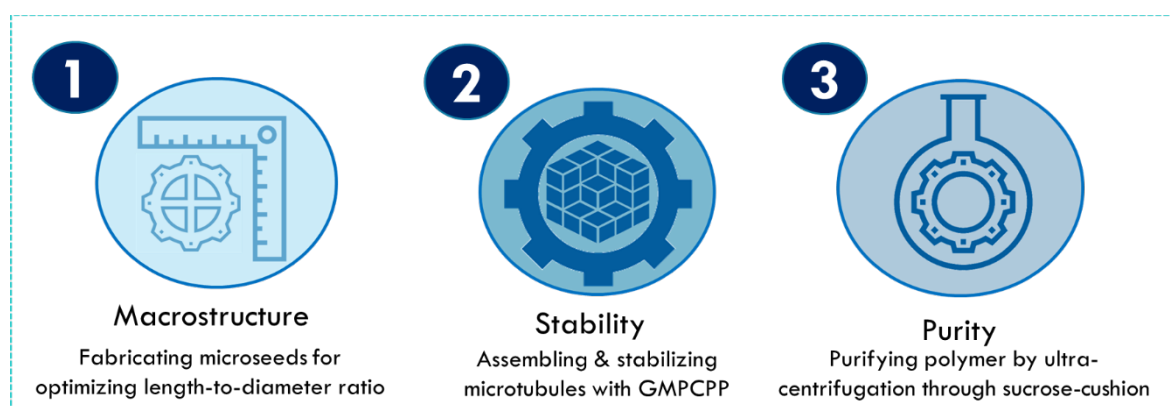


Figure 4.1A: Design principles to rationally engineer microtubule architecture.

4.2 Results and discussion

4.2.1 Miniature microtubules

The first step towards the miniature GPC-MT was to generate TX-stabilized microtubules and identify ways to control their length (Figure 4.1). Upon noticing the unusually uniform length of MTs, it was suspected that this was a result of the pipette tip diameter, which might break down the polymers to similar lengths. Therefore, the tip was cut to enlarge the diameter for probing if this would affect the length MTs (to be longer).

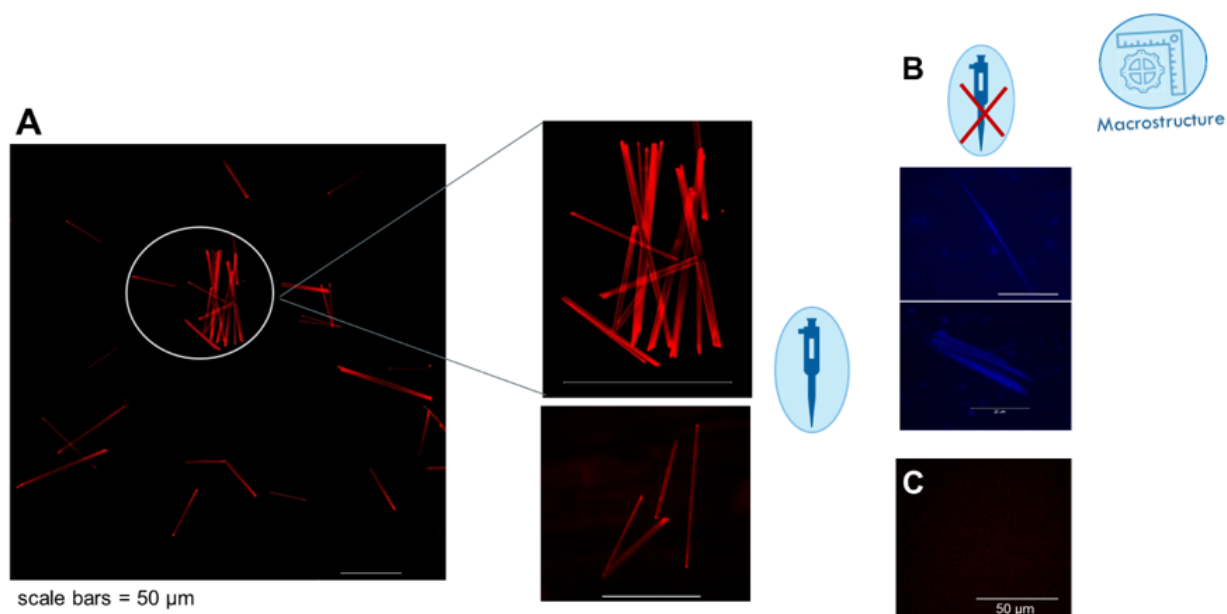


Figure 4.1: Controlling microtubule length by pipette tip.

A) Rhodamine-labelled uniform-length microtubule polymer, mounted from standard P200 pipette tip (0.6mm diameter) **B)** TRITC-labelled tubulin polymer, mounted with cut, large-diameter pipette tip (estimated diameter 0.25-0.5cm) **C)** Control without polymerization agent. Scale bars = 20µm, unless otherwise indicated. D) Scale bar 20µm (left) and 50µm (right).

Panel A of Figure 4.1 shows regular microtubule (MT) lattices grown and stabilized with Paclitaxel (purchased from Cytoskeleton Inc). A greater resolution makes apparent the near uniform length of the MTs of approximately 50µm. This seems unusual and a greater degree of variation was expected and MTs appeared short in comparison to literature.

As the MTs were polymerized in a tube and pipetted onto an imaging glass slide from there and not polymerized on the glass slide itself (without the restriction), it was hypothesized that it could be the pipette tip, which cuts the MTs down to this length.

Panel B of Figure 4.1 demonstrates that the MTs now indeed reach a greater size of approximately 100-150µm and again, the length appears uniform. This time the tip was cut to extend the diameter 2-4fold (not measured). This suggests that MT length can be easily controlled with varying the tip diameter.

This further indicates that the pipetting step poses a limit to the length of MT polymer due to the breakage. On the other hand, they can grow much longer on a glass slide. This was also tried for Paclitaxel-polymerized MTs, but did not work successfully (results not shown). C shows a negative control without enhancer present and confirms that a polymerizing agent is required to produce MTs.

Results (Figure 4.1) have shown that MTs are cut down to a certain length by the tip through pipetting. The next step was to actively shorten the MTs for the miniature version as previously described (Jeune-Smith and Hess, 2010). The same observation was applied: a microliter Hamilton syringe with ultra-thin needle (320 μm inner diameter) was used to shear the MTs, as it turned out, this is a known procedure to produce short MTs (Figure 4.2). The expectation was to see shorter, fully intact microtubules from first assembling, then shearing them.

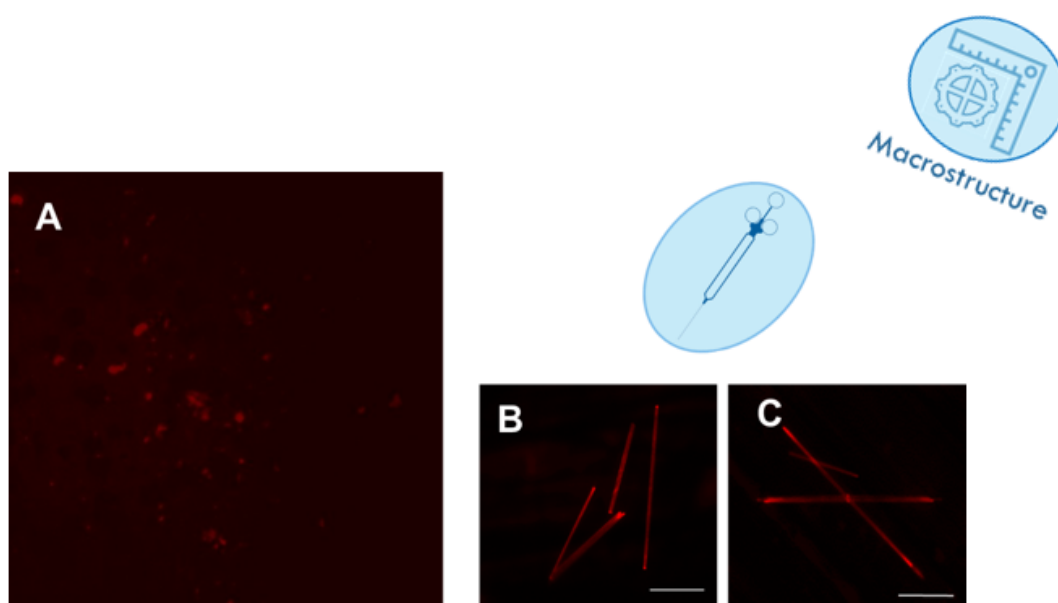


Figure 4.2: Controlling microtubule length by shearing.

A) Aggregates from miniature MT's sheared by micro-syringe **B)** and **C)** polymerization control with medium-length MT sheared with standard P-200 pipette tip. X-Rhodamine-labelled tubulin was polymerized with Paclitaxel and the assay was repeated three times with the same result. Scale bars= 20 μm .

In figure 4.2, A depicts the results of shearing the polymer: but it does not appear as microtubules and shorter versions of the control lattices in B and C. Instead, the protein seems to have formed aggregates. This indicates that the micro-syringe methodology might not be effective for this purpose in this case and that possibly the MTs are not stable enough for the shearing.

Because the micro-syringe method did not yield miniaturized MTs and induced aggregation (Figure 4.2), an alternative approach was pursued. Instead of artificially shortening the MTs, the growth time was limited. In the initial phases, polymer length is a function of growth time. By limiting polymerization and terminating the process after a short duration (by placing the tube at RT), it could therefore be possible to produce miniature MTs (Figure 4.3).

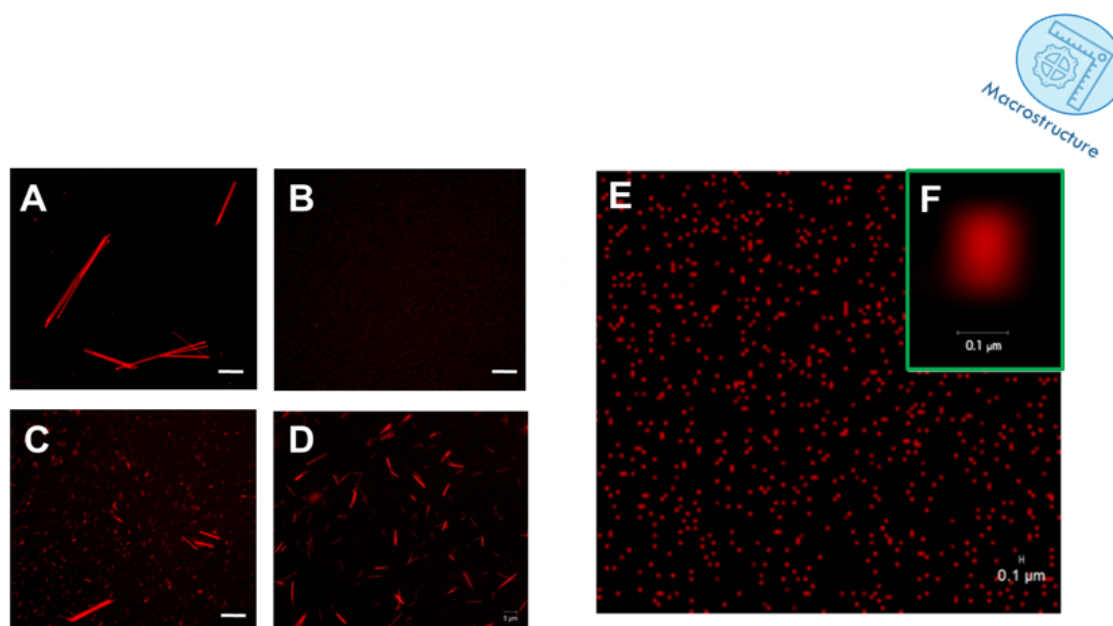


Figure 4.3: Controlling microtubule length by limiting growth time.

A) Rhodamine-labelled uniform-length microtubules mounted from standard pipette tip and polymerized with Paclitaxel for 60mins **B)** Negative control without polymerization agent **C)** MT at 25mins **D)** MT at 10mins **E)** MT at 5 mins **F)** Zoom of a single micro-MT / elongated seed. The assay was repeated two times with similar results. Scale bars = 20μm, unless otherwise indicated

In Figure 4.3, by restricting polymerization from 60mins, 25mins to 10mins, the microtubules become visibly shorter at each time point, as expected. E shows that growing them for just 5 minutes produces the desired micro-MTs: slightly elongated seeds and 'minimal microtubules' of approx. 0.1μm length, as envisioned. These results demonstrate that restricting growth times to just around 5 mins can yield the desired elongated seeds.

4.2.2 GPC-stabilization

After MTs were miniaturized by generating elongated seeds instead of shearing full MTs, all of the first design specifications was achieved – the next was exchanging Paclitaxel with GPC to assemble stable MTs with GPC and examine whether the morphology would be representative (Figure 4.4).

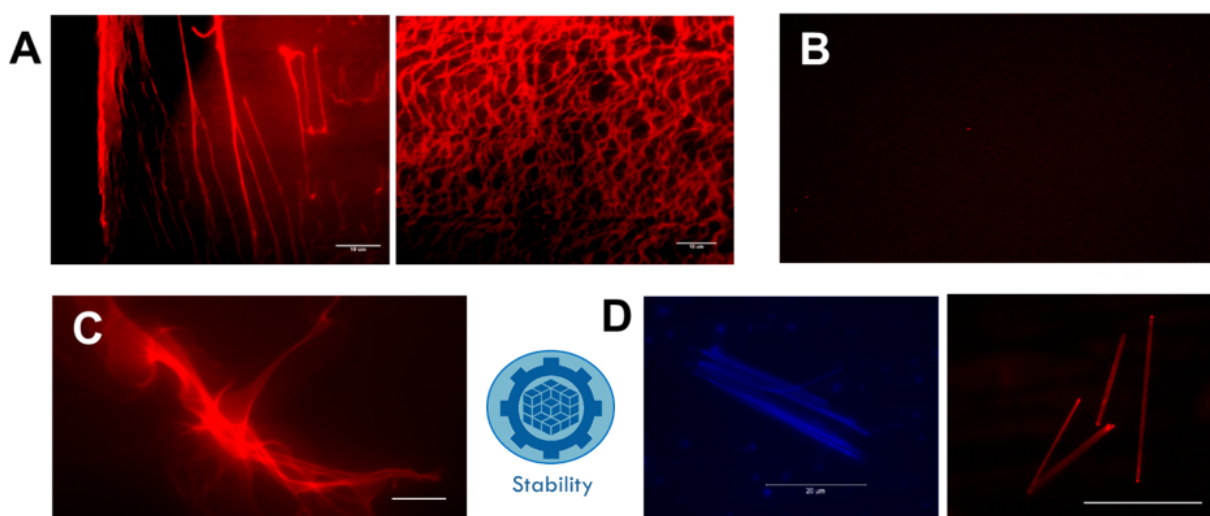


Figure 4.4: GPC-stabilized microtubules assembled on glass slide.

A) Morphologies of Rhodamine-labelled tubulin polymerized with GPC on a glass slide matrix **B)** Tubulin polymerized with Paclitaxel (TX) on glass slide **C)** Morphology of GPC-polymer assembled in reaction tube **D)** Morphologies of TRICT-labelled TX-polymer as control, assembled in reaction tube. Scale bars= 50µm, unless otherwise indicated (also 50µm in D)

Figure 4.4 A shows morphologies of GPC-microtubules compared to (D) for TX-microtubules: both forms appear drastically different from one another. These MTs were polymerized on a microscopy glass slide in a microscopy chamber warmed to 37°C. They represent a network-like structure or very long, atypical microtubules, visually reminding of roots and cytoskeletal structures.

In contrast, B shows that on the glass plate, the TX-tubulin does not polymerize, since only background is visible with a few bright red dots indicating seeds. This was attempted multiple times with a heated imaging stage at 37°C (as suggested by the manufacturer) and via heated 37°C heated microscopy chamber, to no avail.

C shows GPC-MTs polymerized in the reaction tube. They can be assembled successfully in the tube, but again, assume a vastly different morphologies than the TX-MT control in D. The GPC-polymer appears to lack the distinct cylindrical protofilament structure of compared to TX-microtubules (necessary to expose the taxoid site).

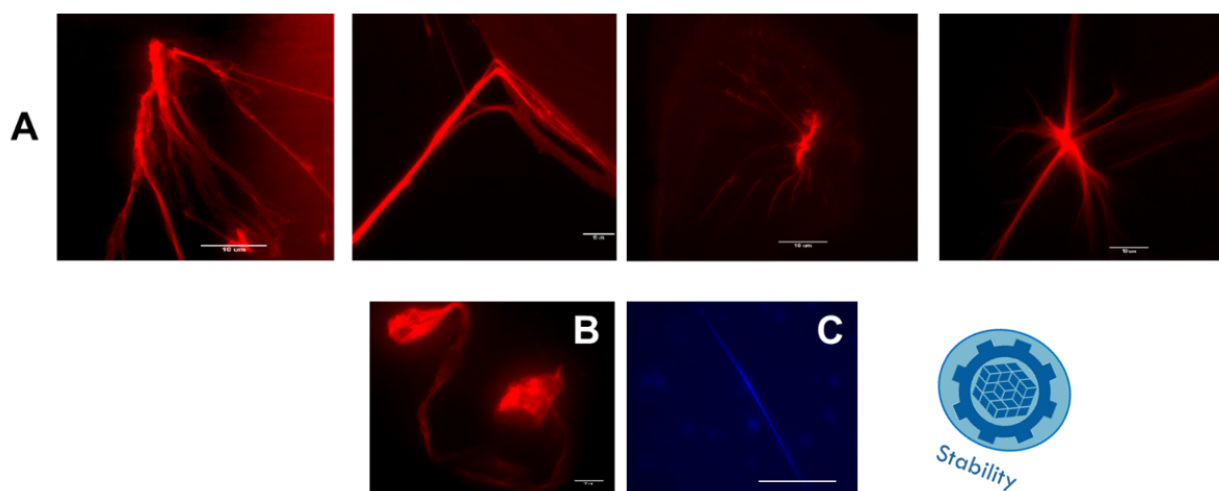


Figure 4.5: GPC-stabilized microtubules assembled in reaction tube.

A) Further morphologies of GPC-polymerized microtubule morphologies from glass slide matrix **B)** GPC-tubulin morphology assembled in reaction tube **C)** control polymer morphology polymerized with TX in Eppendorf tube. Scale bars= 50 μ m (C), otherwise as indicated. The assay was repeated twice with similar results.

Figure 4.5 A depicts the general GPC-MT morphologies from polymerization on a glass slide and showcases the great diversity (and lack of reproducibility) of these polymers. Sample B was assembled in the reaction tube and resemblance with TX-MT in C, it is equally very different. These results demonstrate that MTs can be assembled with GPC indeed, but that morphologies differ greatly.

These findings indicate that polymerization with GPC results in an alternate morphology of tubulin under these conditions and structures representative of the control could not be formed by either assembly condition (reaction tube or glass slide). The GPC-polymer does not appear sufficiently representative of the general structure under these conditions and distinct morphologies were reported also elsewhere (Yajima *et al.*, 2012).

Microtubules lattices can vary in their structure, between stabilized and dynamic forms and taxol and GMPCPP-stabilized forms (Alushin *et al.*, 2015). However, GCP used rarely and therefore, this niche is not yet understood well. Further investigation would be required to generate GPC-polymer that is more representative of TX-microtubules.

GPC-stabilized MTs were generated, but resulted in vastly different morphology in fully polymerized form. This assay investigated whether the same was true for GPC-miniature seeds (assembled on glass slide), since they would be the final *in vitro* selection target, not the grown microtubules (Figure 4.6).

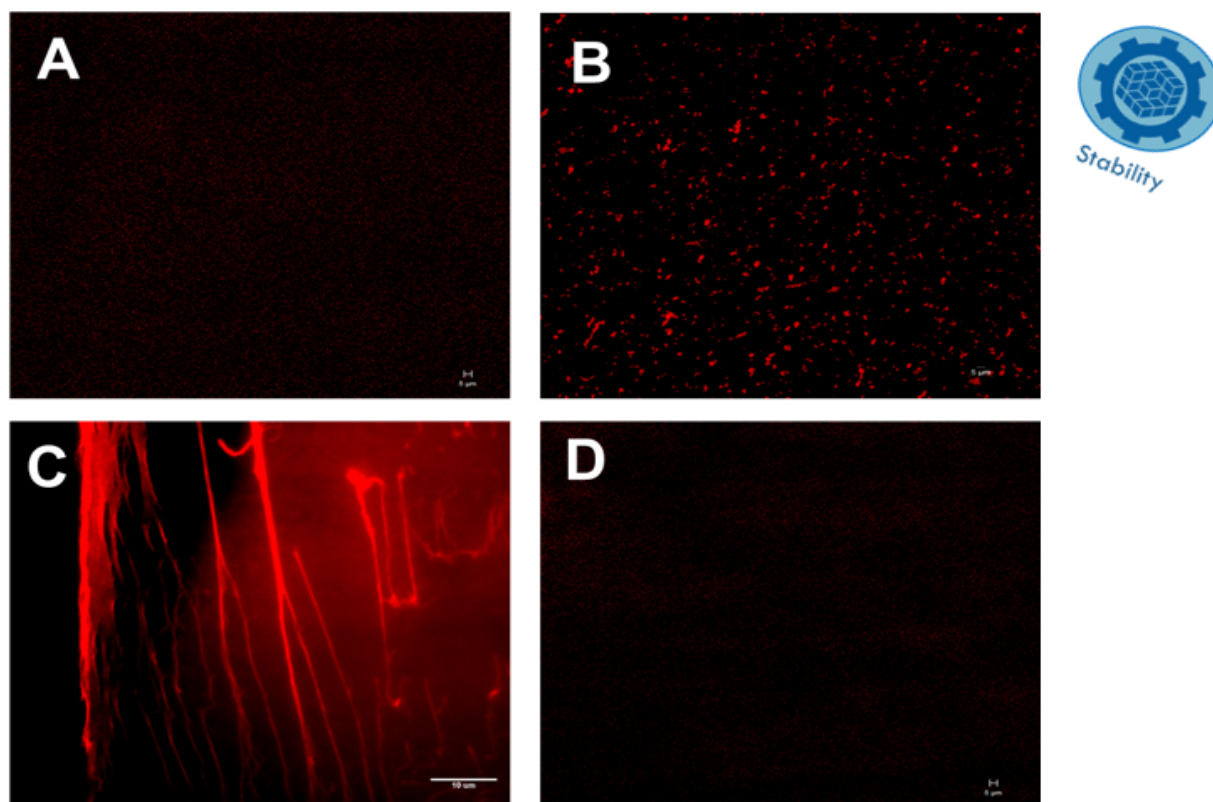


Figure 4.6: GPC-assembled miniature microtubules.

A) GPC microtubule seeds at 5mins **B)** GPC miniature microtubules at 25mins **C)** GPC full-length microtubules at 60mins **D)** Negative control (no polymerization agent). The assay was repeated twice with similar results.

In Figure 4.6 at 5mins, (A) no polymer is visible yet, but at 25minutes (B), seeds appear in figure B) and grow into long MT's at 60mins (C). GPC is not as strong an enhancer as TX, therefore polymerization is progressing more slowly. At 60minutes, ultra-long microtubules have grown on the glass slide, as previously observed (Figure 4.4, Figure 4.5). Without enhancer (D), no polymer forms.

These results suggest that miniature microtubules can indeed be grown as GPC-stabilized form and appear more similar to typical morphologies in their short form, but still not as closely related to TX-miniature microtubules (see figure 4.3), as desired.

In stability tests, GPC-miniature MT's and TX-miniature MTs remained stable for approx. 48 hours and 73 hours, respectively (results not shown). This is expected, as GPC is a GTP analogue, which slowly hydrolyzes over time, while TX is a highly efficient, clinically active stabilizer and does not hydrolyze over time.

In summary, the stability would be within a good range to work with for in vitro selection. But since also the GPC-mini-MT morphology is different, it raises the concern that they might not share the essential structural features with TX-MT's and therefore, access to specific binding sites, such as the taxoid site, might be restricted. Therefore the approach seems limited to generate highly representative macrostructures with GPC.

4.2.4 Purification

Regardless of whether the selection target would be GCP-or TX-MTs, a last challenge remained: purifying the polymer from the mixture to recover the pure selection target. Off-target structures, like denatured protein, unpolymerized subunits or aggregates would represent additional targets, which have to be removed for a polymer-specific selection. AMA's which bind to the dimers typically inhibit the reaction, whereas binders on assembled tubulin act as enhancers - the aim of the selection.

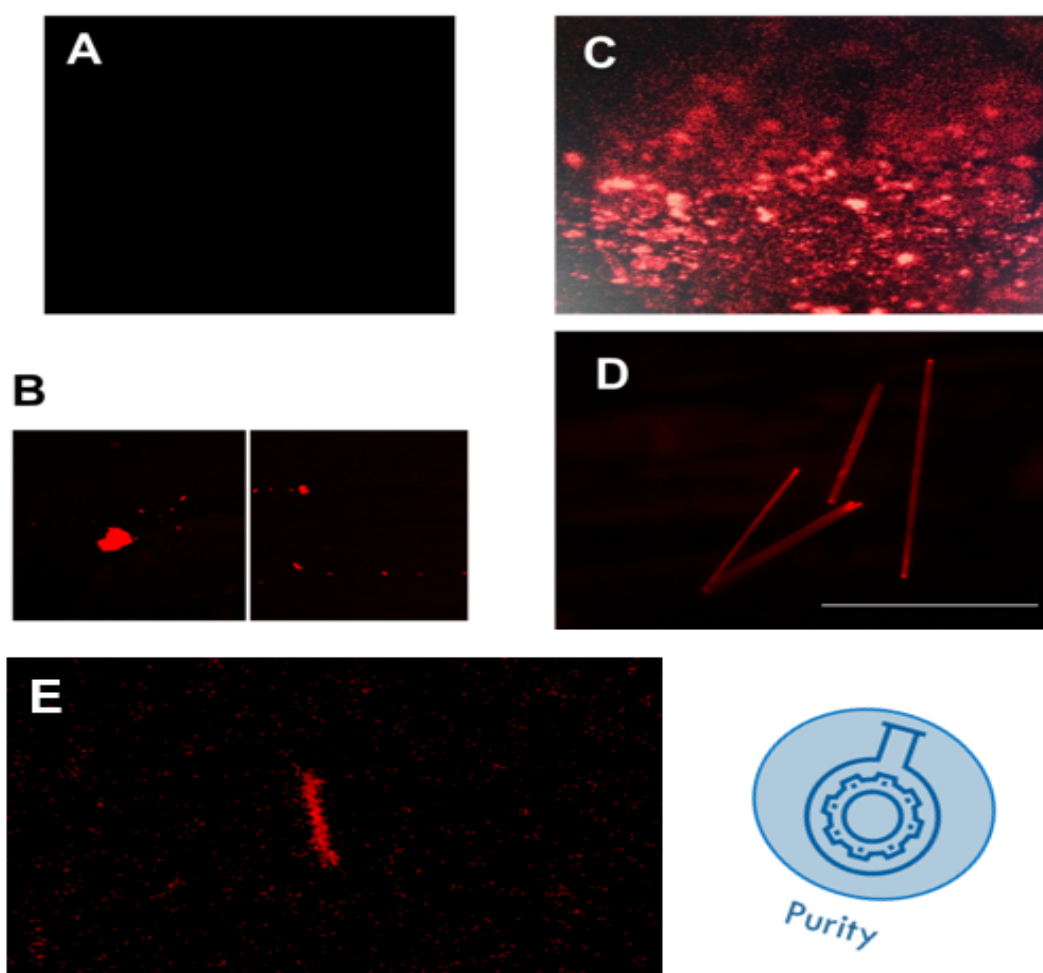


Figure 4.7: Purification of polymer by ultra-centrifugation.

A) Supernatant **B)** Aggregate pellet **C)** Aggregate pellet, high-resolution (screenshot) **D)** Control morphology of short microtubules **E)** Microtubule polymer and non-polymer tubulin background. The assay was performed with Paclitaxel-stabilized microtubules and repeated twice with the same result. Micro-ultracentrifugation was carried out with a sucrose cushion in solution. Scale bar=50µm

This experiment (Figure 4.7) was an attempt to isolate polymer from the mixed background by ultra-centrifugation through a sucrose-cushion, which would preserve its polymeric structure by providing a soft cushion in solution and avoiding the shearing at the bottom of the tube by the centrifugal forces.

E shows the polymer target with background and demonstrates that the mixture consists of varied tubulin species. This contains tubulin subunits (not visible due to lack of signal), as polymerization is not absolute (100%) and also depends on the time allowed for assembly, while some may be denatured.

A shows the supernatant from the separation, which contains the subunits. The lack of bright red fluorescence suggests that no seeds (polymer) are present in this sample. Subunits or dimers on their own do not emit fluorescence, (Cytoskeleton Inc).

B depicts the pellet with the polymer fraction. The bright fluorescent signal indicates the presence of polymeric tubulin and visualizes its macrostructure. Compared to the control TX-MTs, the isolated target structure would be expected to look similar, as the microtubules formed were of the same type (D). However, they appear aggregated – a high-resolution zoom-in (C) confirms this. This indicates that the highly unstable protein has aggregated and disassembled during the ultracentrifugation (at RT) and that the microtubular structure was not preserved under these conditions.

4.3 Discussion

In this chapter, a polymeric microtubule (MT) target was fabricated to test feasibility of an advanced in vitro selection (covered in future outlook next). Three central design considerations were devised and implemented:

1) Shortening the lattice length to create miniature MTs, which offer improved access the taxane binding site inside the MT lumen.

Following the general observation that the diameter of the pipette tip affects the length of the microtubules (through shearing, although not systematically established), further shearing of the MTs with a micro-syringe needle was attempted, but the protein aggregated. Ultimately, the best approach was to limit the polymerization time to just a few minutes (or around 20mins for the GPC form). This produced polymeric, elongated seeds, both with Paclitaxel and GPC (ultra-short MTs).

2) Stabilizing the MTs to preserve the polymeric form with an alternate agent to TX, to keep the taxane binding site remains unoccupied.

Tubulin was polymerized with GPC and grown into long MTs both on an imaging glass slide and in a reaction tube. Both morphologies appeared starkly different from the TX control. Next, the miniature form was explored to see if the morphology would look more representative. Miniature GPC-microtubules were assembled successfully (as mentioned above) and appeared more closely related to the control, but still not sufficiently similar to represent a suitable selection target. GPC miniature MTs were stable for two days and the TX form for three (data not shown).

3) Purifying the miniature-microtubule from the background mixture to separate the polymer.

The polymers were micro-ultracentrifuged through a sucrose-cushion and two distinct fractions were obtained, one containing subunits and the other the pellet. But the microtubules (TX-stabilized, long form) had disassembled and aggregated. Therefore, the polymer structures could be isolated in their preserved form under these conditions.

4.4 Conclusions and prospects

A polymeric selection target for could be an exciting and novel way to generate potent, taxol-like binders. The compounds which most effectively impact tubulin dynamics are stabilizers (discussed in the introduction). Therefore, a three key design thinking specifications were devised to rationally engineer the optimal macromolecular architecture.

However, use of GPC-polymer as target could pose certain limits on selection efficiency. As it is less stable than the TX-equivalent (2 days vs 3 days), many time-consuming cycles of fabrication and purification would have to be undertaken routinely – every other day (due to cold-induced disassembly, polymers cannot be stored cold or frozen). Since the centrifugation step has to be carried out at remote facility of another institution (2-3 hours commute), this would likely take an entire day and slow down selection immensely. This comes in addition to the fact that nitrocellulose filter selection takes many rounds.

In contrast, this target might be more suitable for a shorter selection, like with a CE instrument or as proposed in future work based on CE/NGS (see below). Furthermore, polymer concentration would be difficult to measure or estimate, thereby selection conditions would be only vaguely known and could not be precisely adjusted. This might be acceptable in some cases, but is not ideal for a first selection.

As next steps, GPC-assembly could be studied more extensively with electron microscopy to understand the morphologies in more depth. This could help probing varying assembly conditions to investigate whether more taxol-like MTs could be produced. Since this a niche field of foundational research, it lays outside the main motivation of this thesis and is typically researched by tubulin-oriented labs.

Once CE or CE/NGS selection could be carried out (a matter of equipment availability), further exploring the target would make sense, as these selections

would require only a few rounds. This work could yield valuable and unprecedented insights into tubulin-aptamer interactions and reveal epitope-specific sequence clusters for further study and shed additional light into this under-explored field. This is covered in greater detail in future work as an alternative selection.

Towards taxoid-site binders with NGS selection and cluster analysis

A novel selection scheme was devised to capitalize on two distinct microtubule morphologies observed in this research seeking to generate Paclitaxel-like tubulin binders with an epitope-specific selection.

NGS is a potent, relatively novel sequencing technology of tremendous potential in selection experiments (Schuetze et al., 2011, Blank and Blind, 2005).

Acting in concert with data sequence data analysis, a variety of selection methodologies would be suitable to identify putative consensus sequences reflective of the epitopes after a few rounds of selection.

As two distinct morphologies of tubulin have been fabricated (please see chapter 5), both forms likely possess distinct epitopes. As described, emergence of the taxoid binding-site is highly specific to regular lattice formation of the microtubule. As observed, the GmPcPP-assembled microtubule, however, lacks this distinct structure under the conditions probed. Therefore, it might serve as an alternate target lacking accessible taxoid sites. Taking advantage of NGS, toggling between TX-tubulin and GMP-tubulin in alternation might prove promising to map sequence clusters specifically to the taxoid epitope.

For instance, a round of GPC-selection would generate binders interacting with varied epitopes (except for the structure-specific taxoid epitope). In a following, alternating round, binders specific to GPC-tubulin would be eliminated, leaving binders to the TX-tubulin – similar to group theory or a simple Venn diagram. Taking advantage of NGS and sequence clustering, the relative distribution of the clusters could be analyzed to map binders to either form. A workflow like this could advance the knowledge of tubulin binding site interactions with aptamers to inform further selection experiments and development.

5

***In vitro* selection of tubulin-binding aptamers**

5.1 Introduction

In vitro selection (SELEX) is a procedure to generate high-affinity nucleic acids against a given target from a pool of random sequences (reviewed in chapter 1). Titer plates, magnetic beads and nitrocellulose filters are widely used traditional methods. The FluMag method involves fluorescence-based DNA quantitation and magnetic beads (Stoltenburg *et al.*, 2005).

A constraint of beads is that they require immobilization of the target, typically by incorporation of biotin or streptavidin. The modified target represents a non-native protein conformation, due to the incorporation of the modification and a higher complexity of the selection, due to the presence of non-target molecules. For protein targets, nitrocellulose membrane filtration is an excellent partition method, since proteins typically have a high affinity for the membranes, as the majority of proteins have a net positive charge, whereas nitrocellulose filter paper is negatively charged. Therefore, they do not require immobilization. Additionally, filters represent equilibrium-binding conditions, as the target remains in its preferred native state for the incubation step. Filters exhibit low background binding, which can be even further reduced by alkali treatment (Gopinath *et al.*, 2006).

Anti-protein aptamers are therefore typically selected with nitrocellulose filters (summarized in Table 7.1). Examples include a 2'-OH modified anti-VEGF aptamer (Burmeister *et al.*, 2005) and toggle-selex against both human and porcine thrombin (White *et al.*, 2001), Tenascin C-binders with cell-selex and purified protein (Hicke *et al.*, 2005) and new thrombin-binding aptamers containing a modified nucleotide (Latham *et al.*, 1994).

Further, filters have been used in automation of sequence acquisition (Cox *et al.*, 2002) and aptamers against bovine factor IXa, which were so specific they were able to distinguish between the closely related human and bovine forms (Gopinath, 2006).

Table 5.1: Protein-binding aptamers generated by nitrocellulose filter partition

Target	Reference
T4 DNA polymerase	Tuerk and Gold, 1990
HIV-1 Rev	Bartel <i>et al.</i> , 1991
Reverse transcriptase (HIV-1)	Tuerk <i>et al.</i> , 1992
Bacteriophage R17 coat protein	Schneider <i>et al.</i> , 1992
Rev-binding element of HIV-1	Giver <i>et al.</i> , 1993
Reverse transcriptase (avian myeloblastosis and Moloney murine leukemia)	Chen and Gold, 1994
Protein kinase C β II	Conrad <i>et al.</i> , 1994
Human α -thrombin	Kubik <i>et al.</i> , 1994
Vascular endothelial growth factor (VEGF)	Jellink <i>et al.</i> , 1994
HIV-1 Rev	Jensen <i>et al.</i> , 1995
HIV-1 Integrase	Allen <i>et al.</i> , 1995
Rous sarcoma virus (RSV)	Pan <i>et al.</i> , 1995
Human IgE	Wiegand <i>et al.</i> , 1996
Elongation factor SelB	Klug <i>et al.</i> , 1997
Keratinolytic growth factor	Pagratis <i>et al.</i> , 1997
Human rIFN- γ protein	Kubik <i>et al.</i> , 1997
Nucleo capsid (NC) protein of HIV-1	Berglund <i>et al.</i> , 1997
NS3 of hepatitis C virus	Kumar <i>et al.</i> , 1997
NS3 of hepatitis C virus	Urvil <i>et al.</i> , 1997
Coat protein of alfalfa mosaic virus	Houser-Scott <i>et al.</i> , 1997
HIV-1 gag polyprotein	Lochrie <i>et al.</i> , 1997
Human activated protein (APC)	Gal <i>et al.</i> , 1998
HIV-1 Tat	Yamamoto <i>et al.</i> , 1998
Vascular endothelial growth factor (VEGF)	Ruckman <i>et al.</i> , 1998
Human nonpancreatic secretory phospholipase A2	Bridonneau <i>et al.</i> , 1998
Rex fusion protein	Baskerville <i>et al.</i> , 1999
Ribosome-inactivating protein, Pepocin	Hirao <i>et al.</i> , 2000
Ricin A chain	Hesselberth <i>et al.</i> , 2000
Human factor VIIa	Rusconi <i>et al.</i> , 2000
HIV-1 Tat	Kawakami <i>et al.</i> , 2000
Bacteriophage MS2 coat protein	Shtatland <i>et al.</i> , 2000
Hepatitis C virus nonstructural protein 3 protease	Fakuda <i>et al.</i> , 2000
Drosophila transformer 2 (Tra2)	Brunel <i>et al.</i> , 2001
Endothelial regulatory protein pigpen	Blank <i>et al.</i> , 2001
Human coagulation factor IXa	Rusconi <i>et al.</i> , 2002
Human epidermal growth factor receptor-3	Chen <i>et al.</i> , 2003
Angiopoietin-2	White <i>et al.</i> , 2003
HCV NS3 helicase domain	Nishikiwa <i>et al.</i> , 2004
Histidine utilizing protein (HutP)	Kumarevel <i>et al.</i> , 2004
HA of B/Johannesburg	Gopinath <i>et al.</i> , 2005
2006 [14] HA of A/Panama	Gopinath <i>et al.</i> , 2006
Mouse prion protein	Sekiya <i>et al.</i> , 2006

Adapted from Gopinath, 2007, not exhaustive

Another consideration is how to generate ssDNA. That is, because after one selection round, incubation with the target molecules, elution and nucleic acid purification to recover binders, the ssDNA, the pool is PCR-amplified, which produces dsDNA. To generate the pool ready for the next round, the duplex DNA has to be separated into single strands first.

Traditional methods include PAGE separation, streptavidin-coated magnetic beads (with biotinylated antisense strands) or enzymatic digest with Lambda Exonuclease (with phosphorylated antisense strands). Magnetic beads are a widely applied method to separate duplex DNA into single-strands, are not without pitfalls: for instance, streptavidin has been found to dissociate in high temperatures or during alkaline treatment (Paul *et al.*, 2009). Furthermore, streptavidin presents an additional target, as aptamers are exposed to it as part of the selection mixture. Streptavidin is highly aptagenic -_prone to bind nucleic acids (Stoltenburg *et al.*, 2005, Srisawat and Engelke, 2001, Tahiri-Alaoui *et al.*, 2002). This requires time-consuming extra counter-selection steps, which increase the complexity of the selection. Meanwhile, separation with PAGE is cumbersome and associated with high loss of sequences (Svobodová *et al.*, 2012).

Lambda Exonuclease digestion has emerged as a well-understood, efficient method for this vital task. Comparative studies (Svobodová *et al.*, 2012) analyzed yields, sequence recovery and time and cost for the most common ssDNA generation methods. The exonuclease digest has produced the best results. It has also been thoroughly studied and optimized for the task (Avci-Adali *et al.*, 2009). Production methods for ssDNA have been evaluated thoroughly and have supported these findings (Civit *et al.*, 2012, Marimuthu *et al.*, 2012).

The work furthermore presented the combination of the enzymatic digest with asymmetric PCR (to yield more sense strand) as excellent method for ssDNA generation. Asymmetric PCR was used in the original selection for anti-tubulin aptamers (Fukusaki *et al.*, 2001) and reviewed in detail by Citartan *et al.*, (2012). Further, error-prone PCR polymerases or NRR (non-homologous random

recombination) can be advantageous to introduce greater diversity to the pools through mutations (Bittker *et al.*, 2002).

Lambda Exonuclease is a protein from bacteriophage λ which possesses 5'→3' exodeoxyribonuclease activity and catalyzes removal of 5'mononucleotides from dsDNA. In the PCR-amplification step, use of a phospho-modified reverse (RV) primer yields the 5'-phosphorylated antisense strand. The enzyme digests the phosphorylated strand efficiently and selectively, while it has greatly reduced activity on the non-phosphorylated original strand and on single-stranded DNA (Li and Elledge, 2007, Subramanian *et al.*, 2003). This generates the next ssDNA pool.

A challenging selection was anticipated, as tubulin is an attractive, yet underrepresented target in literature. The only published work on anti-tubulin aptamers stems from relatively early days of the field (Fukusaki *et al.*, 2001) and presented only weakly binding aptamers (45.0 μ M and 19.4 μ M for T1 and T2, respectively). The two repeat sequences are both T-rich, which tend to be 'sticky', non-specific, while no counter-selection steps were reported to correct for this. Indeed, when tested in in vitro tubulin polymerization assays, the aptamers appeared to specifically interact with tubulin with small effects (see chapter 4). Therefore, the selection steps were carefully optimized to facilitate further refinement and potential troubleshooting.

On the level of library design, considerations are how to enhance formation of sufficiently complex secondary structure and how to best utilize the sequence space. As selection is time-consuming and cumbersome, effective library design is paramount. Since introduction of the SELEX method, only a few hundred aptamers have been generated, while thousands of new antibodies were produced.

In this effort, skewed incorporation ratios towards GC or AT nucleobases can allow for greater abundance of non-canonical secondary structures, such as G-quadruplex motifs. G-rich oligonucleotides (GRO's) can possess anti-proliferative properties (Bates *et al.*, 1999) and have shown promise for preferential receptor-mediated cell internalization (Hovanessian *et al.*, 2010, Bates *et al.*, 2010). In screening results

(see chapter 4), it was the T-rich aptamers, to emerged as the most promising tubulin inhibitors of the aptamer population (see chapter 4).

Libraries with predefined structures were investigated, where the coverage of sequence space was expanded with parent/daughter libraries of reduced complexity (Borer and Pike, 2015). Design of the random region is discussed in terms of length, skewing of nucleobase composition, degree of randomization and optimization of the PCR (Hall *et al.*, 2009). Other works have focused on expansion of the genetic alphabet of libraries to further explore sequence space (Kimoto *et al.*, 2013) and using a doped pool (partially randomized) for selection of RNA aptamers against human influenza virus hemagglutinin (Misono and Kumar 2005).

5.2 Results and Discussion

5.2.2 PCR optimization

To optimize the PCR amplification, a 90-mer (the library size) is used as test input sequence to determine optimal cycle number and annealing temperature (T_a). First, an asymmetric amplification was used to minimize antisense strand accumulation, an undesirable side-product.

This experiment aimed at verifying specificity at cycle numbers ranging from 4, 8, 12, 20 and 25 amplification cycles and for a 90mer Dummy representing a library N=54 nucleotides random region (R=54nt).

Here (Figure 5.1), the optimum cycle number and annealing temperature was determined for asymmetric PCR. Asymmetric PCR is less efficient than symmetric PCR, but it produces more sense strand.

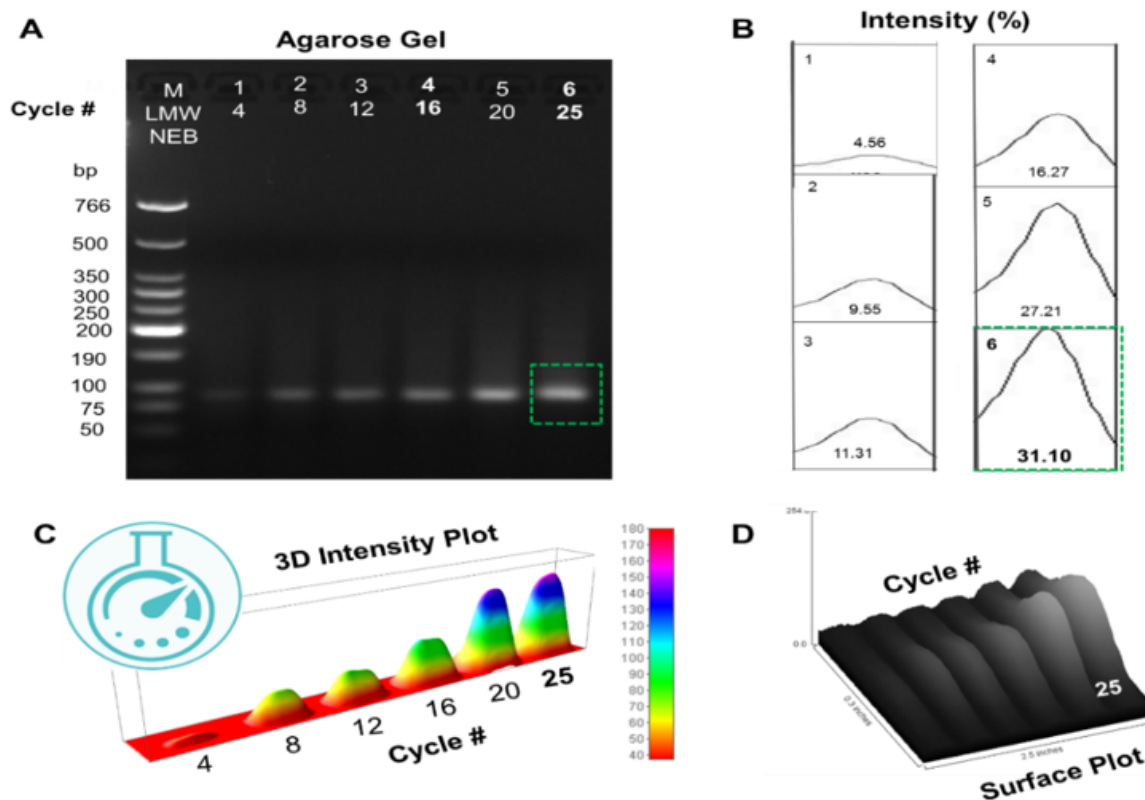


Figure 5.1: Asymmetric PCR for cycle number optimization.

A) 3% Agarose gel with PCR amplicons **B)** Band intensity plots (a.u.) **C)** 3D band intensity plot **D)** Band intensity surface plot. Cycle numbers were increased from 4 to 25 cycles and the assay was repeated twice with very similar results.

In Figure 5.1 for cycle numbers, result are as expected: With increasing amplification cycle numbers, an intensity increase for product is observed, while non-specific amplicons are not observed. At 4 cycles, vanishingly small amounts of amplicons are obtained at 4.5% relative intensity (a.u.). This increases to 9.5% at 8 cycles and to 11.3% at 12. A further increase to 16.3% follows at 16, to 27.2% at 20 and to 31.10% for 25 cycles. This suggests that these asymmetric PCR conditions seem suitable to generate specific products efficiently. PCR cycles from 12-15x should suffice even in early selection rounds.

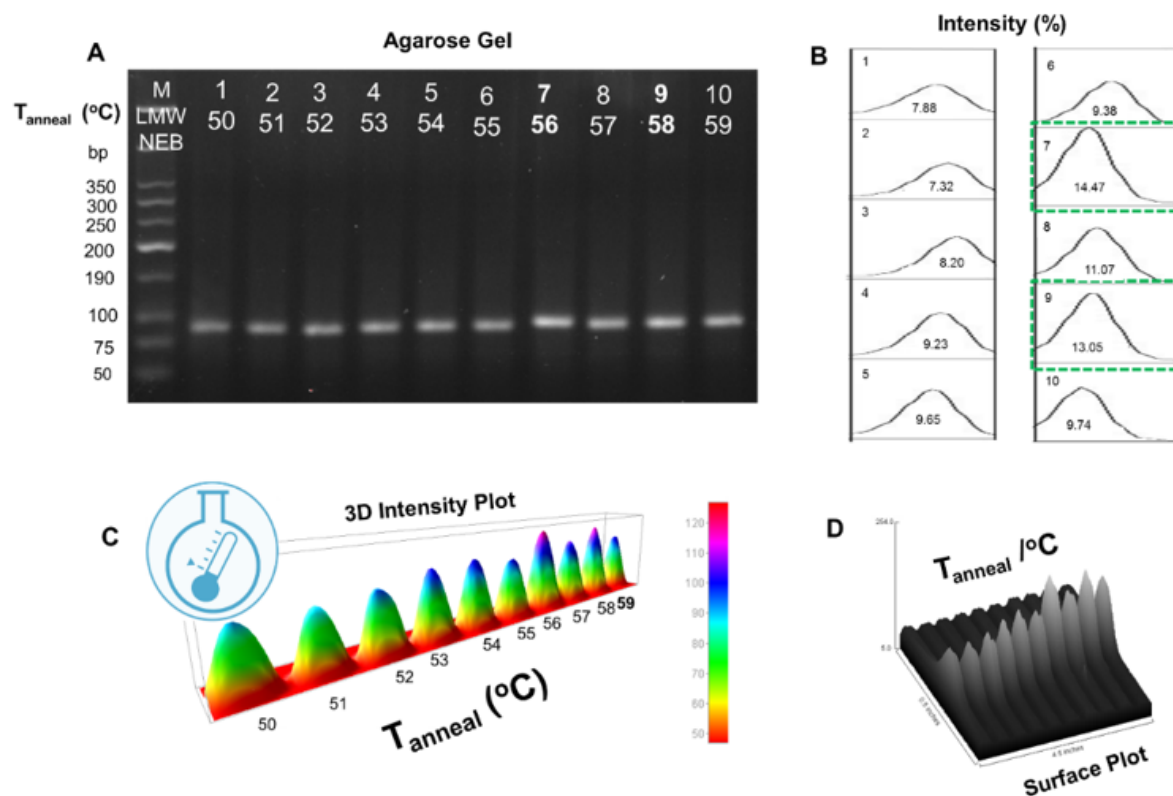


Figure 5.2: Asymmetric PCR for annealing temperature optimization.

A) 3% Agarose gel with PCR amplicons **B)** Band intensity plots **C)** 3D band intensity plot (a.u.) **D)** Band intensity surface plot. Temperature range tested was 50-59°C and the assay was repeated twice with very similar results.

Figure 5.2 reveals the ideal annealing temperature. At low temperatures from 50-52°C, weak intensities of 7-8% are observed and the peaks emerge at 56°C at 14.47% and 58°C at 13.05%. Side-products are not observed under these conditions. This suggests the PCR remains specific at lower temperatures, but is not very efficient, while annealing seems optimal at 56-58°C.

Next, the same parameters were optimized for symmetric PCR with equimolar primer concentrations. Symmetric PCR is more efficient, but more prone to generating non-specific products.

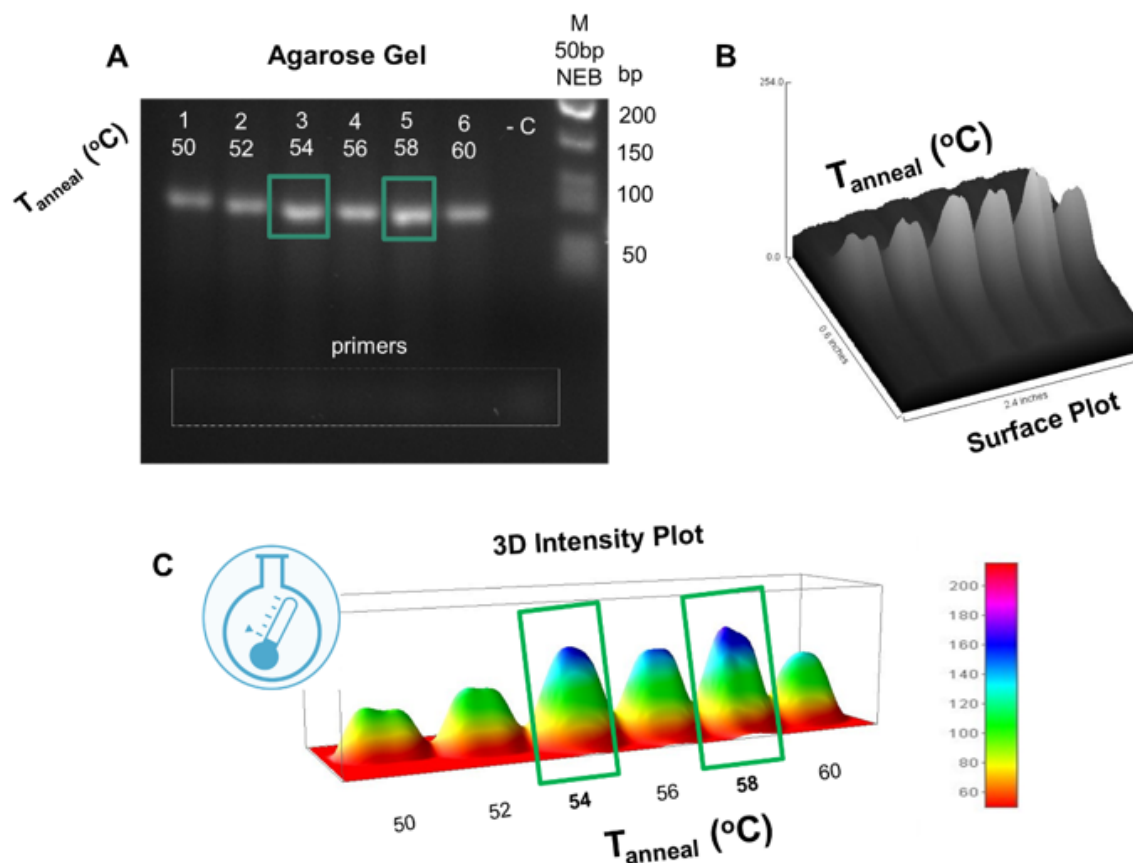


Figure 5.3: Symmetric PCR for annealing temperature optimization.

A) 3% Agarose gel with PCR amplicons **B)** Band intensity plots **C)** 3D band intensity plot (a.u.) **D)** Band intensity surface plot. Temperature range tested was 50-60°C and the assay was repeated twice with very similar results.

Figure 5.3 shows peaks at 54°C and 58°C on the surface plot and 3D intensity plot for the symmetric PCR conditions, suggesting the optimum annealing temperature range. This is visible as well on the surface plot (B) and the 3D intensity heatmap (C). Negative controls were included regularly to verify no contaminations occurred.

Specificity of the PCR reaction is known to vary with template concentration. Higher concentrations of input DNA typically yields more unspecific bands. Since enrichment of aptamers in later rounds would represent a sharp concentration increase, these conditions are tested here with 15 cycle numbers at 56°C annealing.

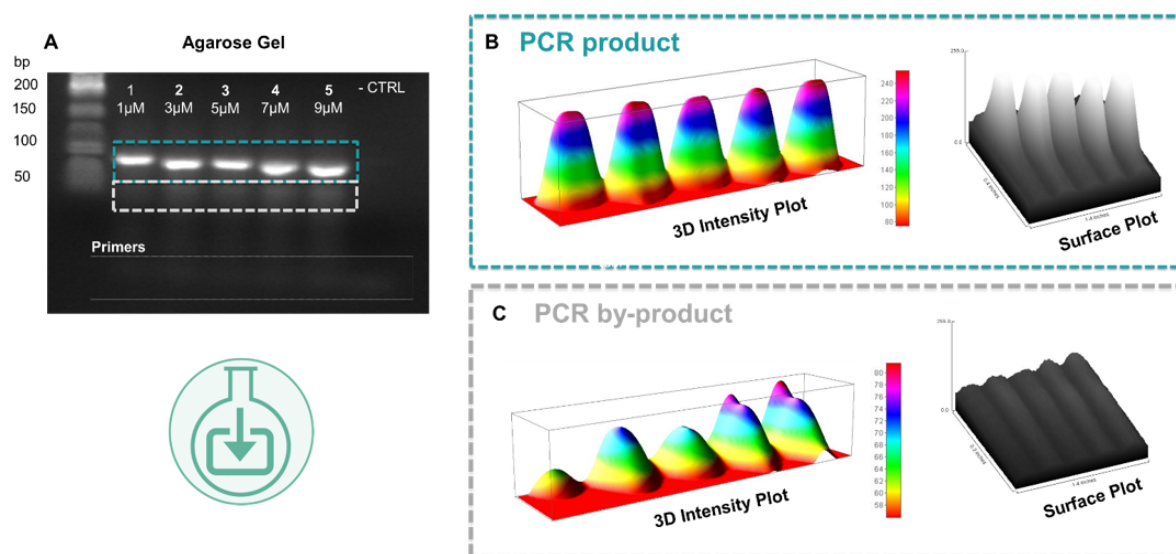


Figure 5.4: Specificity across template concentration gradient.

A) Agarose gel with PCR amplicons and negative control **B)** PCR products as 3D intensity plot (left) and surface plot (right) **C)** PCR byproducts as 3D intensity plot (left) and surface plot (right). Symmetric PCR was performed.

Results in figure 5.4 show that as template concentration increases, the intensity of the amplicon increases proportionally, as expected (lane 2 intensity appears to be a pipetting variation, as the repeat assay did not show the drop). As suspected, at increasing template concentration a non-specific band appears indeed in proportionally elevated intensities. This demonstrates that upon enrichment in later rounds, an additional 50bp band might emerge as side-product, possibly among others.

Non-specific products can both be shorter or longer or be in a close size range to the original band. This serves as an additional distinguisher to select the correct aptamer band for gel excision for sequencing and suggests that PCR under these conditions would not be problematic and still sufficiently specific.

5.2.3 ssDNA generation with lambda exonuclease

Following incubation, partition and amplification, a critical subsequent step is generating ssDNA from double-stranded PCR product for the next pool. For this, the antisense strand has to be eliminated - otherwise remains hybridized to sense strand (the aptamer) and prevents it from folding correctly.

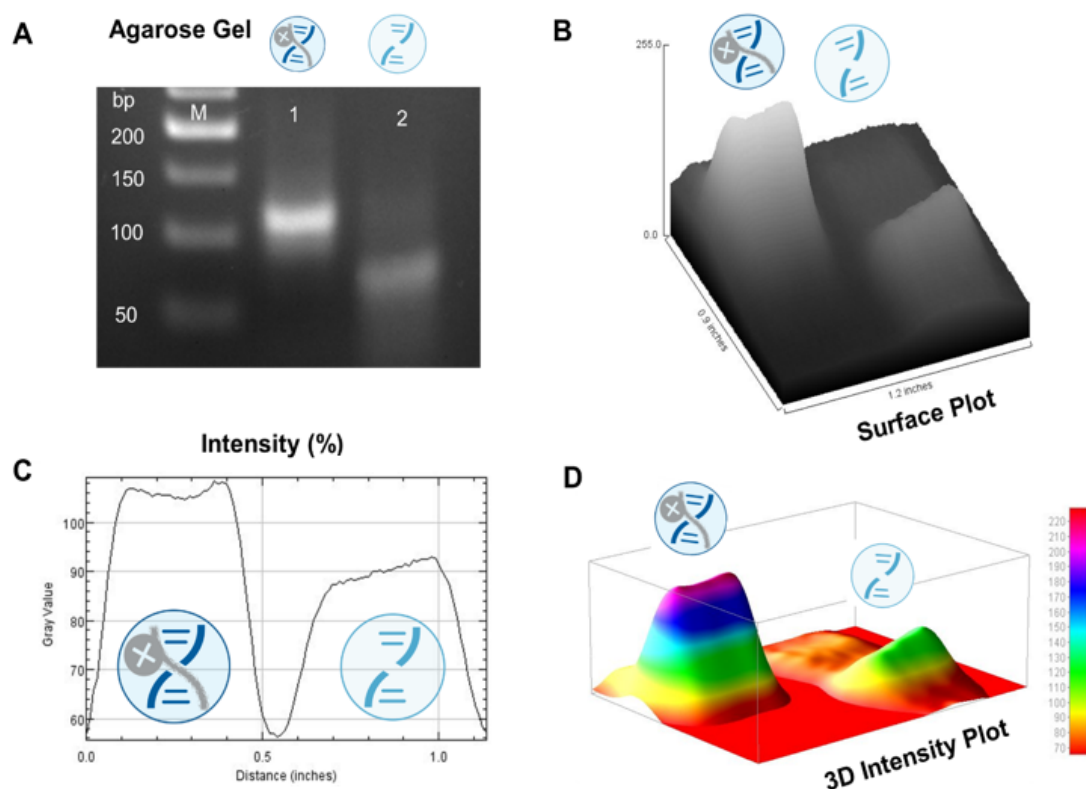


Figure 5.5: Lambda digest of PCR product for ssDNA generation.

A) Agarose gel with ds PCR product and ssDNA lambda digest **B)** Intensity surface plot of PCR product and ssDNA digest **C)** Quantitative intensity plot of amplicon and digest **D)** 3D intensity plot of amplicon and digest

In Figure 5.5 A-D show consistently that the high-intensity dsDNA band (~110bp) has disappeared and the lower intensity ssDNA band has appeared instead (~70-80bp). The single-stranded DNA band runs at lower intensity, as expected, (Sriprakash *et al.*, 1975). This is also visible in (C) as drop in intensity from ~ 150a.u to ~90a.u. This suggests the digest has been successful and complete.

5.2.4 Filter partition efficiency

Another integral part of the selection protocol is the partition to efficiently separate binders from the non-binders. Although background binding for nitrocellulose filters is low, alkali treatment further reduces it to a minimum (McEntee *et al.*, 1980). Therefore, this assay aimed at determining the potential background levels following the treatment to ascertain the efficiency of this step.

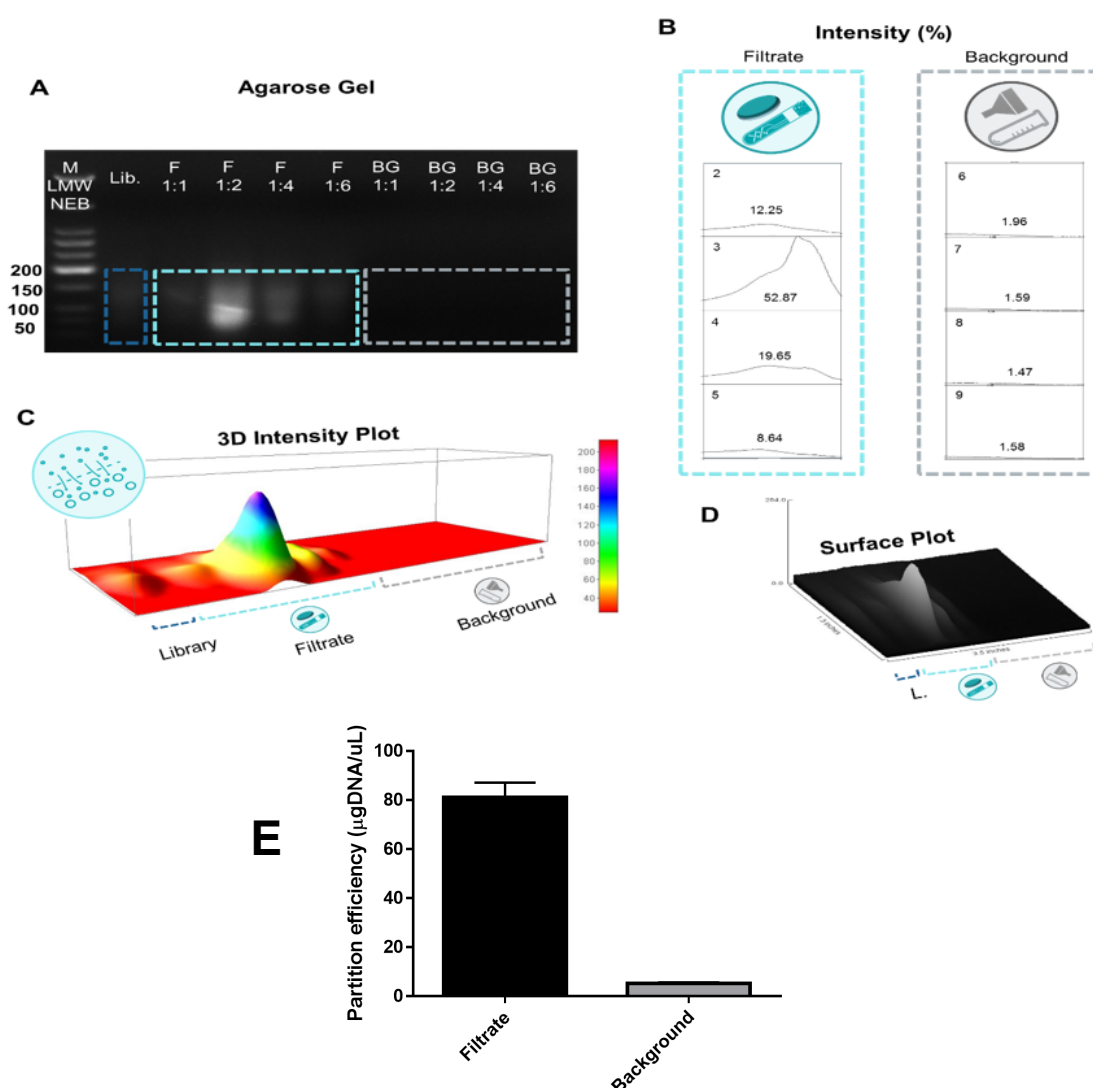


Figure 5.6: Filter partition efficiency

A) 3% agarose gel with dilution series of filtrate and eluate of background **B)** Band intensity plots
C) Band intensity surface plot **E)** Partition efficiency plot by concentration

The amount retained on the filter (the undesirable background fraction from non-specific adsorption) was 5.3ng per μL and the flow-through was 81.2ng per μL of the sequences, which demonstrates the small fraction of non-specific background. A small dead volume within the syringe was observed a few times, as droplets can get trapped.

Panes A-D in Figure 5.6 show the filtrate vs the background from eluted filter-binders. Results indicate that the library, a smeared band due to conformational diversity of ssDNA, is collected successfully in the flow-through, as the gel shows no detectable background from the fraction eluted from the filter. This confirms that the alkali-treated membranes partition the pool efficiently and that background remains below the detectable limit (however, the background will never be zero). This suggests that the partition works sufficiently well. For the selection therefore, only protein-binding sequences will be retained in the eluate and non-binders will be eliminated in the flow-through.

5.2.5 Selection protocol

After verifying the efficiency of the core steps of the selection protocol, the last step was to confirm successful progression from PCR-amplification, ssDNA generation, partition and nucleic acid purification as a cycle. The assay aimed at re-amplifying the fragment after the last of all the steps to investigate whether the original, correct band would be generated again.

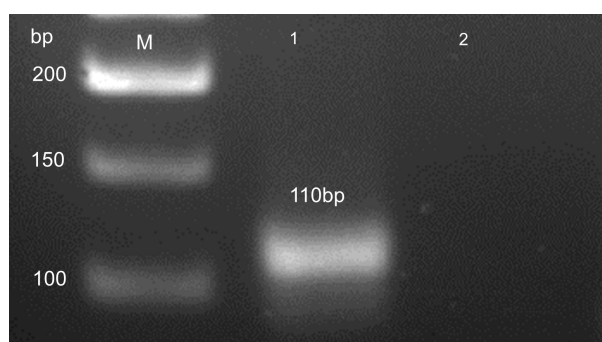


Figure 5.7: *In vitro* selection full cycle protocol test.

1) Correct-sized PCR amplicon (EtOH purification) **2)** No band appearing after PCR (Phenol-Chloroform extraction + EtOH purification). To test the protocols for one round selection, dsDNA PCR product was converted to ssDNA with lambda exonuclease enzyme, purified with EtOH precipitation and re-amplified to determine band. The assay was repeated twice with similar results.

Two purification methods for nucleic acids were tested: EtOH precipitation only (lane 1) and Phenol-Chloroform extraction and subsequent EtOH precipitation (lane 2). After PCR, a band of correct size appears 110bp (90nt aptamer + HEGL-spacer) in lane 1, but no band is observed in lane 2. This suggests that the prior method was successful and EtOH purification suffices, while it appears the pool has been lost with Phenol-Chloroform extraction. As EtOH was sufficient to isolate the DNA and correctly amplify it subsequently, this was deemed the best method to apply to the selection.

5.2.6 *In vitro* selection results

After thorough optimization and verification of the critical steps, the *in vitro* selection against tubulin was attempted (purchased >99% pure from Cytoskeleton, Inc). These results present the first and second trial.

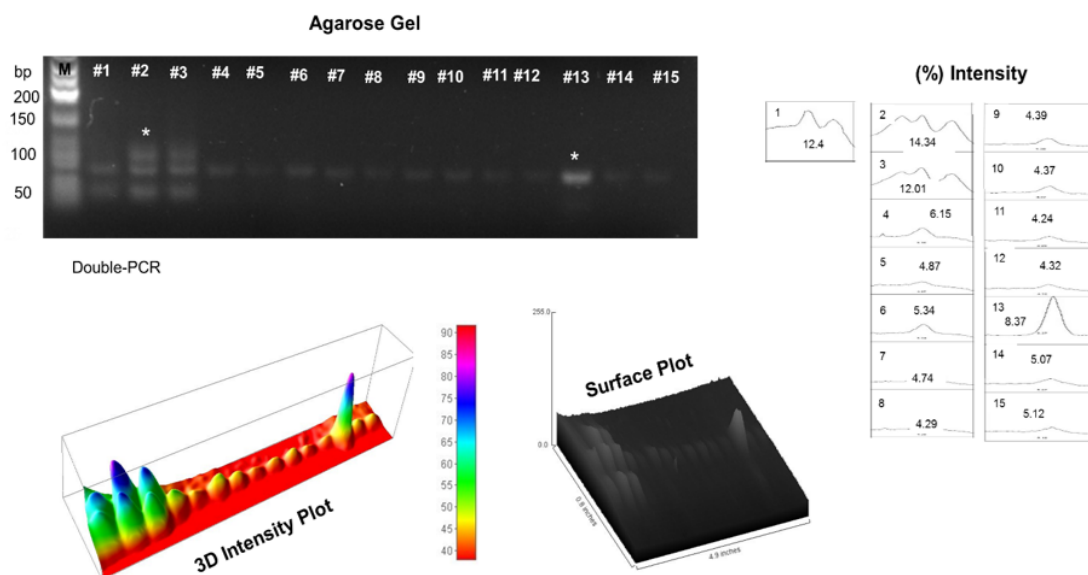


Figure 5.8: Band intensities of selection #1 (rounds 1-15).

Band intensities per selection round of selection #1 visualized on 3% agarose gel and quantitative band intensity plot, 3D and surface plot. Asterisks indicate counter-selection.

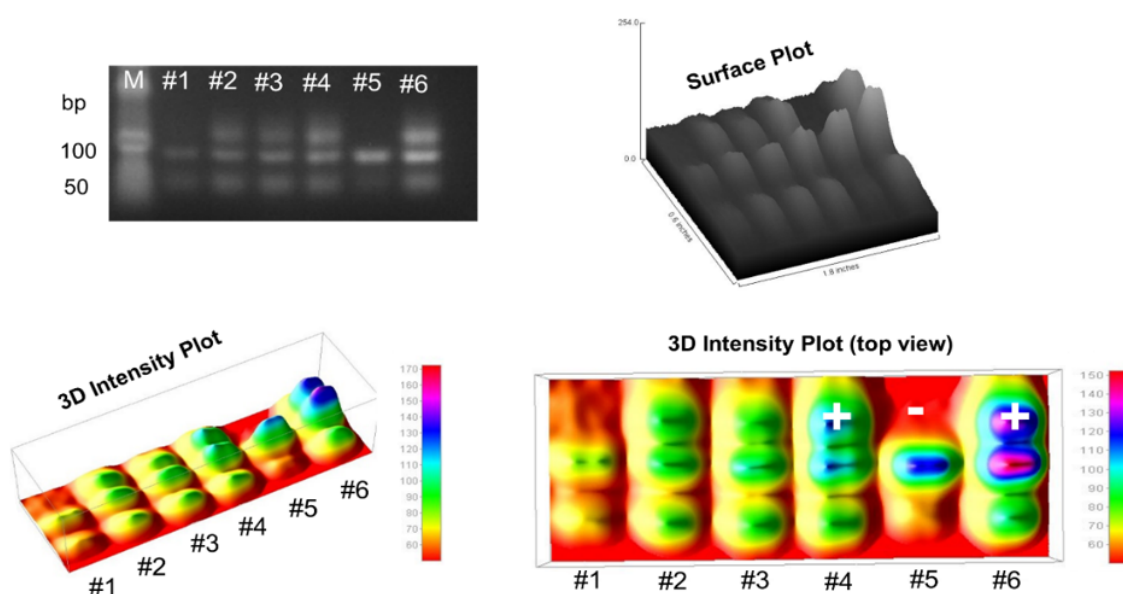


Figure 5.9: Band intensities of selection #2 (rounds 1-6).

Band intensities per selection round of selection #1 visualized on 3% agarose gel and quantitative band intensity plot, 3D and surface plot. Asterisks indicate counter-selection.

The set of Figure 5.8 for the first selection show PCR-amplified 90bp aliquots from pools #1 to #15. The intensities remain constant throughout the rounds, no strong increase, typical of enrichment, is observed as it would be. In lane #13, the 90bp band appears at higher intensity, because the previous round was another counter-selection step (against empty filters). This results in an intensity increase, because the entire fraction is PCR-amplified this time.

Non-specific bands are observed only in round #2 and #3, which then disappear, as observed elsewhere as well (Pileur *et al.*, 2003). The intensities remain at consistent levels, which indicate lack of enrichment over the 15 rounds.

It can be concluded that the *in vitro* selection was not successful under these conditions, therefore they were altered for the next attempt (section 2.3.4) details the selection scheme).

For selection #2 in Figure 5.9, no enrichment is observed, either. After counter-selection, the upper and lower band disappears, as in the previous selection. These could be artifacts from PCR, duplexes, 'sticky' non-canonical motifs (which might also bind the protein), or filter-binders, which are carried thorough the rounds until they are eliminated in a counter-selection step. Incubation times, concentration ratios and filter washing steps were slightly altered.

These results present the third and fourth trial.

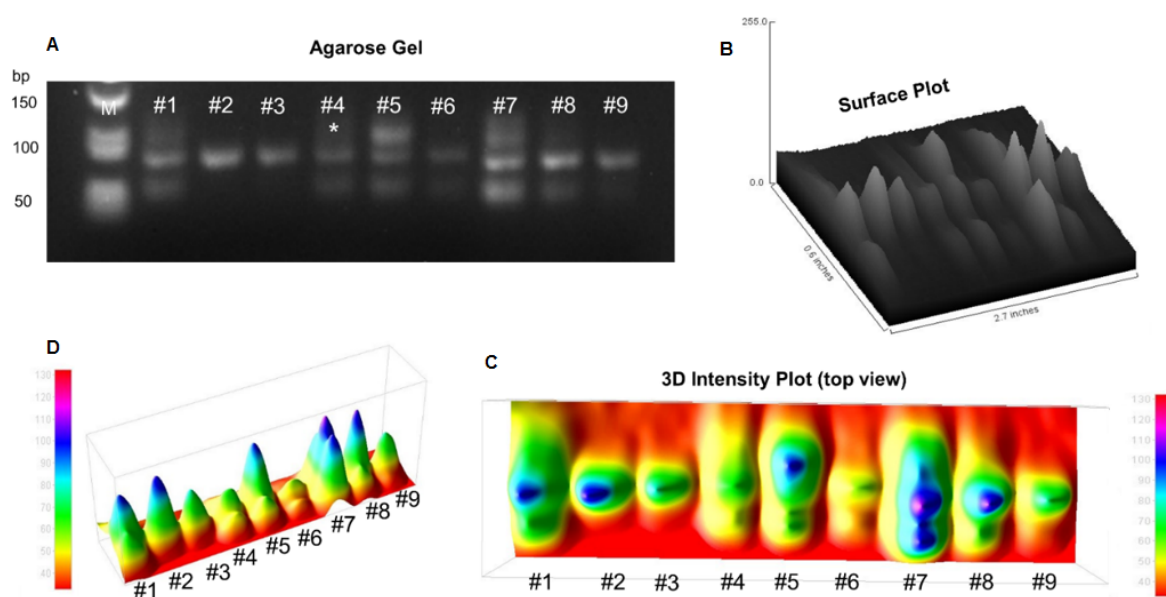


Figure 5.10: Band intensities of selection #3 rounds (1-9).

Band intensities per selection round of selection #3 visualized on 3% agarose gel and quantitative band intensity plot, 3D and surface plot. Asterisks indicate counter-selection.

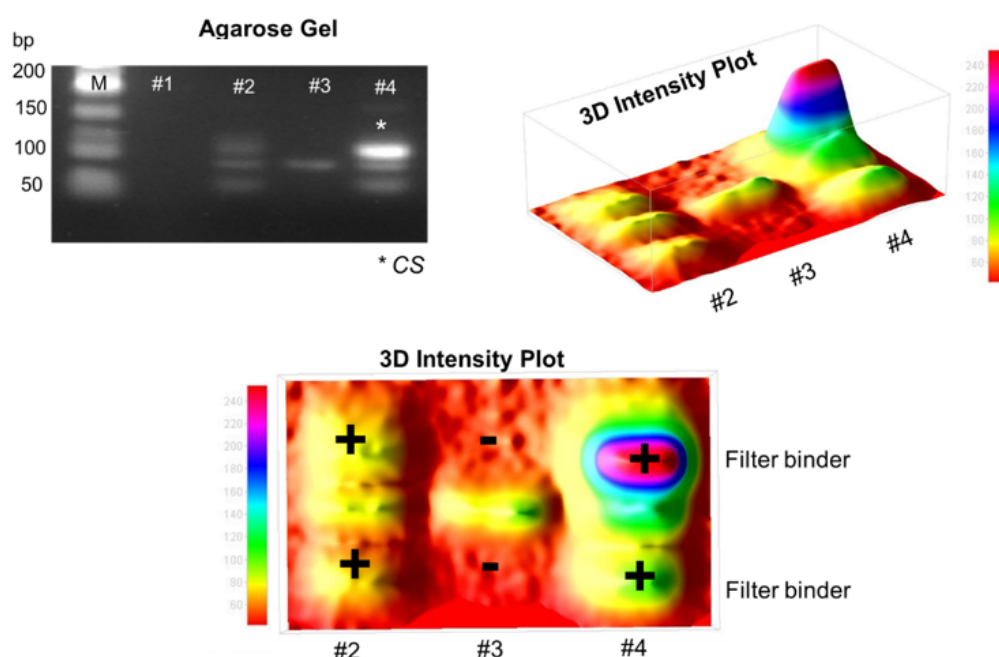


Figure 5.11: Band intensities of selection #4 (rounds 1-4).

Band intensities per selection round of selection #4 visualized on 3% agarose gel and quantitative band intensity plot, 3D and surface plot. Asterisks indicate counter-selection.

In selection #3 in Figure 5.10, still no enrichment is observed. The aptamer band at 90bp likely represents a background fraction, while the upper and lower band may represent PCR by products and/or 'sticky' filter-binders. This can be observed from the disappearance of these bands after counter-selection in round #1. Counter-selection was done against the filter without target. The round after, however, these bands appear again, as some may be retained in the pool and be re-amplified over several rounds. Incubation times, concentration ratios and filter washing steps were slightly altered.

Figure 5.11 for selection #4, results draw a very similar picture: strongly visible bands appear only after counter-selection, but were lost afterwards, likely due to the enrichment being only temporary (an artefact of counter-selection). Therefore only a few rounds were performed. Incubation times, concentration ratios and filter washing steps were slightly altered.

5.4 Discussion

In this chapter the *in vitro* selection protocol was optimized. The motivation was to ensure the best conditions for work with a challenging target that has not yielded high-affinity binders before. Several selections were attempted.

Lying at the core of *in vitro* selection, PCR conditions were tested thoroughly to maximize amplification efficiency and ensure specificity for symmetric and asymmetric PCR. Lambda exonuclease digestion of the phosphorylated antisense strand of dsDNA was optimized to generate ssDNA. Further, partition efficiency with alkali-treated filters was confirmed, and the nucleic acid was re-amplification with PCR to verify smooth progression through the steps of PCR, ssDNA generation, partition and purification/precipitation, representing an entire selection round.

Afterwards, four selections were attempted, but under the conditions tested, tubulin-binders could not be enriched. A first selection over 16 rounds (15 rounds shown) was followed with some variations with another three attempts of 10, 6 and 4 rounds without the desired results or further insights. Despite filter pretreatment, background increased after counter-selection, much as described by other groups (Pileur *et al.*, 2003). The first and only selection was done at 4°C on high-binding titer plates and non-binding aptamers were washed out (Fukusaki *et al.*, 2001).

Results confirmed that background amplification remained very low, as filter-binders became only visible after double-PCR-amplification of an aliquot. After counter-selection, this band became visible directly on an agarose gel, due to amplification of the entire pool, as expected. But this band disappeared over the next rounds in each case, therefore it did not represent a true enrichment of the aptamers

From analysis of the amplified aliquots of each pool, the appearance of non-specific bands of ~100bp and ~50 bp were observed, which both disappeared after counter-selection against the filters. Possibly, these could be PCR artifacts, filter-binders or sequences which bind both the protein and the filter, because they are eliminated in

counter-selection step (this was performed by collecting the flow-through, instead of the fraction bound to the filter).

In summary, each step of the selection was thoroughly tested and confirmed before the selection. This lends further evidence to the likely non-aptagenic nature of the target and the challenge of this selection. As suspected, this set of results suggests that selection of tubulin-binders is not straight-forward and that methods other than the ones applied here may be more promising to explore in the future.

This is coherent with the fact that since the initial publication (Fukusaki *et al.*, 2001), no follow-up experiments were reported and no other tubulin-binding aptamers were successfully selected in the context of published work.

In addition to this, the original aptamers had atypically low binding affinities, while also being T-rich, generally 'sticky' sequences. Results of chapter 4 confirmed that the aptamer have only modest-to-small effects, consistent with these binding affinities. This indicates the remarkable challenge of the selection target, but also the unparalleled potential to contribute to the field in significant ways.

5.5 Conclusions and prospects

Tubulin is among the most actively researched pieces of the cellular puzzle. Its role as key component of cell cycle and the most prominent cancer drug target makes it a compelling selection target. In combination with tightly-binding aptamers, it could offer an unprecedented, versatile platform for foundational and applied research on mitosis, tumorigenesis as well as proliferation and drug discovery.

Aptamers have been selected against many proteins. Therapeutically relevant targets are usually pursued with much excitement and fierceness and add momentum to the thriving narrative of aptamers therapeutics. Yet, one guest did not make it to the party: tightly-binding aptamers against tubulin. They remain notably absent from the enthusiastic reports on new anti-protein aptamers. The lack of published work in this area stands in striking contrast to the pressing need for better cancer therapeutics and significant role of the protein. This concurrently points to a great challenge and untapped potential.

This chapter focused on a thorough, detailed set up and optimization of the selection protocol in anticipation of a challenging selection. This would facilitate troubleshooting and further attempts, if required. Indeed, four selections were attempted and carried out carefully with slight variations each time, but aptamers could not be enriched.

The common theme for these attempts was the loss of the bands after they had initially been enriched (as an artifact) after the counter-selection round. This manifested as an entire fraction of bands being eliminated (on the heatmap) after counter-selection. This suggests that this fraction was likely non-specific filter binders, which were accumulated until the counter-selection step and then disappeared. The other bands were likely background amplification, which remained at low and constant levels throughout the rounds.

As observed in the partition efficiency assay, a very small background fraction remains on the filters, which was propagated and made visible only by double-

amplification. On the other hand, 'sticky' binders which might bind the protein, but also the filter, were eliminated in counter-selection.

This highlights the challenging nature of this project and a limitation of filter-selection. Furthermore, the target appears to lack of aptagenic properties. Perhaps the warm temperature was not ideal for the unstable protein. Possibly another library could have been tried. For future outlook, a few suggestions are discussed next.

For further work, other methods could be applied to this selection. Magnetic beads are an efficient method for both partition and ssDNA generation. Filter membranes are excellent for anti-protein aptamers, but suffers from a 'blind spot' in its spectrum: G- or T-rich or otherwise 'sticky' aptamers. Replicating the original publication with the filters is challenging, as similar aptamers would likely bind the filter membrane and be lost in counter-selection (Gong *et al.*, 2012). Counter-selection against the matrix and/or other proteins are important to ensure specificity of the binders.

Magnetic beads on the other hand, would allow to carry forward these sequences and hence, may offer a greater spectrum to select binders. Noteworthy is that the original work has resulted in precisely such sequences (T-rich, G-rich and A/T-rich), therefore changing partition methods would be reasonable.

Use of an RNA library, creating a different library and vary the length could be tried. RNA aptamers offer greater structural variability and stability than DNA aptamers – a new subfield termed architectonics has emerged (Jaeger and Chworos, 2006) and the RNA approach was also encouraged by other researchers in personal discussions.

Tubulin is an unstable and dynamic protein, which may pose unique challenges for selection. Incubating the pools at cold or ambient temperature, instead of 37°C, might hinder dynamic movements of tubulin and reduce rapid denaturation or aggregation (as noticed first-hand in chapter 3 and 5, tubulin is prone to form aggregates).

Ultimately, alternative cancer-related targets are attractive, even though high-affinity aptamers may already exist. It can be fruitful to build upon existing work in an actively researched area, expanding upon existing knowledge to reduce the risk (yet also the novelty) and draw from a more established knowledge base. A consideration for selecting the thesis topic and the target was the ease of testing: the workflow of generating and derivatizing aptamers in combination with an established drug discovery screen like *in vitro* tubulin polymerization had appeal. The idea of the nucleic acid acting directly as therapeutic, (as opposed to a delivery vehicle or detection agent) also played into the choice as well as the novelty of the research.

For instance, the nucleolin-binding aptamer is an exciting target (Reyes-Reyes *et al.*, 2010, Girvan *et al.*, 2006). The aptamer (formerly AS1411) acts as drug-like molecule, by destabilizing Bcl-2 mRNA as a decoy (Soundararajan *et al.*, 2008) and has advanced into clinical trials (Le Trinh *et al.*, 2015).

In addition, the success of Herceptin (covered in chapter 1) shows the excellent promise of the HER2 receptor target and the therapeutic feasibility, as multiple HER-2 aptamers have been generated and applied (an anti-HER2 aptamer was originally described in the research proposal). Traditional binding assays, like EMSA, ELONA and use of SPR would serve the purpose equally well and this could be followed by cell internalization studies and drug delivery in the longer term.

6

Post-selection optimization

6.1 Introduction

Post-selection optimization comprises steps to enhance aptamer properties after selection. This may involve chemical modifications for stabilization and to tailor pharmacokinetic properties, like exo—and endonuclease resistance (Lupold *et al.* 2002) or renal clearance rates (due to the typically low MW), such as PEG-conjugation onto a hydroxyl-group either directly (Veronese and Pasut., 2005) or via a spacer link (Langer and Tirrell, 2004). This can greatly enhance plasma permanence and bioavailability of aptamers or facilitate their cellular internalization. This is because the PEG group modulates the negative charge of their phosphate backbone. Another example is to reversing the sugar stereochemistry to non-natural L-chirality (L ribose) to yield enantiomeric spiegelmers of greater stability (reviewed by Keefe *et al.*, 2010).

As aptamers can be more easily modified than antibodies, other approaches aim at improving binding affinity or at investigating the sequence space further with the potential to discover high-affinity motifs. For instance, mutagenic PCR was used to improve streptavidin-binding aptamers (Tahiri-Alaoui *et al.*, 2002), RNA aptamers against Hepatitis C protease (Fukuda *et al.*, 2000), a ribozyme for alcohol dehydrogenase (Tsukiji *et al.*, 2003), and a DNAzyme for applications as endonucleases and in a biosensor (Lu, 2002). Sequence space was explored as well using microarrays (Katilius *et al.*, 2007) and by the step-wise mutagenesis and post-selection screening for an anti- PSMA aptamer (Savory *et al.*, 2010). Other approaches are reviewed by Klug and Famulok, 1994. The arsenal of post-selection modifications at disposal is unique to aptamers and an attractive means to further explore their potential.

6.1.1 Sequence minimization

Within a given aptamer sequence, not all nucleotides are necessarily essential to achieve the target-binding conformation, or participate in binding (Hesselberth *et al.*, 2000, Kim *et al.*, 2010, Burgstaller *et al.*, 1995). Motivation of this thesis is exploring aptamers as versatile, improved and more cost effective therapeutics. The length of the

sequence, along with its complexity and modifications, is among the critical factors for cost of synthesis. Therefore, sequence minimization is a commonly sought after selection and aims at identifying and eliminating the non-essential nucleotides. For instance, with mfold assistance, aptamers were shortened in a crawling base deletion pattern (Gopinath *et al.*, 2016). Shortening has also been pursued for a streptavidin-binding aptamer (Le *et al.*, 2014), Tenascin C aptamer (Hicke *et al.*, 2001) and aptamers selected from tumor cells, which have been minimization and stabilized (Shangguan *et al.*, 2006). Another minimization approach was by non-homologous random recombination (Bittker *et al.*, 2002).

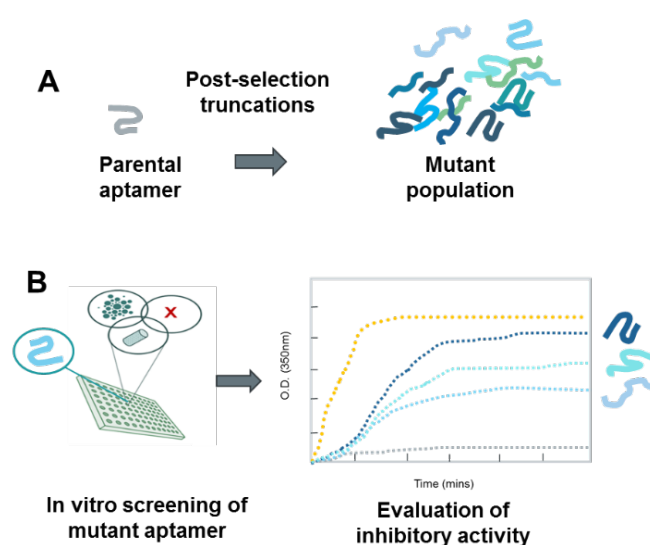


Figure 6: General principle of post-selection modification with tubulin polymerization-based activity screening.

A) The original aptamer (parental sequence) is modified in different approaches, such as minimization, single nucleobase deletions and creation of chimeras to design a population of mutants **B)** Mutants are screened *in vitro* and their inhibitory activity levels are compared.

6.1.2 The aptamer clock: a rapid prototyping template

Sequence truncations serve to further investigate promising sequence space. This has been explored for minimization and truncates (Le *et al.*, 2014), truncation of internal loops for the anti VEGF 165 aptamer (Kaur and Yung, 2012), a PDGF aptamer, (Vu *et al.*, 2016), a dimeric aptamer (Lin and Jayasena, 1997), and an RNA aptamer that binds human complement C5 (Biesecker *et al.*, 1999). Truncations approaches can be informed by boundary experiments (Sayer *et al.*, 2004) or computational structure

modeling combined with secondary structure predictions, as in the case of RNA aptamers against PSMA (Rockey *et al.*, 2011) and was also applied to elucidate the relationship of structure-sequence-activity (SAR) (Fischer *et al.*, 2008).

Changes of only a few single bases, such as incorporation of modified nucleotides, can have profound effects (Shigdar *et al.*, 2013, Mallikaratchy *et al.*, 2011). Mfold is a computational secondary structure prediction method based on folding energy minimization (Zuker *et al.*, 1999 and Zuker, 2003). It serves to rapidly predict the secondary structure, enthalpy and other basic parameters of aptamer folding *in silico* and is used prominently. The workflow sought here was similar to previously described work with mfold, (Gopinath *et al.*, 2016). The central 'clock'-like stem-loop of a parental sequence was used to create 1nt deletion mutants with altered (predicted) structure, whereas the sequence remains the same, save for 1nt less. This 'aptamer clock' was used as rapid design and testing platform to act in concert with secondary structure predictions and the activity-based *in vitro* drug discovery screen (reviewed in chapter 1, used in chapter 4) to generate motifs with improved activity profiles. Structure motifs were rationally engineered from modifying the parental sequence (Hoinka *et al.*, 2012). In this section, predicted secondary structure features were deleted, rearranged or altered through deletions in the parental sequence to yield a population of structure variants and changes in activity were monitored. This served as a first step to further elucidate the Structure-Activity-Relationship (SAR) of these tubulin-binding aptamers and inform future doped/patterned libraries.

6.1.3 Controlling aptamer activity with switches and antidotes

Controlling levels of aptamer activity is an attractive prospect in the therapeutic field, as dosages are often experimental and patient-specific. Innovative works demonstrated antidote-mediated control of an anticoagulant aptamer against coagulation factor IXa *in vitro* and *in vivo* for the first time (Rusconi *et al.*, 2002, Rusconi *et al.*, 2004). Fueled by this promising success, similar studies soon presented aptamer antidotes against anticoagulation drug Bivalirudin (Martin *et al.*, 2013) and to inhibit thrombin generation (Nimjee *et al.*, 2006).

Another approach to control aptamer activity has been demonstrated by incorporating photo-inducible nucleotides, such as caged thymidine nucleobases (Heckel *et al.*, 2005) or another anticoagulant, endowed with a light-sensitive trigger (Heckel and Mayer, 2004). Further, a caged antidote chimera (Held *et al.*, 2006) was designed bearing two distinct functional regions: a protein-binding domain (anti-thrombin) and a regulatory domain, while the caged antisense antidote region is by default inactive. Irradiation of the chimera triggered its activation and successfully suppressed aptamer activity. A limit to the appeal of this approach is posed by a 50% loss of activity. This was measured in a blood-clotting assay with the chimera and the original sequence. Most such post-selection constructed switches suffer from decreased activity compared to the original.

Finally, the direct selection of a structure-switching aptamer addressed this limitation (Nutiu and Li, 2003, 2005). The selection design targeted the capability to undergo conformational change from self-hybridization to target-binding form (duplex-to-complex) upon target-addition. The structure-switching activity was monitored as change in fluorescent signal using labelled aptamer and an antisense DNA bearing a quencher. Chimera in drug delivery are reviewed thoroughly elsewhere (Kanwar *et al.*, 2011).

In this section, the first idea was to utilize a tubulin-binding DNA aptamer (Fukusaki *et al.*, 2001) and an arginine-binding DNA aptamer (Harada and Frankel, 1995) – (also exists RNA aptamer (Geiger *et al.*, 1996) -to create a similar post-selection chimera prototype. Affinities are moderate in the former and rather weak in the latter case (low micro-and millimolar range, respectively). The difference here is that the proposed

ligand-induced conformational changes would be monitored as changes in inhibitory activity on tubulin polymerization.

The second project endeavored to inactivate structure motifs through partial or complete hybridization with antisense antidotes similar to the anticoagulant-antidote pairs discussed. This approach could serve to identify the essential motifs involved in target-binding. Changes in binding affinities are monitored as activity levels with the *in vitro* inhibitory screen.

This subset of work focused on shedding additional light into how to tightly control nucleic acid therapeutics with the approach to modulate tubulin inhibition as proof of concept that can be expanded upon in the future.

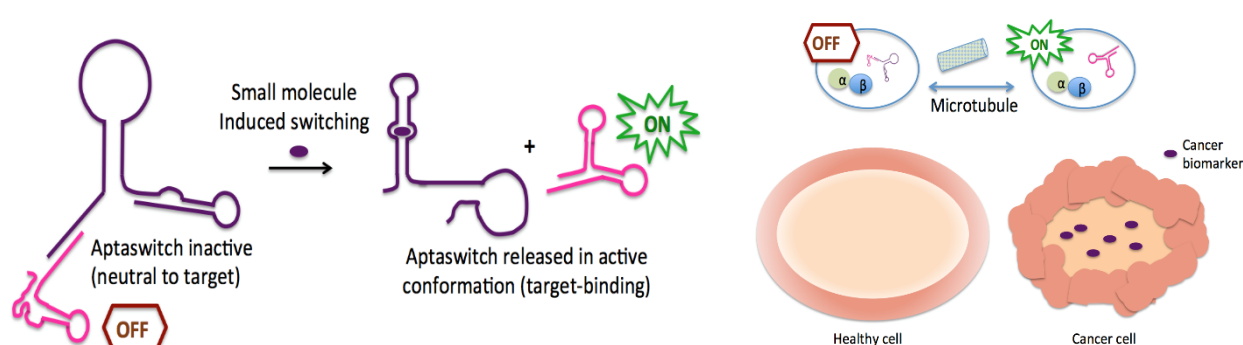


Figure 6B: Concept of a biomarker-induced therapeutic chimera with on/off switching capability.

An ideal chimera would by default be in OFF mode through self-complexation. Upon addition of a ligand (here Arginine), the ligand-binding domain, part of the self-hybridizing region, would bind Arg instead with strong affinities. This would release the target-binding domain (anti-tubulin) which would not bind the target and inhibit the polymerization reaction (left). In the longer term, this chimera could be reprogrammed to respond to intracellular tumorigenesis biomarkers instead, such that the drug-like nucleic acid agent is activated when in a cancer cell and is benign in healthy cells (right).

6.2 Results and discussion

6.2.1 Aptamer minimization

Tubulin-binding aptamers (characterized in chapter 4), the ‘Tuda’ population, were optimized for length by eliminating both flanking regions. Activity of the minimized versions, M1-M16 were then analyzed. Here, polymerization, depolymerization and phase I (nucleation) duration are measured.

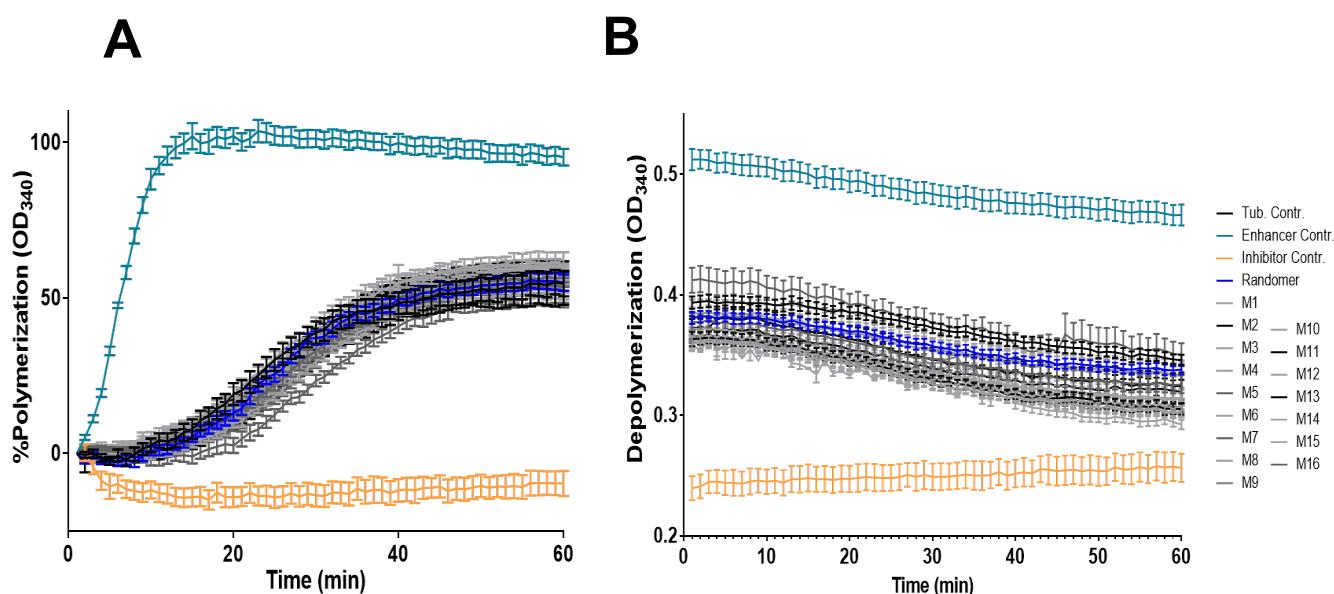


Figure 6.1: *In vitro* polymerization and depolymerization of minimized Tuda aptamers.

A) Assembly slopes **B)** Disassembly slopes. Curves represent TX enhancer (turquoise), VC inhibitor (orange), Tubulin (black), randomer (blue), Tuda aptamers (grey) and Numbers denote the Tuda aptamers and R stands for randomer, TX for Paclitaxel (enhancer) and VC for Vincristine (inhibitor). Absorbance (O.D. 340nm) was measured at 37°C and while cooling down the plate for depolymerization in 1 minute-intervals for 60mins. O.D. values are expressed as normalized %O.D. Values represent the average of three independent experiments performed in triplicates and error bars express SEM.

In Figure 6.1. (A) gives the overall polymerization activity profile of the minimized M aptamers. The M-series oligos do not appear to affect the kinetics significantly, except for one of them: M10 (analyzed in further detail in next section). In general, phase I seems to extend longer with the aptamers. This is visible from the delayed onset of polymer growth.

The aptamers have only small effects on depolymerization (B) and slopes remain in the range of the randomer control. In a few cases, the aptamers increase velocity of the disassembly to the effect that more microtubules have depolymerized at the end compared to tubulin and randomer, as suggested by the decreased O.D.

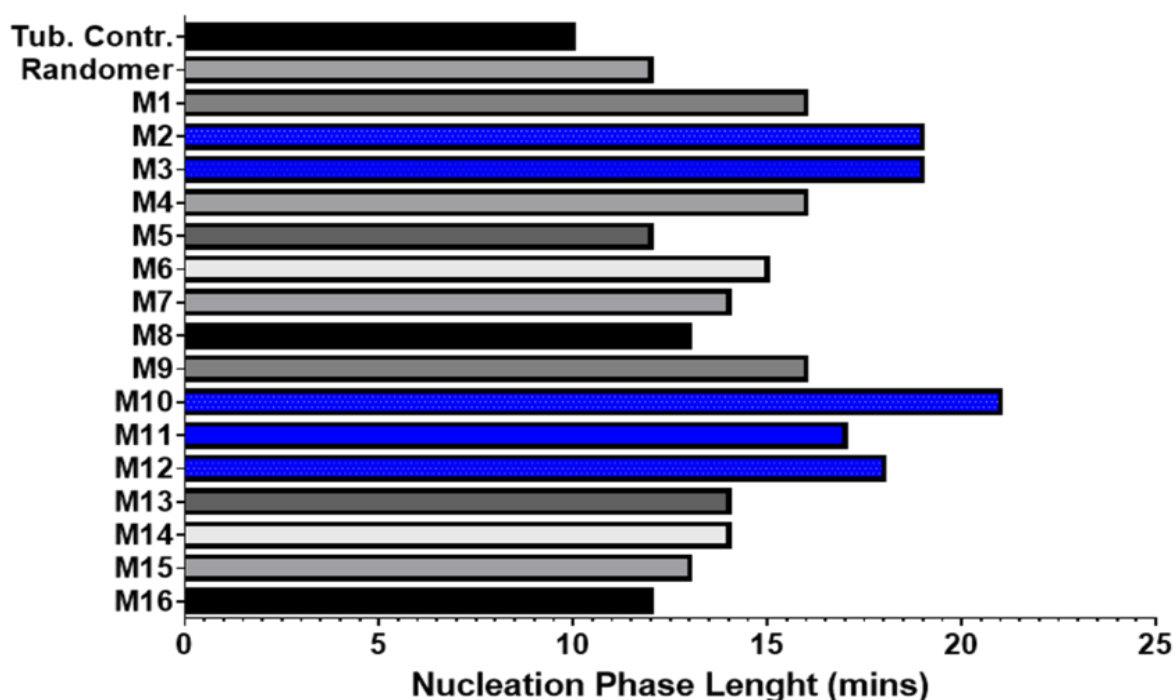


Figure 6.2: Nucleation phase duration (mins) of minimized aptamers.

Nucleation phase duration was determined semi-quantitatively from reaction curves by measuring duration until onset of MT polymer growth. Bars highlighted in blue denote the subgroup analyzed. Values represent the average of three independent experiments performed in triplicates.

The nucleation phase depicted in Figure 6.2 is not affected significantly. However, a group (M2, M3 and M10-12) perturbs nucleation for a few minutes beyond the randomer control, suggesting specific effects. M10 delays phase I progression to 21mins from 12mins and 10mins, for randomer and tubulin, respectively. With M10, this more than doubles.

Here, absorbance levels at each phase are measured at 10mins, 25mins and 60mins.

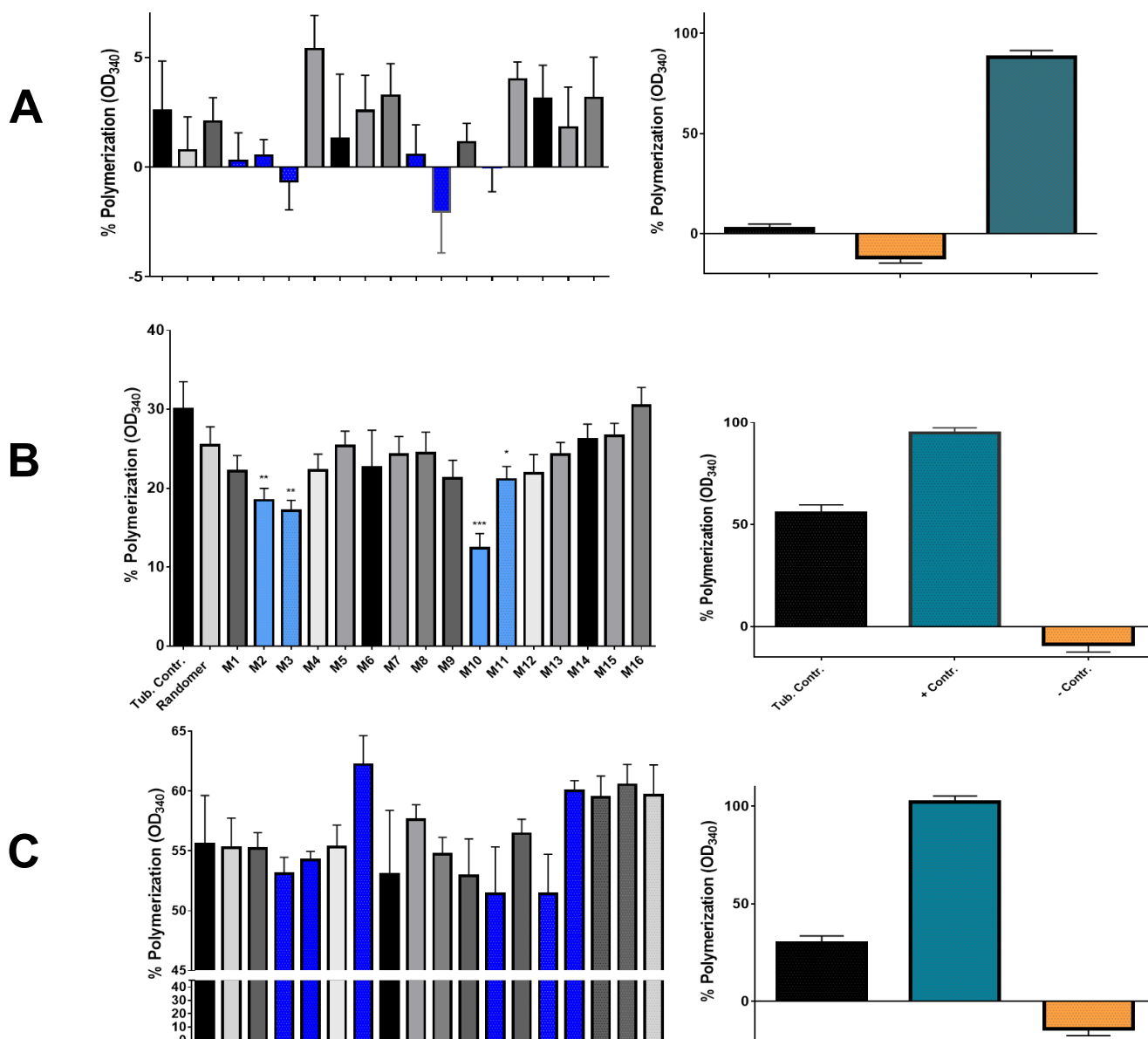


Figure 6.3: Microtubule mass across critical reaction time-points.

A) Nucleation (10mins) **B)** growth (25mins) **C)** steady-state (60mins) with enhancer and inhibitor graph for scale (right). Bars highlighted in blue denote an interesting subgroup. Absorbance (O.D. 340nm) was measured at 37°C in 1 minute-intervals for 60mins and compared per time-point. O.D. values are expressed as normalized %O.D. Values represent the average of three independent experiments performed in triplicates and error bars express SEM.

Figure 6.3 shows that most of the minimized aptamers do not inhibit phase I significantly at the 10mins mark (A). The most strongly reduced levels are observed for the oligos M2-M4 and M9, M10, but fall within the range of the randomer control. This suggests these are general effects. B shows that around mid-point of the reaction (25mins), microtubule levels decrease statistically significantly by adding M2, M3, M10 and M11, whereas no significant change occurs for the rest of the panel. Addition of oligo M10 to the polymerization mix has the effect that only about half the microtubule polymer mass forms compared to the tubulin control. At endpoint (C) of the reaction (60mins) no significant differences are observed.

Next, the polymerization reaction slopes were then generated per each minimized oligo (M1-16) to facilitate comparison with the original version (T1-16).

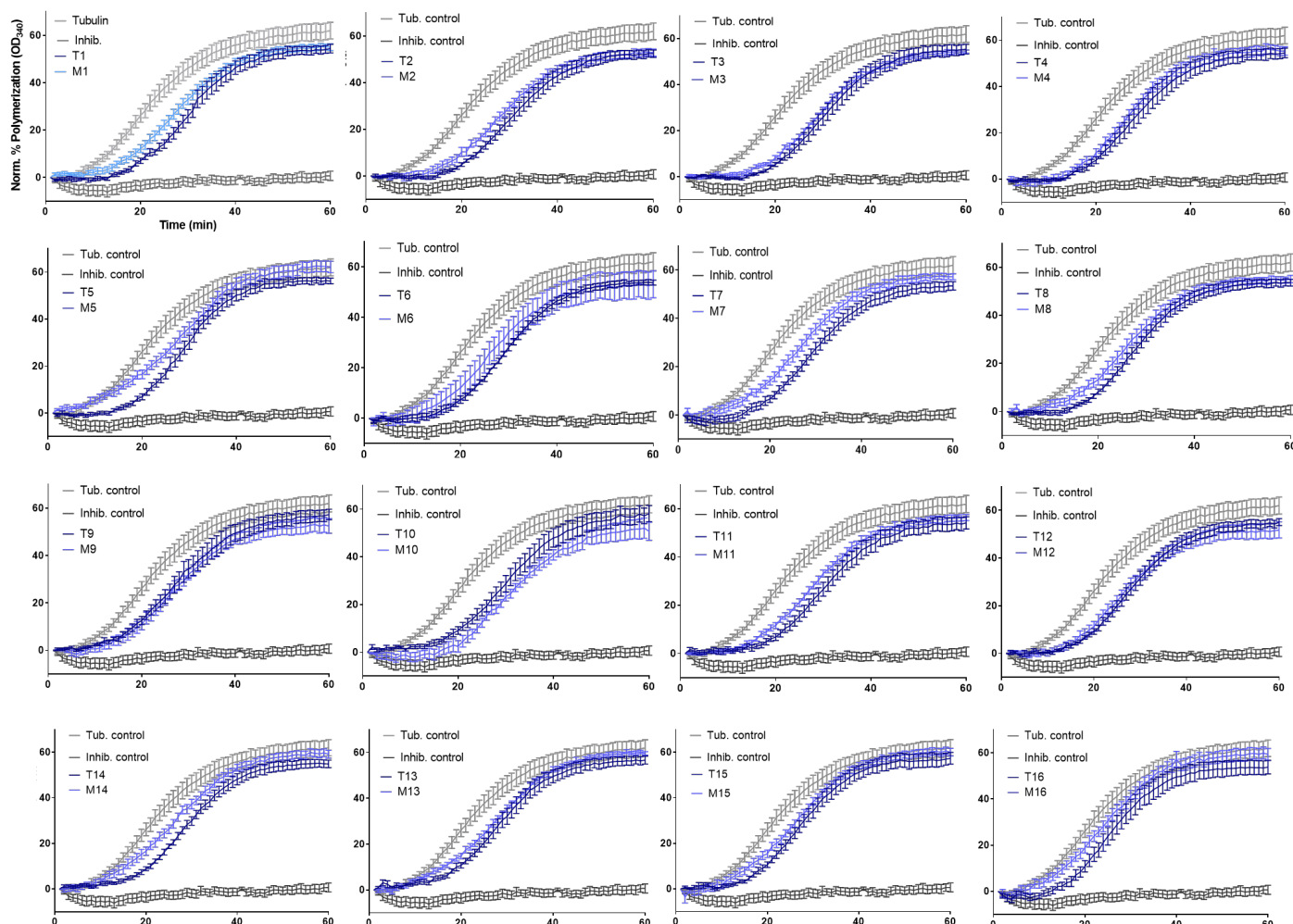


Figure 6.4: Assembly of minimized and full-length aptamers.

Slopes represent the minimized aptamer (light blue) and original (dark blue) and the average of three independent experiments performed in triplicates. Error bars express SEM.

An overall trend is that the minimized oligos M1-M16 appear to possess either retained or slightly diminished activity on the assembly reaction. In general, the shortened sequences show reduced nucleation phase inhibition compared to the full-length aptamers. An exception seems to be the M10 oligo, which has a stronger effect than the original full oligo T10. This suggests the elimination of the flanking regions has likely eliminated nucleobases that were essential. In contrast, it has facilitated a more effective conformation in the case of M10.

Then, absorbance values were compared between the originals (T) and the minimal versions (M) at 25minutes in growth-phase (Figure 6.5).

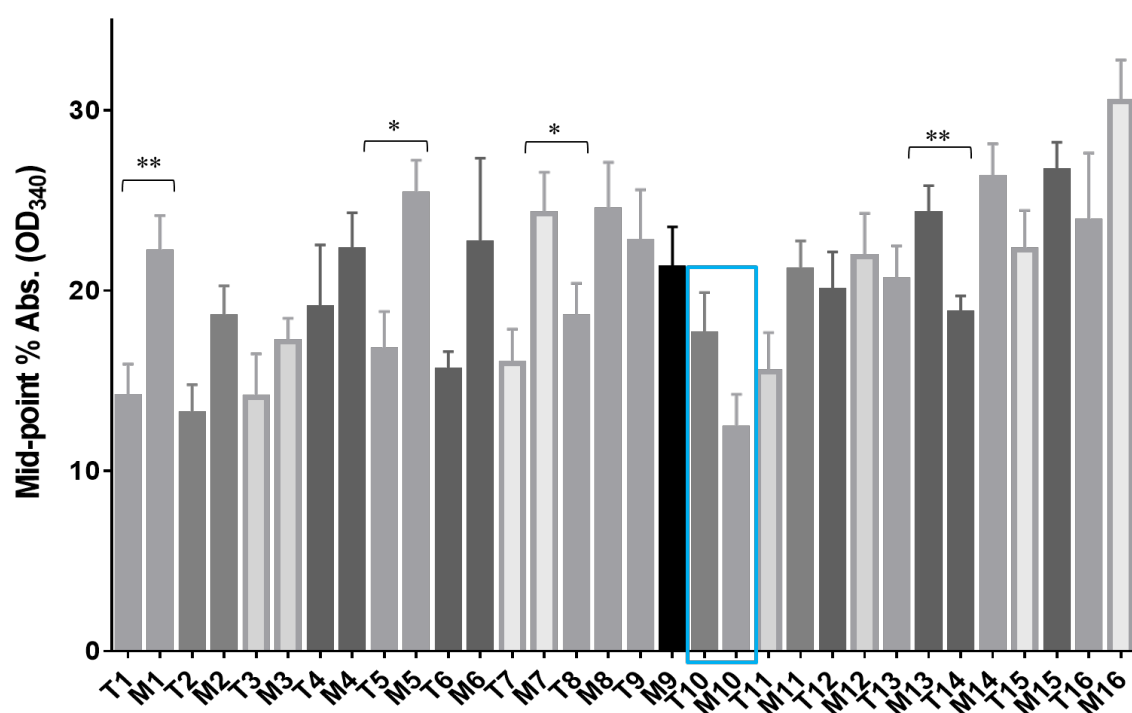


Figure 6.5: Midpoint microtubule mass of minimized and full-length aptamers.

Timepoints in growth-phase (25min) were compared. Cyan box indicates the promising improved oligo M10. %O.D.Values represent the average of three independent experiments performed in triplicates and error bars express SEM.

Absorbance values were compared between the originals (T) and the minimal versions (M) at 60 minutes at steady-state (Figure 6.6).

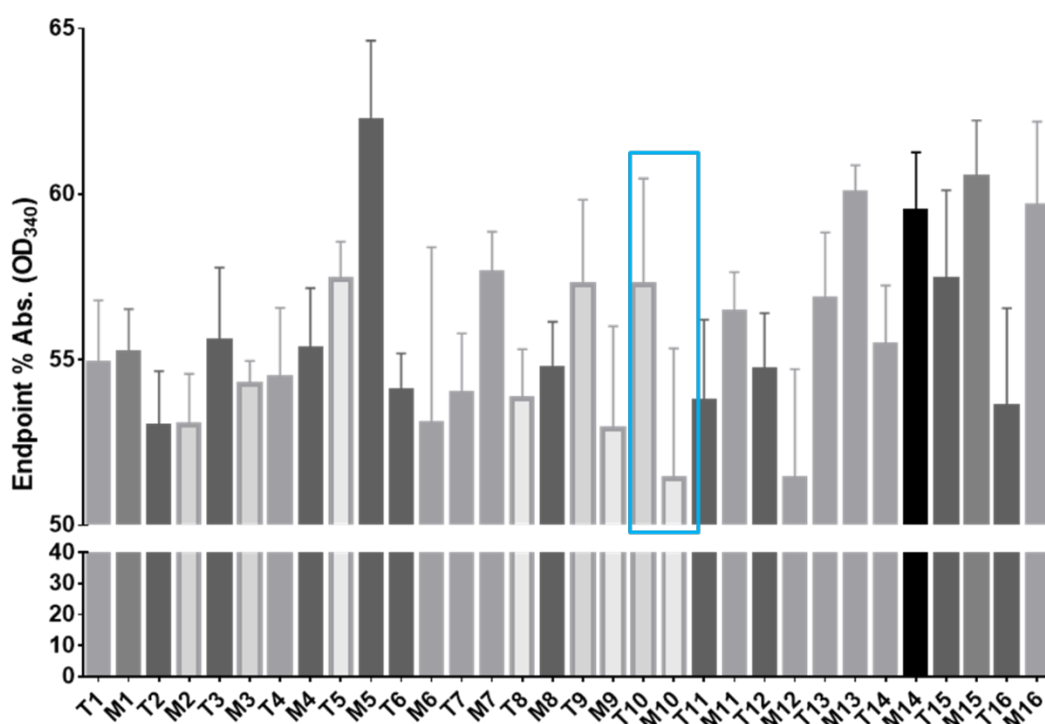


Figure 6.6: Steady-state microtubule mass of minimized and full-length aptamers.

Time-points at endpoint (60mins) were compared. Cyan box indicates the promising improved oligo M10. %O.D.Values represent the average of three independent experiments performed in triplicates and error bars express SEM.

In a general, the minimized aptamers (M) do not reach the (however small) inhibitory effect of the full aptamers (T). This can be inferred from the higher microtubule levels at the reaction mid-point compared to the full sequences. For many of the pairs, the differences are vanishingly small, but more significant for others.

An interesting exception is M10, the minimized truncate of T10: It has greater effects than the full parental sequence, suggesting that elimination of the nucleotides has enhanced the conformation and improved protein interaction (to a small extent). At endpoint similarly, the minimized sequences generally appear weaker, as indicated by higher absorbance values. Equally, the M10 minimal sequence seems improved compared to the original at this point.

V_{max} of the five most promising minimal candidates were calculated and reaction slopes visualized.

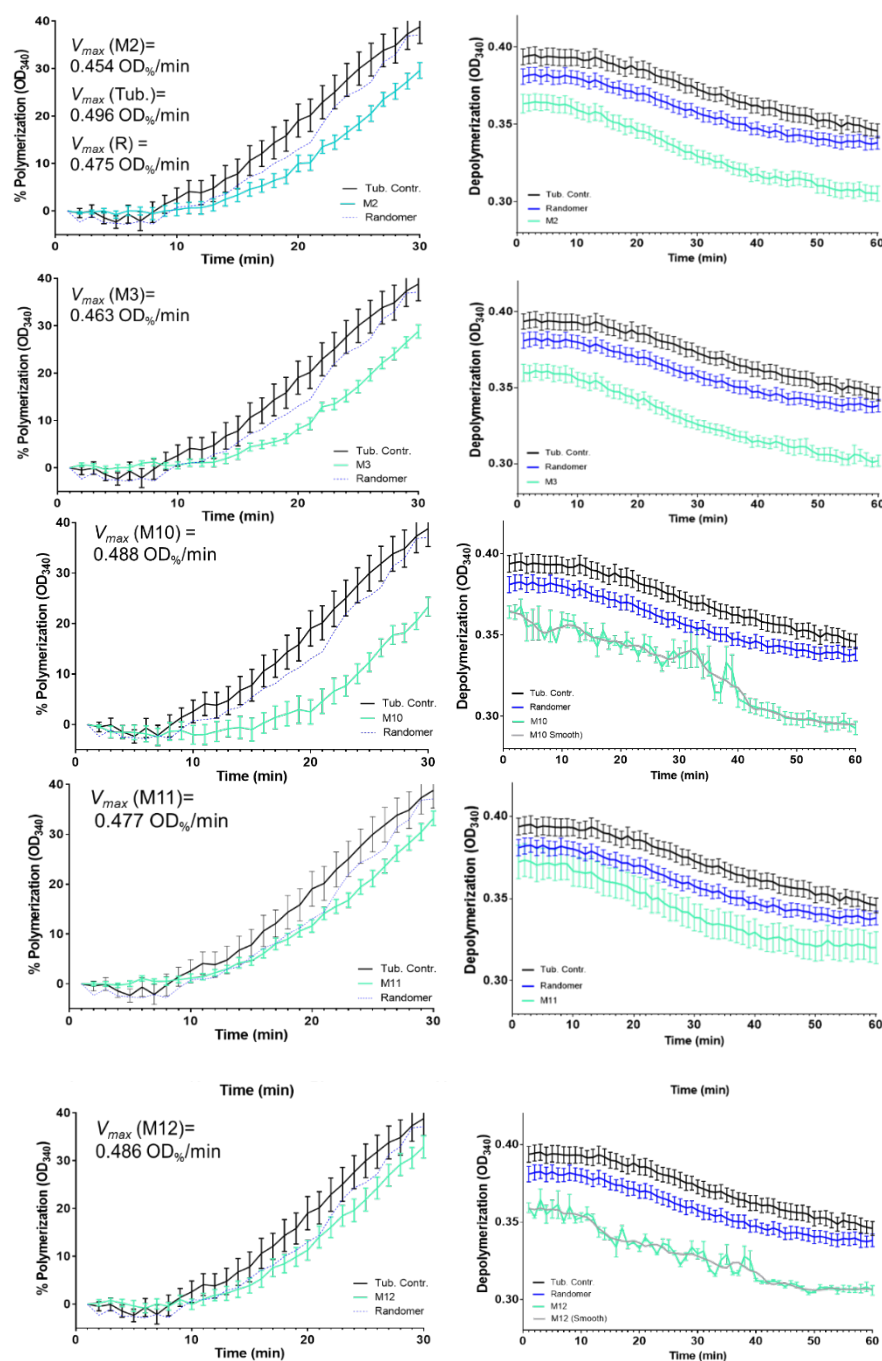


Figure 6.7: Polymerization and depolymerization with V_{max} .

V_{max} was determined with linear regression over 20 time-points of the growth phase and the top aptamers of the panel were determined across reaction parameters. Error bars represent SEM.

Secondary structures were predicted with mfold.

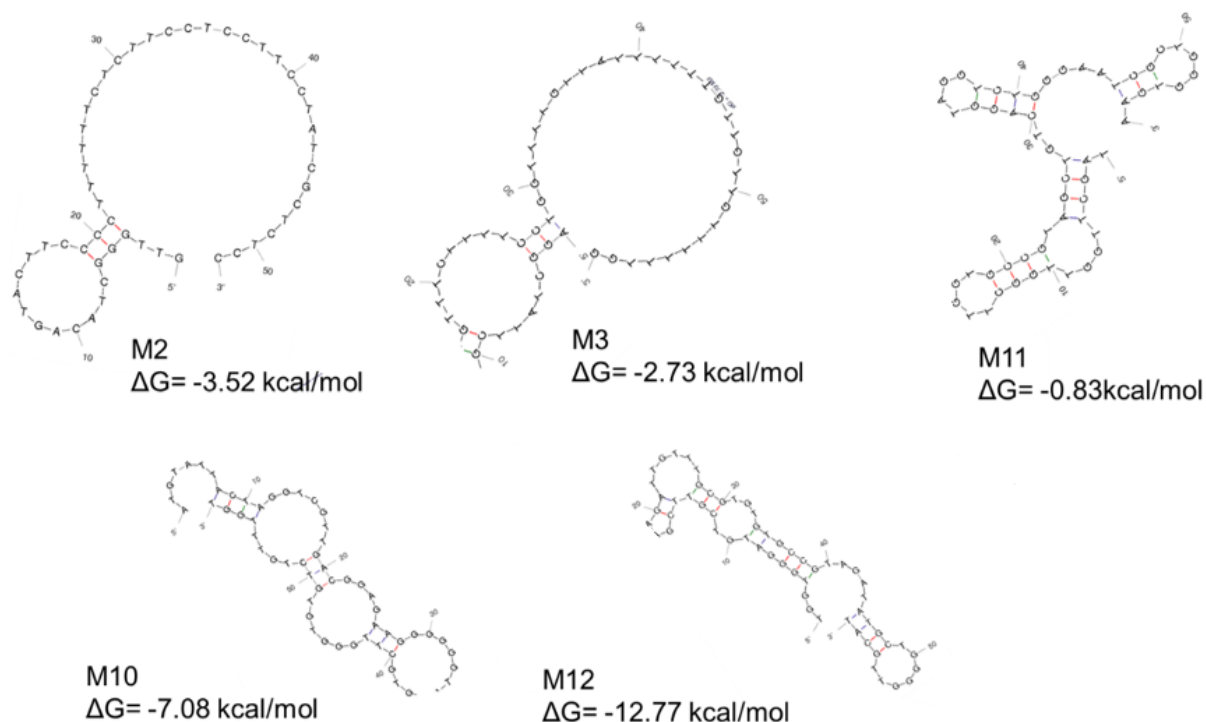


Figure 6.8: Predicted secondary structures of minimized aptamers.

Secondary structures and enthalpy values were predicted with the mfold webserver based on energy minimization under approximate folding and estimated ionic strength of tubulin buffer.

In the reaction curves of figure 6.7, M2 and M3 appear to have the best results among the sub-population. M2 and M3 reduce V_{max} to 0.454 and 0.463 OD/min, compared to 0.496 and 0.475 OD/min for tubulin and randomer controls, respectively. However, differences are only small and are therefore not deemed statistically significant. The aptamers appear to enhance microtubule depolymerization visibly, as steady-state absorbance is visibly decreased for M2, M3 and M12, whereas the randomer does not affect the reaction.

Figure 6.8 depicts predicted secondary structures (mfold). Assuming similar conformations, M2 and M3 both bear a large loop and smaller stem-loop. Also M10 and M12 appear similar, as they both contain four small loops, whereas M12 carries an additional 5nt stem-loop. According to the enthalpy calculation, M12 is the most stable among them. The 18nt stem-loop motif of M2 forms instead a 21nt stem-loop motif with a 1nt bulge in M3, whose loop is smaller than that of M2 and less stable. M10 and M12 have 3 stem-loops of slightly different configuration and M12 contains a 7nt bulge.

Interestingly, M2 and M3 both slightly decrease V_{max} and assembly dynamics, while they also are similar in structure. These motifs, such as the large stem-loop might therefore be a (faint) hint to be more promising features than the rest of the group and could be investigated in follow-up assays.

6.2.1 Discussion Summary

In this assay, post-selection minimization of a series of aptamers was undertaken by deleting primer-binding regions on both ends. Primer-binding regions may or may not participate in binding and/or structure formation and nucleotides might be removable (Kumbar *et al.*, 2014, Vu *et al.*, 2016).

This minimization strategy has generally resulted in aptamers of retained or reduced activity. This was observed across multiple reaction parameters, from polymerization progression of the distinct phases, assembly kinetics and microtubule levels and their activity levels. From the post-SELEX modified panel, M1-3 and M10-12 seemed the most promising within a small range.

For one group (M3, M4, M9, M11, M12, M13 and M15) removal of flanking regions did not significantly alter effects. This indicates that these bases are non-essential and neither significantly impede nor enhance conformation and/or binding. For another (M1, M2, M5, M6, M8, M14 and M16) removal of the flanking regions has rendered them less effective. This indicates that the bases on either side or both sides facilitate formation of secondary structure features or participate directly in binding. Therefore, these bases cannot be removed without loss of activity. An exception is M10. This minimal version of T10 has shown significantly greater potential to inhibit nucleation phase, and decrease V_{max} and polymer levels throughout the reaction.

This could suggest that in this case, the deleted bases may have previously interfered with effective folding. In this case, this optimization strategy yielded an aptamer of reduced size, which is cheaper and faster to synthesize than the original, an ultramer, while demonstrating improved activity across key reaction parameters.

Overall results suggest that the flanking regions are required to form target-binding, sufficiently stable secondary structures. In the case where the effect remains very similar, the deleted sequences are most likely not directly involved in target binding. The mixed results, ranging from less to similarly active, to one significantly improved oligo, shows that the strategy affects each sequence differently, which corresponds well to the general trial-and-error nature of this work.

The weak range of effects of the original aptamers pose a limitation to effective assay design to probe these modifications, since activity changes occur within a narrow range. As outlook, further truncation strategies are best explored based on a more tightly-binding parental sequence to allow for a clearer distinction of before and after inhibitory effects.

The minimization strategy was effective in the case of M10. Upon removal of the flanking regions, the oligo achieved greater inhibition of microtubule formation. This oligo could serve as optimization template for further truncations.

6.2.2 Aptamer clock structure derivatives

Structure derivatives were designed by deleting a single nucleotide from the original sequence and the algorithm was applied to the new sequence to reveal whether the structure had changed or not. Not all deletions resulted in a structural change, hence the process involved some testing. Furthermore, only structural experiments can confirm the ultimate structure and their differences, but for the intention of the pilot study towards SAR insights described here, mfold was an excellent tool.

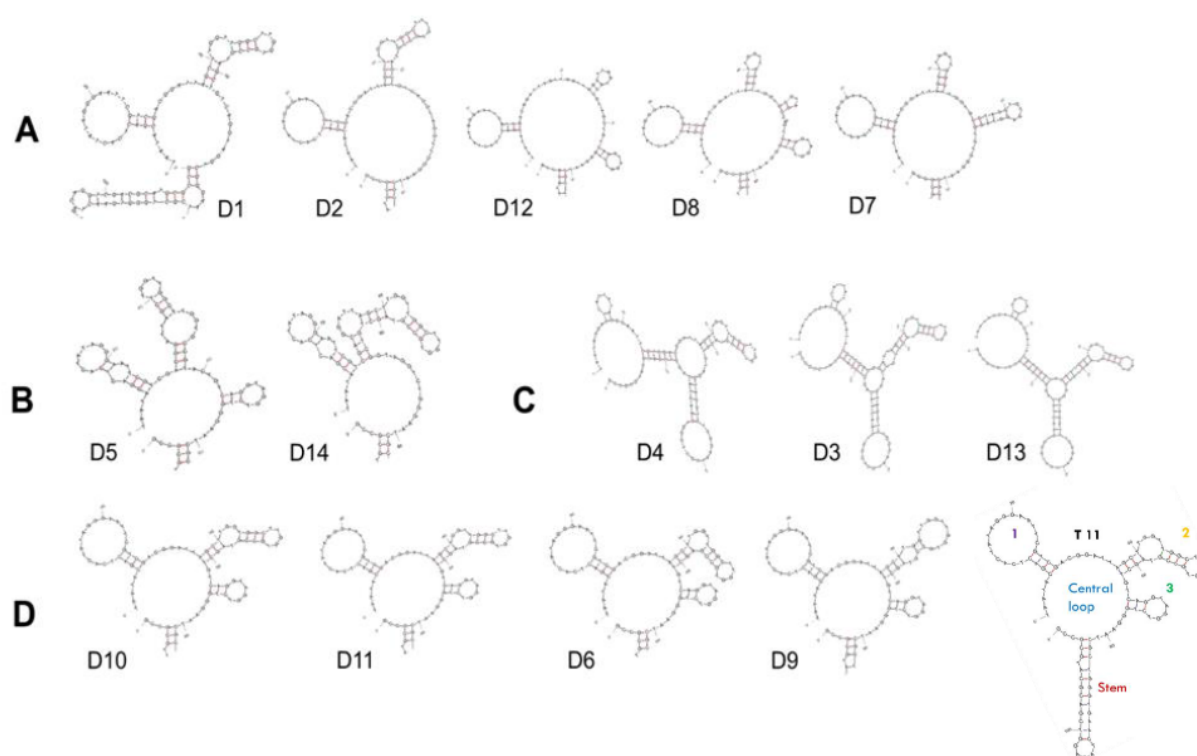


Figure 6.9: Predicted secondary structures of ‘aptamer clock’ truncates.

Groups A)-D) represent truncates created by nucleotide deletions based on the parental sequence (T11). Secondary structures were predicted with the mfold webserver based on energy minimization. Approximate folding conditions and ionic strength in tubulin buffer were used for the prediction.

Reaction slopes were measured for polymerization and depolymerization and nucleation phase duration was compared.

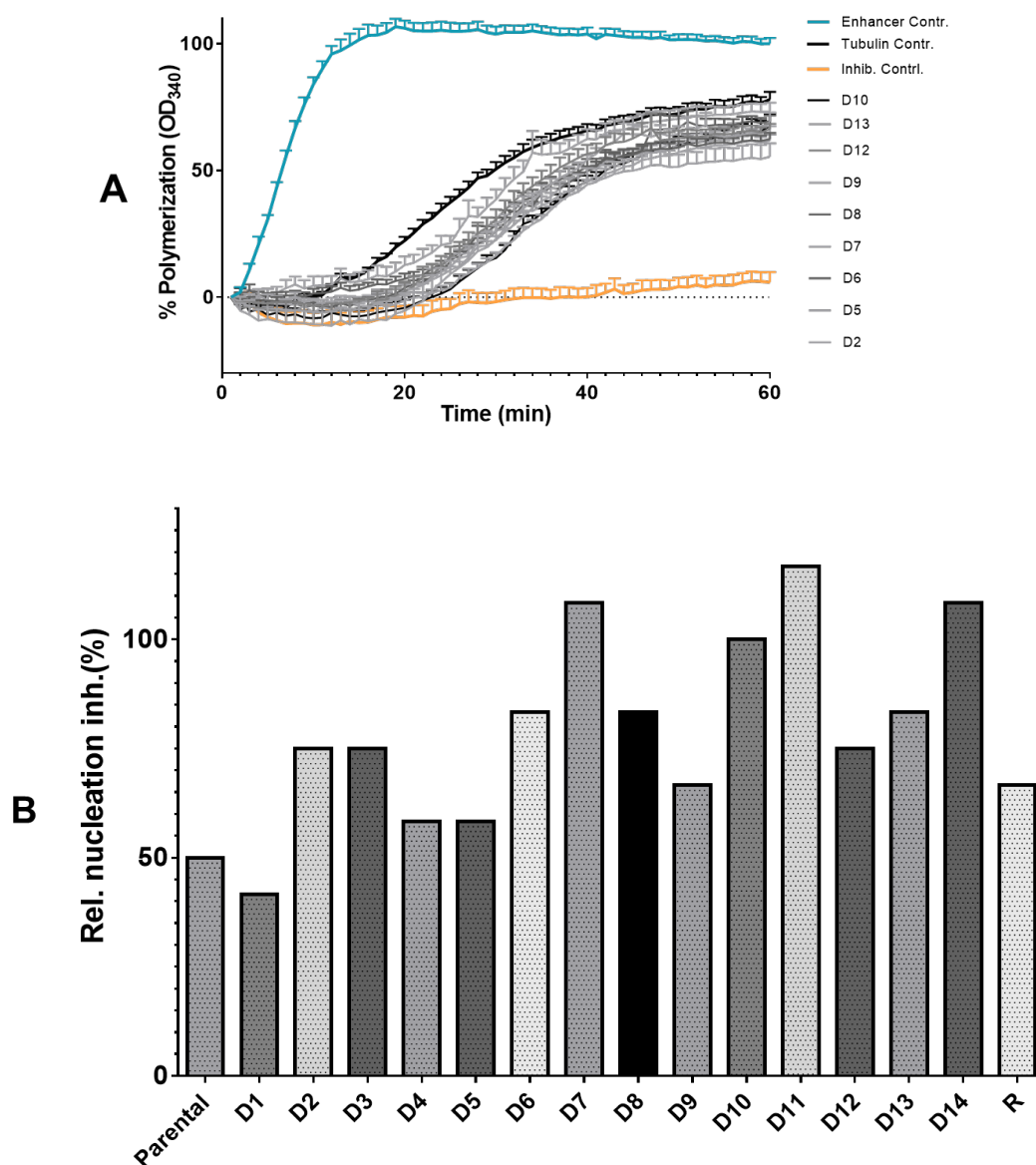


Figure 6.10: Polymerization and microtubule mass of clock truncates.

(A) Polymerization and depolymerization slopes **(B)** relative nucleation inhibition. Error bars represent SEM and values represent the average over three independent experiments performed in triplicates.

Figure 6.9 presents the predicted secondary structure motifs of all the variants. They were grouped by pattern similarity. Group A bear variations of motifs 1-3. Group B contain varied motifs 2 (or lack motif 3), group C are altered completely and D bears motif 2 variations.

In Figure 6.10, panel A shows the assembly reaction and the overall range of effects for a sense of scale. The variants clearly decrease overall MT levels throughout the reaction and some of them starkly so – figure 7.11 provides the best overview.

Relative nucleation durations (%) are shown in (B). Here, a similar pattern emerges with D7 and D11 emerge as the most promising and more than double nucleation time. D14, D10 considerably reduce the reaction dynamics in phase I.

Polymerization slopes per structure variant were compared (Figure 6.11).

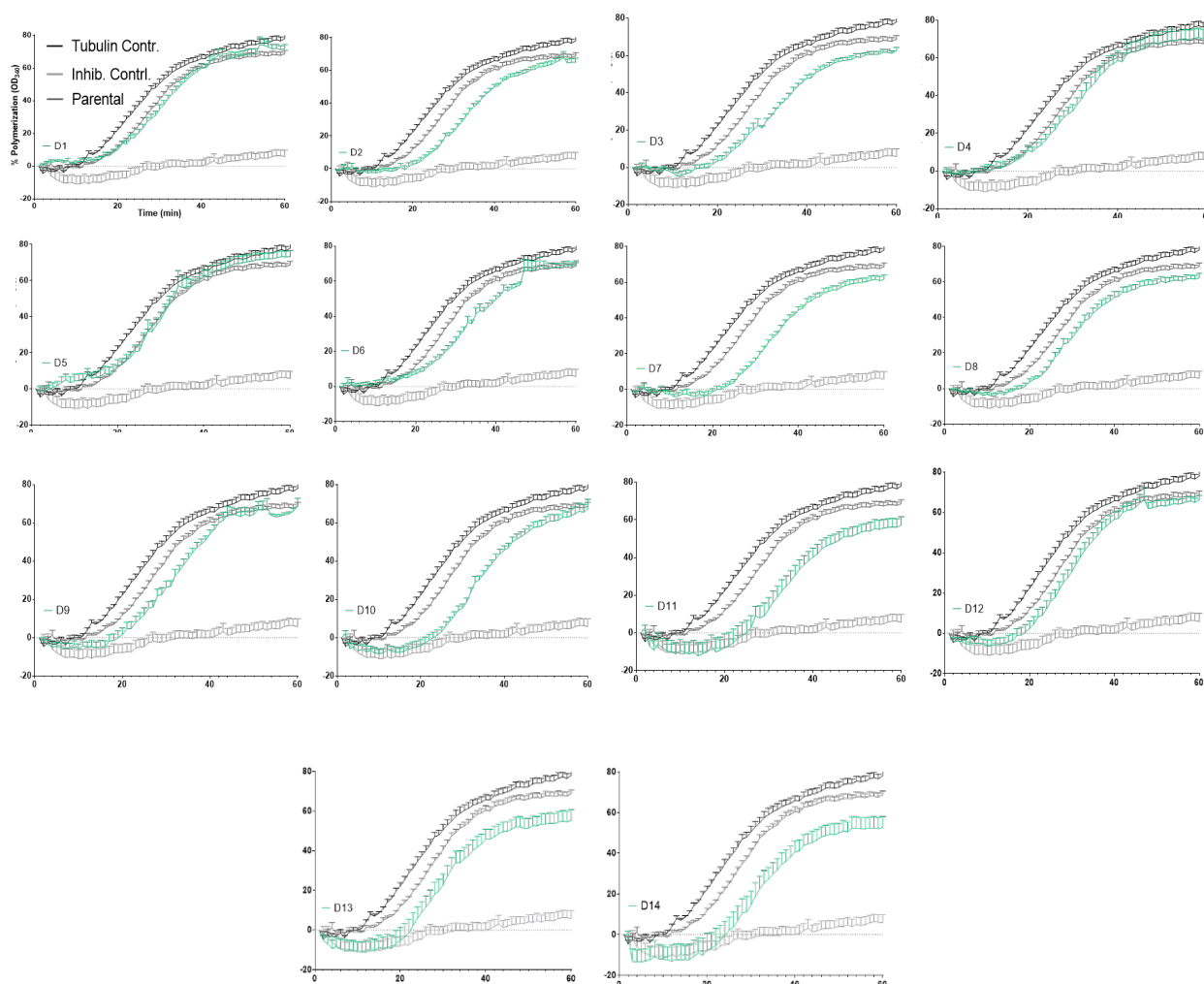


Figure 6.11: Polymerization of clock truncates and parental sequence.

Colors represent tubulin (dark grey), parental sequence (medium grey), inhibitor (light grey) and truncates (cyan). Absorbance (O.D. 340nm) was measured at 37°C in 1 minute-intervals for 60mins. O.D. values are expressed as normalized %O.D. Values and represent the average of three independent experiments performed in triplicates and error bars express SEM.

In Figure 6.11, slopes of some deletion truncates closely match the parental sequence, (D1, D4, D5, D9, D12), which may indicate that the particular deletion did not significantly alter binding affinity.

Only small changes are observed for D2, D3, D6 and D8. The group D7, D10, D11, D13 and D14 shows considerable improvements. This might suggest that the deletions have enhanced folding for tubulin-binding and that they might more strongly interact with the target. This observation excellently lends itself to a follow-up experiment. Secondary structures of the subgroups are shown in figure 7.12.

These truncates represent 1nt-deletion mutants. Interestingly, the sequence remains practically the same, yet the latter group exhibits visibly and significantly improved aptamer activity, which highlights the exciting opportunities to probe SAR for anti-protein aptamers.

A subgroup of the most promising variants was analyzed. First, their secondary structures were predicted with mfold (Figure 6.12).

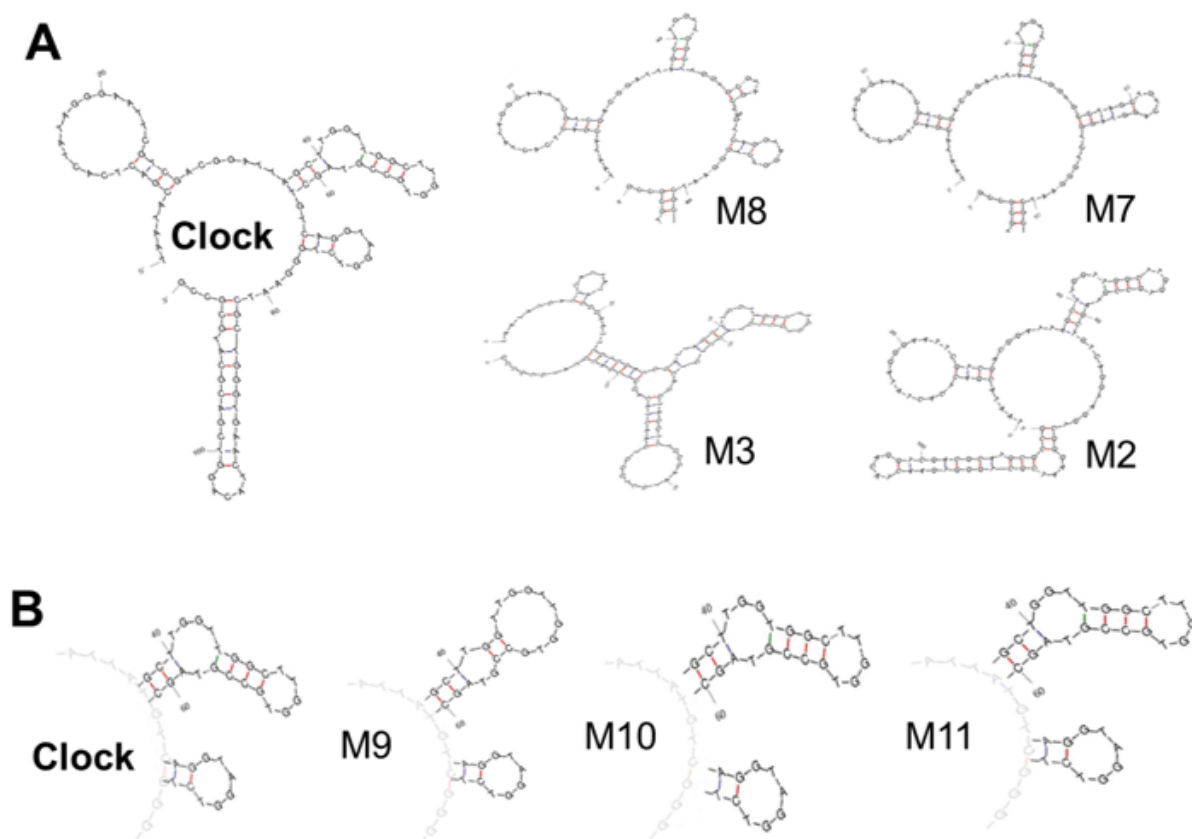


Figure 6.12: Predicted secondary structures of truncates per design group.

A) Group 1 are drawn from various derivatives groups **B)** group 2 are motif 2 and 3 derivatives.

Sequences were created by nucleotide deletions based from the parental sequence (T11). Secondary structures were predicted with the mfold webserver based on energy minimization. Approximate folding conditions and ionic strength in tubulin buffer were used for the

Next, the key reaction features like nucleation parameters, halftime and V_{max} were measured (Table 6.1).

Table 6.1: Comparison of key reaction parameters of phase I and II for

A	Reaction feature	Tub.	Parent.	M2	M3	M7	M8	M9	M10	M11
	$\frac{1}{2}$ time (min)	29	28	33	33	35	31	32	35	35
	% Inh. ($\frac{1}{2}$ -time)	0	3.4	13.8	13.8	20.7	6.9	10.3	20.7	20.7
	Nucleation (min)	12	18	21	21	25	22	20	24	26
	% Inh. (Nucleation)	0	50	75	75	108.3	83.3	66.7	100	116.7

B	Samples	Tub.	Parent.	M2	M3	M7	M8	M9	M10	M11
	V_{max} (mOD/min)	7.3±0.2	6.9±0.2	5.8±0.2	5.5±0.2	5.0±0.3	5.9±0.3	6.3±0.4	5.9±0.4	5.7±0.9
	% V_{max}	100	94.4±2.2	78.9±3.2	75.3±2.9	69.0±3.7	80.9±4.5	86.4±4.9	81.0±5.0	77.7±12.1

A) Phase I features of half-time, nucleation and %Inhibition are compared to tubulin **B)** V_{max} of growth phase. V_{max} was determined with linear regression over 18-20 time-points of the growth phase section with linear growth. All values represent the average of three independent experiments performed in triplicates

Half-time represents the time when half of the total microtubule mass is reached. Halftime duration of the parental sequence and tubulin are 28 and 29mins, respectively. The original aptamer therefore does not considerably affect this parameter. In contrast, an increase to 35mins, a 20.7% inhibition, is observed for M8, M10 and M11. The other oligos have only small effects.

The nucleation phase represents phase I of the assembly reaction until the onset of the elongation phase. M11 increases nucleation to 26 minutes, compared to 18 minutes for the parental sequence, a 116.7% inhibition, while M7 (25mins) and M10 (24mins) also promising.

V_{max} comparisons denote assembly velocity, which is decreased the most for M7 (5.0mOD/min), M3 (5.5mOD/min) and M11 (5.7 mOD/min) compared to tubulin (7.3mOD/min) and is further decreased relative to the parent sequence (6.9mOD/min). The best aptamers were compared via their polymerization slopes overall and microtubule mass (O.D.) during growth of the MTs (25mins) and at steady-state (60mins).

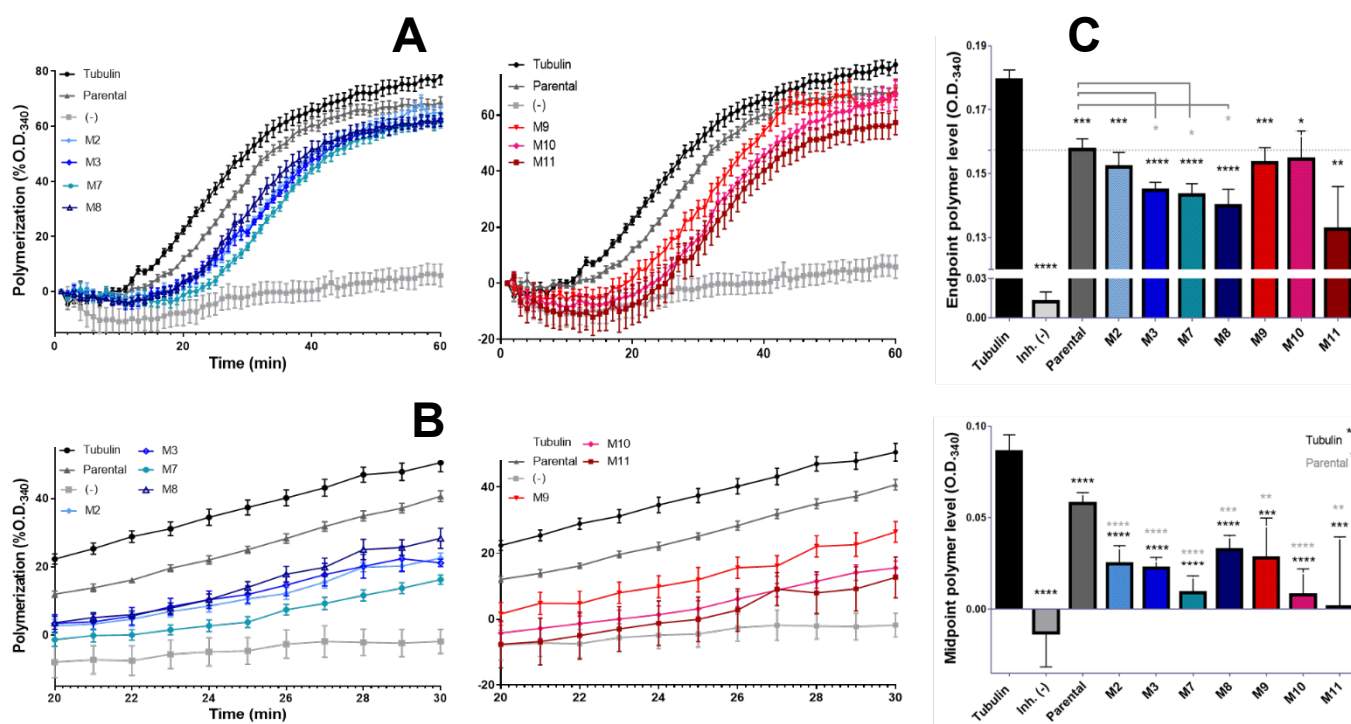


Figure 6.13: Detailed analysis of 'hit' candidate clock truncates.

A) *In vitro* tubulin polymerization curves with turbidity assay **B)** Zoom of growth-phase intersection **C)** Mid- and endpoint microtubule levels (25min and 60min, respectively). All values represent the average of three independent experiments performed in triplicates and error bars express SEM. Pvalues were calculated as one-tailed student-t-tests between samples and tubulin (non-parametric). Pvalues: *=0.05 ** = 0.01 *** = 0.005 ****=0.0001

In Figure 6.13, across (A) and (B) the group M7, M10 and M11 shows the strongest inhibitory potential. For endpoint microtubule mass (C, top), some minor differences are observed relative to tubulin (statistically significant), but not relative to the original for M7, M10 and M11 - the latter also has larger error bars. M3, M7 and M8 decrease endpoint polymer levels to a small, but significant extent compared to the parental sequence.

For midpoint polymer levels (C, bottom), all derivatives slightly, but statistically significantly decrease MT mass compared to both the original and tubulin. Again, the error for M11 is large, therefore M7 and M10 seem the most noteworthy, but the improvements remain in a modest range.

6.2.2 Discussion summary

This assay aimed at exploring the sequence space of a parental aptamer through creating deletion mutants with new structures. Deletion mutants which novel or altered secondary structure features (*in silico* predicted) were tested for their inhibitory activity on purified tubulin.

The experiment revealed effects of varied nature: For a few truncates it remained similar or slightly improved, while for others, activity clearly improved across several reaction parameters (M2, M3, M7, M10 and M11).

This demonstrates, that exploring sequence space in greater depth is applicable to high throughput, rapid prototyping with the ‘clock’ and works in combination with an *in vitro* screen. The ‘clock’ design strategy can be applied to any aptamer and is facilitated when a screening system applicable. This could hold promise as rapid post-selection optimization strategy and has generated improved aptamers for follow-up studies.

A few sequence truncates were further analyzed, but moved to the appendix for brevity.

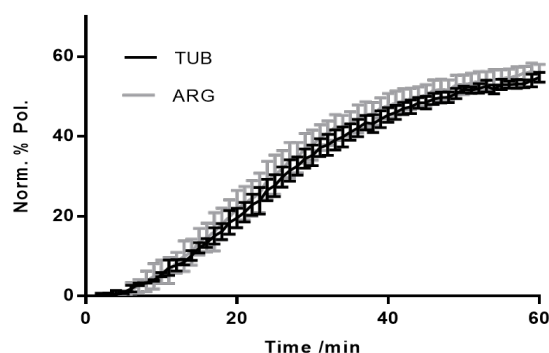


Figure 6.14: Interference assay of *in vitro* polymerization with Arginine.

Reaction slopes was determined with *in vitro* absorbance measurements (O.D. 340nm) of tubulin polymerization at 37°C in the absence and presence of 100μM Arginine. Error bars represent SEM and the assay was repeated twice with the same result.

6.2.3 Aptamer activity: towards a ligand-inducible chimera

With the motivation to control aptamer activity, a chimera was created (in four versions), which contains an anti-tubulin domain (T1 aptamer from Fukusaki *et al.*, 2001) and an arginine-binding domain (Harada and Frankel, 1995). The prototype was used to test whether the inhibitory activity of T1 could be modulated upon ligand addition by inducing conformational change. The arginine-aptamer was selected because it exists as DNA aptamer, has excellent affinity and basic structural information is available. The basic design consisted of a self-hybridizing domain, which, theoretically would undergo a conformational switch when Arginine is added and via induced fit model bind arginine – this would ‘release’ the anti-tubulin domain to bind the target and inhibit microtubule formation. Since T1 has exhibited only modest affinity and effects, the activity difference would only be small and could be further improved upon in future work. A first assay without the aptamer was performed to verify that the ligand on its own did not interfere with the reaction (Figure 6.14).

No visible differences were observed in the absorbance signal or tubulin polymerization. This suggests, Arg on its own at this concentration does not cause artifacts.

The first chimera was tested here in absence and presence of arginine. Activity was analyzed on assembly and disassembly slopes, V_{max} , microtubule mass during growth and steady-state as well as the predicted secondary structure (Figure 7.15).

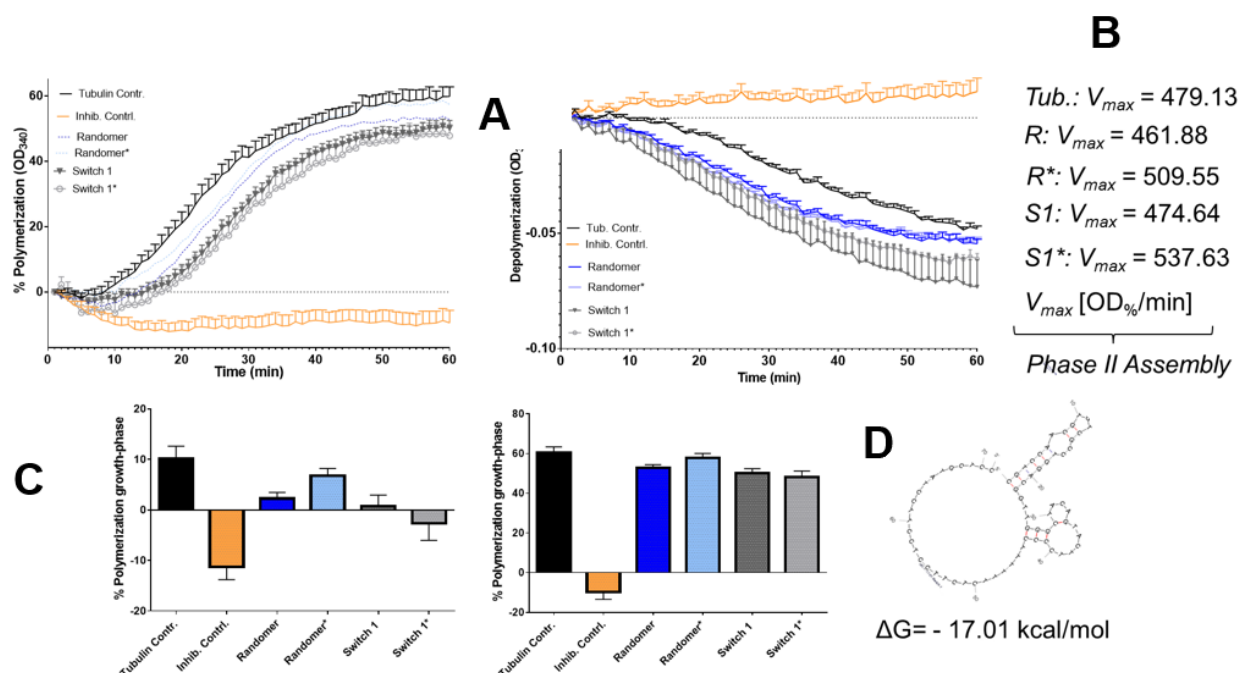


Figure 6.15: Reaction profile of structure-switch chimera version 1

A) Reaction curves for polymerization and depolymerization **B)** V_{max} of assembly **C)** MT polymer mass in phase II and III **D)** *in silico* predicted secondary structure (mfold) and enthalpy under assay folding conditions. Values represent the average of three independent experiments performed in triplicates and error bars express SEM. * means Arg was added at 100 μ M.

For chimera version 1 (Figure 6.15), the assembly (A) and disassembly (B) slopes show no significant change in activity between absence/presence of ligand. The V_{max} span between the chimera is minimally greater than the one between the randomers, but is within the error range of the assay. Again, comparative microtubule levels (O.D.) show no considerable difference between added and absent ligand to the switch. The 'induced' randomer has marginally higher levels (less inhibition), which provides a range of non-specific effects and general variations.

The same analysis was done for chimera 2 (Figure 6.16).

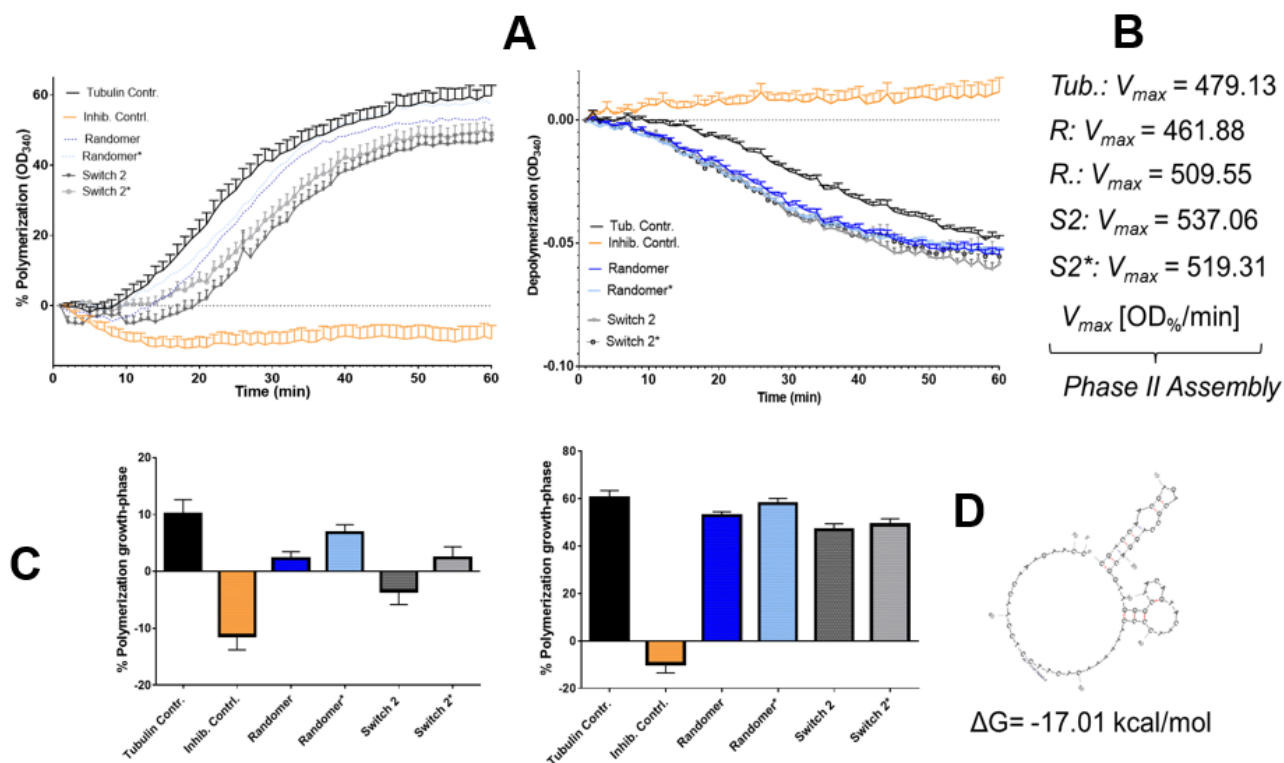


Figure 6.16: Reaction profile of structure-switch chimera version 2

A) Reaction curves for polymerization and depolymerization **B**) V_{max} of assembly **C**) MT polymer mass in phase II and III **D**) *in silico* predicted secondary structure (mfold) and enthalpy under assay folding conditions. Values represent the average of three independent experiments performed in triplicates and error bars express SEM. * means Arg was added at 100 μ M.

For switch 2 (Figure 6.16), no differences between chimera in absence and presence of ligand are observed for assembly (A) or disassembly (B) and changes in polymer levels remain very small and not significant (C) and (D).

The same analysis was done for chimera 3 (Figure 6.17).

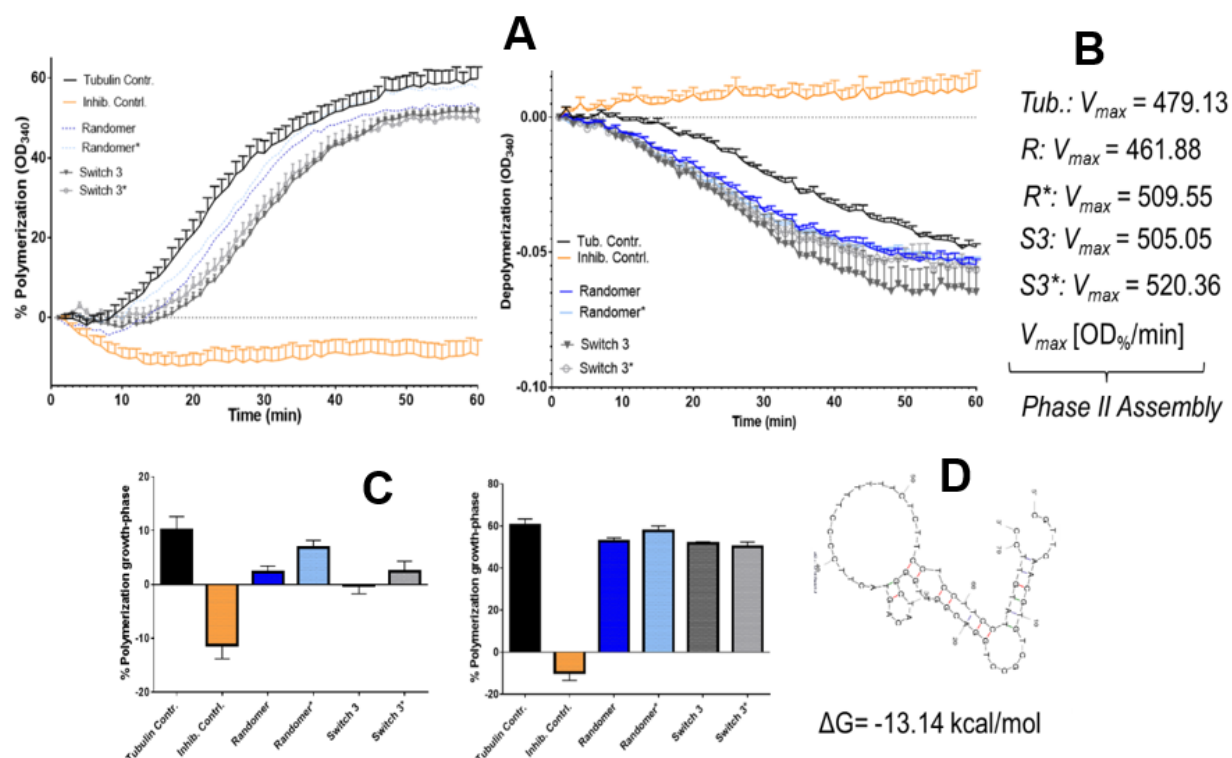


Figure 6.17: Reaction profile of structure-switch chimera version 3

A) Reaction curves for polymerization and depolymerization **B)** V_{max} of assembly **C)** MT polymer mass in phase II and III **D)** *in silico* predicted secondary structure (mfold) and enthalpy under assay folding conditions. Values represent the average of three independent experiments performed in triplicates and error bars express SEM. * means Arg was added at 100 μ M.

In Figure 6.17, chimera version 3 behaves very similarly to version 2. No differences are observed between the sample containing Arginine and the chimera control. V_{max} remain within the same range for both samples (B) and polymer levels do not vary greatly (C). At midpoint, slightly less polymer is formed for the randomer alone, than if supplemented with Arginine and the same is true for the chimera. It can be assumed this is a non-specific occurrence. At endpoint, levels match each other closely.

The same analysis was done for chimera 4 (Figure 6.18).

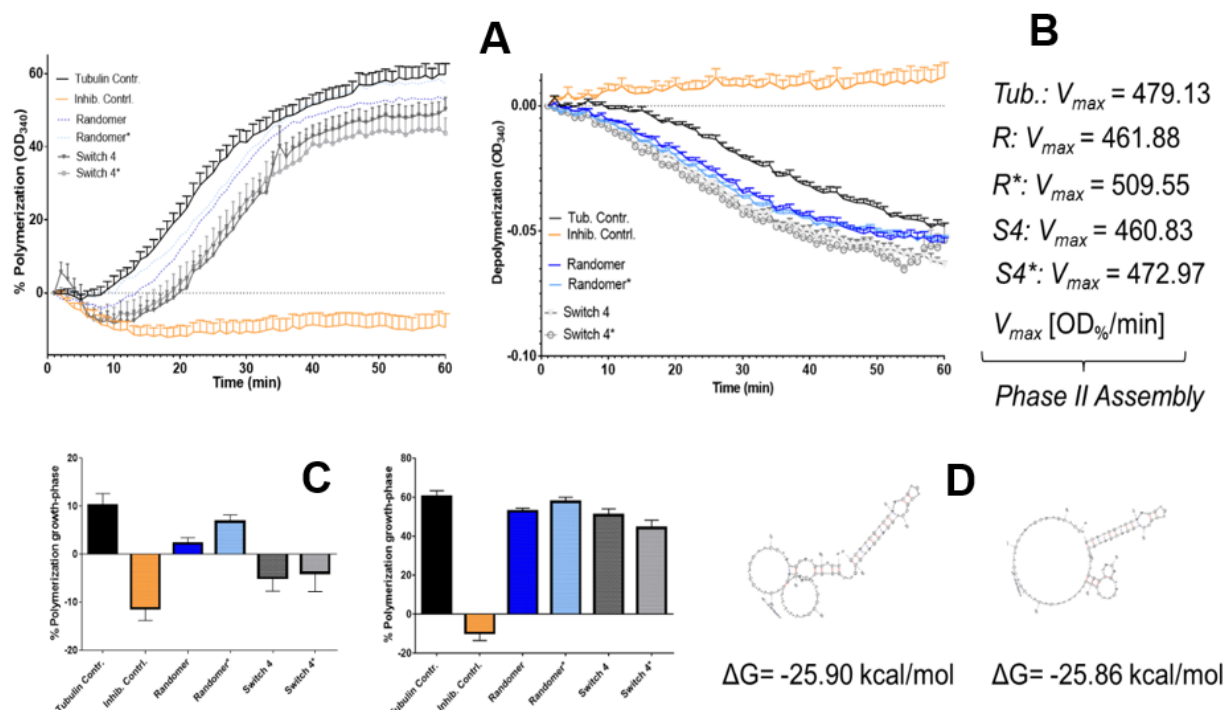


Figure 6.18: Reaction profile of structure-switch chimera version 4

A) Reaction curves for polymerization and depolymerization **B)** V_{max} of assembly **C)** MT polymer mass in phase II and III **D)** *in silico* predicted secondary structure (mfold) and enthalpy under assay folding conditions. Values represent the average of three independent experiments performed in triplicates and error bars express SEM. * means Arg was added at 100 μ M.

Similarly, results for chimera version 4 in Figure 6.18 do not suggest significant changes in activity upon addition of Arginine. Polymerization and depolymerization slopes do not differ greatly (A), V_{max} remains in the same range (B) and microtubule mass does not differ. Although the activity changes are statistically significant, given they are outside the range or error, this is not deemed significant scientifically, as that the randomer shows general variation in the same range.

6.2.2 Discussion summary

In summary, none of the switch versions exhibit any considerable changes in activity upon ligand addition and if so, effects are in the range of the randomer control and hence most likely not specific and part of sample and assay variations. This is a first version of a switch intended as a basic pilot study. For in-depth discussion, preliminary underlying features would require investigation, such as confirming ligand-induced conformational change for the chimera. In addition, more tightly-binding aptamers for both domains would be highly desirable to observe distinct signals.

6.2.3 Aptamer activity: antidotes as hybridization probes

Next, a different approach was pursued originating from the same motivation – controlling aptamer activity through hybridization. Here, an antisense ‘antidote’ was used to partially or fully hybridize to certain regions of the active aptamer and activity was compared before and after (Figure 6.19). Polymerization and depolymerization slopes were compared as well as polymer mass (O.D.) during growth-phase (25mins) and steady-state (60mins). Please see methods section 2.3.3 for more details.

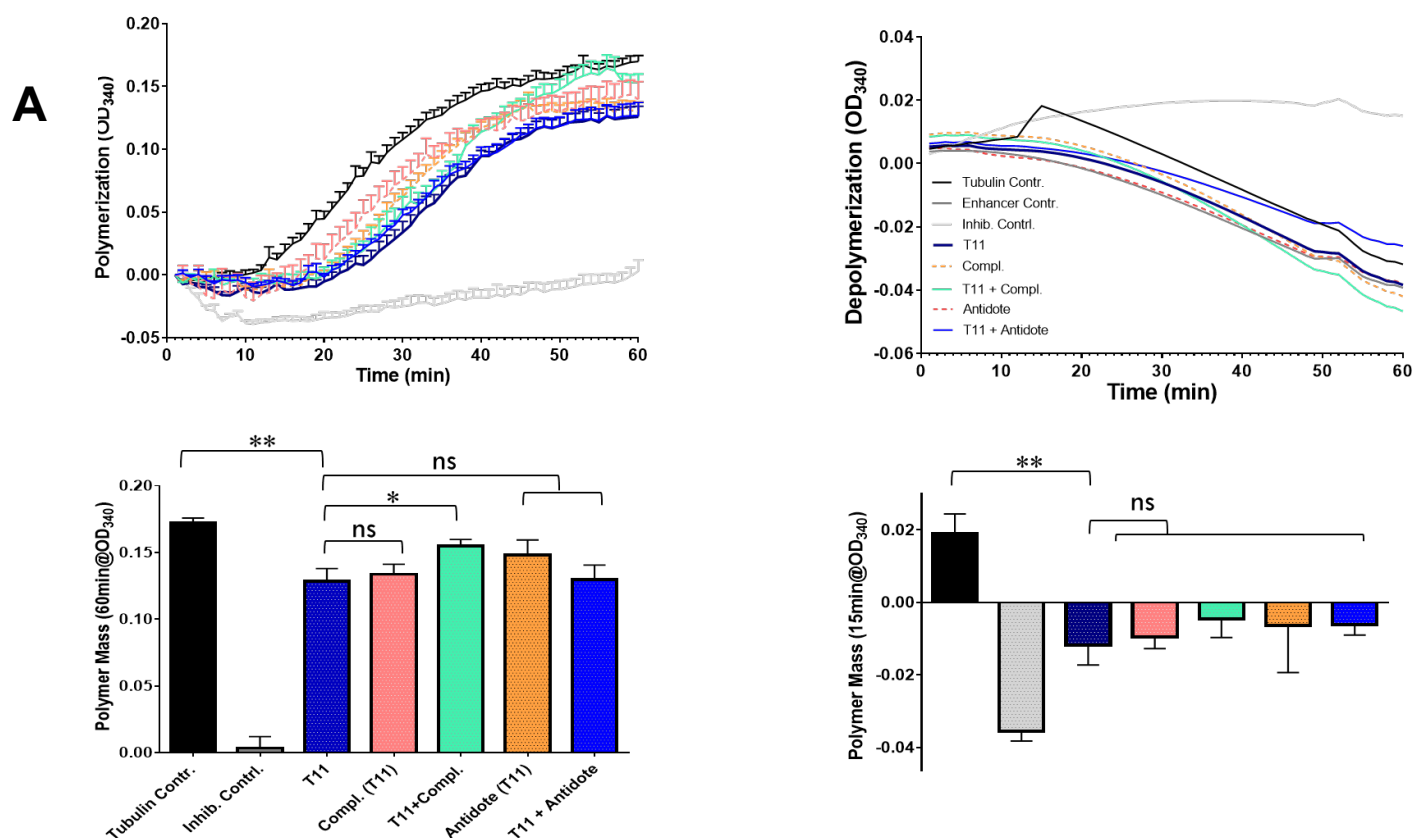


Figure 6.19: Reaction profile of T11 ‘silenced’ by antidote hybridization.

A) Polymerization curve from *in vitro* assay **B)** Depolymerization curve from *in vitro* assay **C)** Endpoint MT mass **D)** Midpoint MT mass. Values represent the average of three independent experiments performed in triplicates and error bars express SEM. Pvalues were calculated as one-tailed student-t-tests between samples and tubulin (non-parametric). Pvalues: *=0.05

Similarly, here a full and partial antisense antidote was used to modulate activity of an R50 oligo, who had shown (small) effects before, which is why R50 was selected for this test (Figure 6.20). This oligo was discovered in another assay series (not shown) and seemed promising.

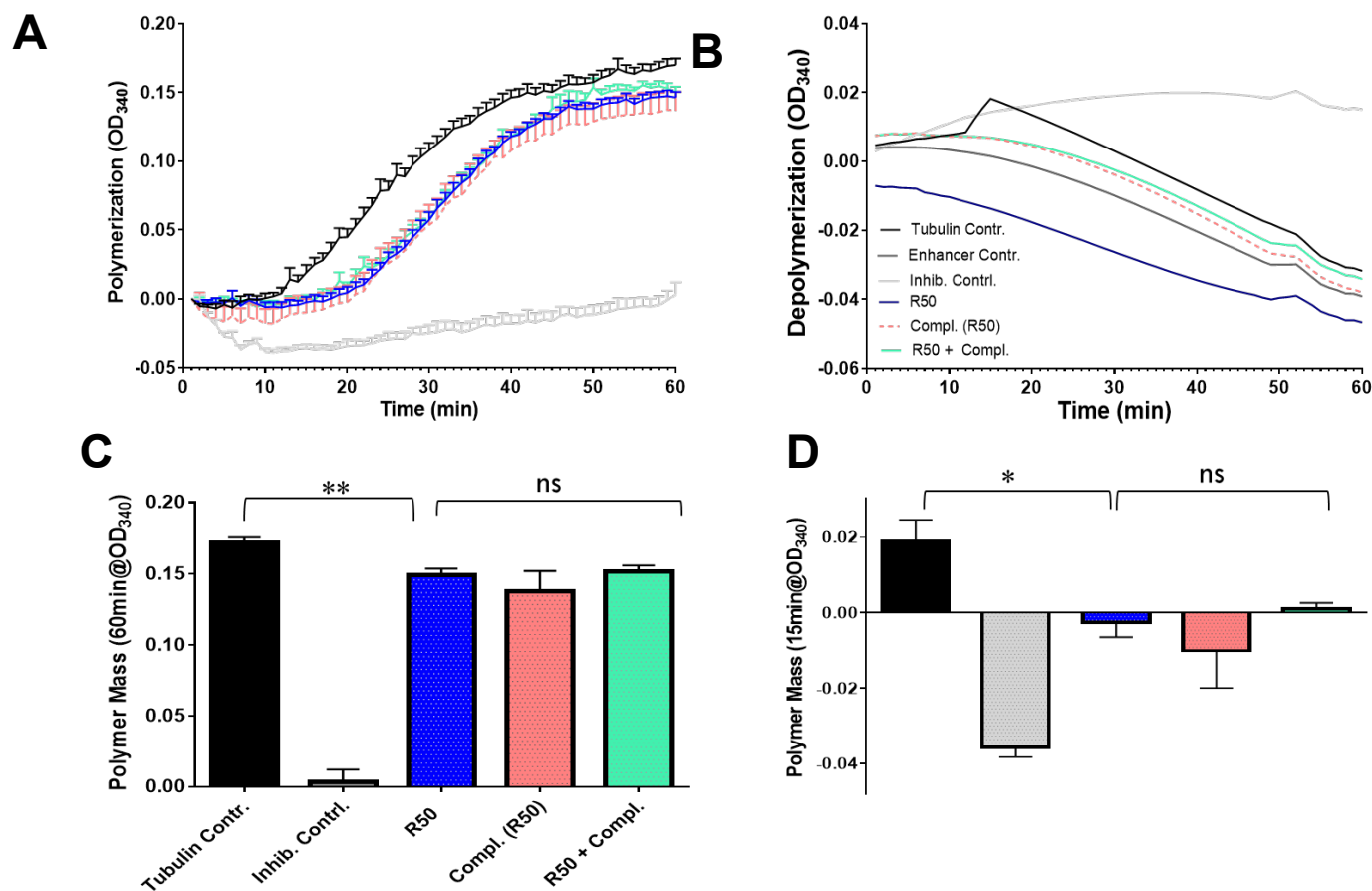


Figure 6.20: Reaction profile of R50 'silenced' by antidote hybridization.

A) Polymerization curve from *in vitro* assay **B)** Depolymerization curve from *in vitro* assay **C)** Endpoint MT mass **D)** Midpoint MT mass. Values represent the average of three independent experiments performed in triplicates and error bars express SEM. Pvalues were calculated as one-tailed student-t-tests between samples and tubulin (non-parametric). Pvalues: *=0.05

Figure 6.19 panel (A) with T11 and the partial motif antidote (T11 + antidote) shows no significant difference to T11 for assembly. For disassembly, the partially 'silenced' T11 (T11 + antidote) shows slightly reduced inhibition activity. But this may be within the error range of the assay. The fully 'silenced' T11 (T11+Compl.) T11+complement appears to decrease inhibition to a small extent, while the disassembly slope remains similar. Comparing polymer levels at reaction midpoint (D), the differences are not significant, while at endpoint (C), the fully silenced T11 shows slightly, but statistically significantly reduced inhibition activity (T11+complement).

For the second assay in Figure 6.20, no difference is observed in assembly between the R50 alone and with its motif antidote (R50 + antidote), nor with the full antidote (R50 + complement). For depolymerization, the R50 oligo alone appears to slightly enhance depolymerization, while the fully hybridized R50 (R50 + Compl.) no longer does. This suggests the fully 'silenced' R50 may have reduced activity on disassembly, but further studies would be required to confirm the effect with complementary methods.

6.2.4 Discussion summary

The antidote for T11 was designed to hybridize with only part of the aptamer in a predicted secondary structure motif seeking to inhibit the motifs activity through hybridization of the antidote. On the other hand, the complement was designed to hybridize with the entire sequence, with the expectations that the effect may be reversed or hindered independently of the particular motifs. The T11 motif antidote did not appear effective, which might indicate that the 'silenced' motif does not take part in binding and hence is not essential for activity. Different and/or longer antidotes could be used to further probe the relationship between different motifs and activity. The full-length hybridization seemed to slightly decrease inhibitory activity for polymerization (in the later parts). 'Silencing' the R50 with the full antidote (complement) appeared to reverse the effect of the oligo for the disassembly reaction, whereas no difference was observed for microtubule assembly.

As differences were only small and/or only came into play in parts of the reaction, they are problematic to distinguish from assay variations. Hence, results are not conclusive and further studies would be required to probe this concept more thoroughly, ideally with a well-understood oligo with strong effects.

6.3 Conclusions and outlook

Results were summarized after each section for the best overview and are discussed here.

6.3.1 Minimization of Tuda aptamers

This assay represented a first step towards creating (modestly) effective, but shorter sequences from the parental Tuda aptamer sequence (see chapter 4). The primer-binding regions (18nt each) of each aptamer were deleted to yield the minimized versions, the 'M-series'. Results varied - most aptamers exhibited decreased activity, some remained the same and one of them, M10, (ATGTATTACTAGGTCGTTGACGGAGAAGGGGGGTAGGTGCTTGGGTGTGTCTGTT TGGT),

emerged with significantly improved activity. This suggests, these flanking regions may have interfered with achieving the best target-binding conformation. Where activity was reduced, this suggests these nucleotides were essential for binding and non-essential where activity remained steady.

A next step might be to investigate the M10 oligo in greater detail for characterization. Further non-essential nucleobases may be removable to further shorten the aptamer and deletions across different regions could be probed. Ultimately, binding affinity measurements with the EMSA, ELONA and/or SPR method would yield further insights. UV/Vis spectra in presence and absence of the target might reveal differences in binding conformation and CD spectra could hint at the presence of non-canonical binding motifs (the G repeats might form a G-Quartet). If promising, more in-depth experiments like NMR could reveal the aptamer structure and yield valuable insights to develop more tubulin-binding aptamers in the future with better binding affinity.

6.3.2 The ‘aptamer clock’ for rapid prototyping

Nucleobase deletions can yield variants with altered conformations, which expose novel or altered binding motifs with potentially improved activity. A panel of ‘structure variants’ was created by targeted single base deletions from a parental sequence (T11) to yield altered (*in silico* predicted) binding motifs.

Some of the derivatives indeed demonstrated improved activity relative to the parental aptamer and in a few cases, even greatly so.

Interestingly, results highlighted the prominent role of the aptamer structure to achieve their effect on the target protein. It demonstrated that small changes, like a single base deletion could improve aptamer activity to a significant extent. New secondary structure features have been designed *in silico* which resulted in better activity *in vitro* in a unique and rapid workflow. For D10 and D11, for instance, the *in silico* structure remained practically the same, with the only difference of one nucleotide involved in a loop, yet both showed significantly improved effects.

A limitation to the assay is the fact that binding motifs are *in silico* predictions, but are best discussed as a total set of features. This is due to possible synergistic, antagonistic and cooperatively effects, which may not be linear upon ‘adding’ or eliminating these motifs. In the future, acquiring experimental structural information would facilitate further Follow-up studies could be performed to investigate the structure and the binding motifs in more detail with traditional affinity and structure characterization assays. This information could add tremendous value to this challenging, non-aptagenic target and could serve to identify a confirmed set of high-potential binding motifs. In future work, these motifs could be featured in patterned libraries for future *in vitro* selections.

6.3.3 Controlling activity: A ligand-inducible chimera and antidote hybridization

Two approaches to modulate aptamer activity on tubulin polymerization were explored in this section with hybridization: Firstly, prototypes of a structure-switching chimera to enable ligand-mediated activity (expectedly within a very small range). Secondly, antisense 'antidotes' to 'silence' binding motifs to control activity.

For the switch chimeras, no noteworthy differences in activity levels were observed between induced and uninduced samples. This could indicate that the post-selection chimera no longer binds tubulin as T1 does and might signify the same for the Arg domain (reported by other researchers, Nutiu and Li, 2005, Held *et al.*, 2006). In both cases, this would not result in any change, as without ligand-binding, the switch would not undergo the proposed structural change and without tubulin-binding, the effect would not be observable.

In future work, it would be invaluable to observe the dynamics of a structural change, determine a concentration gradient to trigger it and confirm that the chimeric form of T1 is still able to bind tubulin in the first place. This could be tested with ITC, CD or EMSA gels and provide a base for further work.

A limitation to the assay is that the suggested conformational change remains to be confirmed. This could be rectified with CD experiments as in the original publication, which confirmed a structural shift in presence of Arginine with CD experiments and gel shift experiments, (Harada and Frankel, 1995). Hence, it might be possible that ligand-binding does not cause significant structure-switching. The molar ratios of aptamer to arginine could be adjusted and concentrations could be increased to 1-10mM as in the original work (Harada and Frankel, 1995).

Another limitation is the rather weak affinity of T1 (45 μ M) and the small range of effects. The target-binding domain could be provided by a more effective tubulin-binding aptamer instead, for instance, clock derivatives D7, D10 or D11, which were designed post-selection and characterized in this thesis. The K_D of 2.5mM of the Arginine-binding aptamer is rather weak as well and the chimera domains were restricted to DNA-based sequences (the RNA aptamer is a more high-affinity binder). Generating high-affinity

aptamers against tubulin would, as attempted and discussed, offer valuable and versatile uses, such as further work on other such chimeras.

It might also make for a 'cleaner' experimental system to use a ligand-binding domain such as a streptavidin-binding DNA aptamer (Wang *et al.*, 2009) or theophylline, (Li *et al.*, 2009) because Arginine is abundant in proteins and other ligands are more orthogonal.

Antidote hybridization assays aimed at probing antidote-mediated control of aptamer activity and revealing critical target-binding motifs by 'silencing' them. The experimental design was geared preferentially towards the latter: the method was intended to enable rapid, iterative series *in silico* design of 'silencing' antidote and *in vitro* testing to quickly track down the features involved in binding tubulin. As oligos are cost-effective (especially short ones) and rapid to synthesize and the screening assay is highly efficient. Projects could be carried out within 48-72 hours cost-and time-effectively. This would be an exciting and original future project and would offer a novel workflow to elucidate aptamer-protein interactions for the tubulin target.

To summarize results, for T11, complete antisense 'silencing' appeared to slightly decrease aptamer-mediated inhibition in disassembly. Meanwhile, the partial binding feature antidote did not show any effects. This could suggest that full-length hybridization in this case might offer the most effective inhibition. It might be the predicted binding motif did either not exist or is not involved in binding or might act in concert with another binding motif. It might be that several, partial antidote might be more effective. The assay could in the future be repeated with more effective aptamer binders for more distinct effects and could be performed with an aptamer where structure and binding motifs are confirmed for the most conclusive results.

For the R50 oligo, no significant effects were observed, except the full hybridization probe appeared to reduce the effect of the R50 oligo on disassembly, but does not appear significant or could be noise. Therefore, further work would required to gain more knowledge. '

7

Concluding remarks and future prospects

7.1 Conclusions

The work presented in this thesis explored new directions in cancer drug discovery with programmable, synthetic bio-polymers in order to overcome limitations of traditional therapeutics, including antibodies. Motivation for this work were the Paclitaxel supply crisis, its astronomical price and severe adverse effects. They stand in stark contrast to the versatile therapeutic potential and low cost of nucleic acids. The thesis therefore aimed at further exploring the intersection of nucleic acids and therapeutics with a yet under-explored selection target, tubulin.

The key projects ranged from high-throughput assay and analysis development for drug-like oligos and screening of tubulin-binding aptamers for the first time. This was followed by design and fabrication of a novel miniature-microtubule target, *in vitro* selection and post-selection optimization strategies, which yielded aptamers with improved inhibitory effects.

Chapter one gave a brief overview on how oncology can benefit from innovations in the medical field in the coming years and reviewed the current state of research on cancer drugs and notable mechanisms of action. It described how anti-mitotic compounds interfere with tubulin dynamics, illustrated the pressing need for new therapeutics and the severe drawbacks of currently used ones. Discussion on the rising of aptamer therapeutics revealed how innovative applications of nucleic acids benefit diagnostics and therapeutics in oncology. This included aptamer-nanoparticle-drug conjugates, to targeted delivery and aptamers as inhibitors of therapeutic proteins, which showcased the promise of using these versatile biopolymers, since, as the molecules of life themselves, they can overcome limitations of antibodies and chemotherapeutics: they offer advantages like non- or low immunogenicity, water solubility and rapid, economical synthesis. It concluded with exciting new developments in the aptamer field, which could benefit this particular topic in the future and proposes how programmable oligo therapeutics could serve as therapeutic protein inhibitors, for instance for the drug target tubulin.

The third chapter applied tubulin-binding aptamers to high-throughput cancer drug discovery to enable activity-based characterization of functional nucleic acids for the first time.

Firstly, it proposed the use of randomer and clinical controls to provide a scale to assess effects. The former served to objectively distinguish non-specific effects from moderate-to weak specific effects and provided a widely applicable benchmark for aptamer-screening experiments. Interesting to note was that even random nucleic acids were found to interact with the reaction, even though the extent was minimal. The latter helped to establish the order of magnitude of activity required for aptamers to reach relevance in a clinical context.

Secondly, chapter four also proposed a comprehensive analytical framework to capture aptamer effects on tubulin dynamics. Lastly, a panel of 16 aptamers of previously published work (Fukusaki *et al.*, 2001) were screened and characterized for the first time by applying established and newly devised analysis methods. It revealed small, but significant inhibitory effects of some of the aptamers. As they possess only moderate to weak binding affinities (where known), it demonstrated the need for strong binding affinities as a prerequisite to impact tubulin polymerization to a greater extent.

Three T-rich aptamers (T1-3), emerged as 'hit' candidates through statistically significant (but modest) inhibition on the assembly kinetics and were identified for follow-up investigation. In comparison with clinical controls, the screening revealed that the aptamers are not strong enough inhibitors at 10 μ M concentration. Further, because the effects fall within a narrow range, inter-assay variability (even if a below average CV was achieved 12% vs 16%), this poses limits to quantitative analysis within this effect range.

The chapter demonstrated sensitivity and robustness of the screen, amenability of oligos to HTS and critiqued the potential and limitations of the applied methods. A few of the analysis methods proved valuable for comparative analysis, such as polymer levels, others remained rather hypothetical, such as nucleus size estimation. Kinetic parameters are derived best from dose-response curves, and therefore served as relative assessment within each assay series only. These insights could greatly enhance future work in this area, for instance with other cancer-relevant aptamers, like GRO's.

The striking gap between the evident relevance of tubulin as a key cellular protein and the lack of publication history suggests that *in vitro* selection against this target may not be straight-forward. Therefore, a new selection strategy based on optimized molecular architecture were explored. Chapter five presented the successful fabrication of a novel target, a GPC-polymerized miniature microtubule, which was executed from an original 3-step design for the molecular macrostructure. It was derived from literature research on Paclitaxel's polymer-binding mechanism of action, literature to support the hypothesis and own reasoning. These results demonstrated how the molecular architecture of a dynamic protein target can be engineered at the level of polymerization. Results have also shown the surprisingly different morphology of GPC-polymer compared to TX-polymer and raised the question of how representative the GPC form would be for a selection - the taxoid site only emerges on precisely structured morphologies, as discussed in chapter introduction).

But having these two morphologies at disposal would enable new, exciting selection strategies, as the one proposed in the chapters' future outlook. For instance, a novel NGS-CE toggle selection could unlock epitope-specific aptamer sequence clusters within 2-5 rounds of selection by use of such a target in alternation with standard (TX-stabilized) MT polymer. It discussed how harnessing advances in high-throughput methods and automation could enable a multitude of insightful studies by tackling sequence clusters and epitopes. These findings could be valuable for selection against other dynamic protein targets, like actin, myosin and receptor-dimers or multimers, as they occur in the ErbB-receptor family, linked to breast carcinoma. The CE/NGS sequence cluster approach could be useful to any selection which explores specific binding sites. Instead of fabricating two distinct target forms, the chapter discussed how the same strategy can be applied to varied targets through the use antibodies or high-affinity binders instead, which can be applied to occupy or release specific binding sites.

The fifth chapter revealed the challenges to enrich aptamers against tubulin with a well-established, conventional methods. It presented use of nitrocellulose filters for partition and lambda exonuclease digest for ssDNA generation and highlighted the challenge to select aptamers against tubulin with these methods. Test assays were carried out to inform future library design with regards to length and nucleobase composition of the

random region. This could save other researchers valuable time (and headaches) and provide a base of insights to draw from for future attempts and in order to accelerate this research.

The sixth chapter covered a number of post-selection optimizations and the subsequent drug discovery screening to characterize the newly developed sequences. This work provided a host of original post-selection optimization strategies as a proofs of concept: This included an aptamer ‘clock’ with a predicted central stem-loop template as rapid prototyping platform and an early version of a proposed ligand-induced structure-switching chimera. Sequence motifs were ‘muted’ by hybridization with antisense ‘antidote’ fragments to explore if tubulin-inhibiting activity could be controlled similarly to work previously described (Rusconi *et al.*, 2002, Rusconi *et al.*, 2004).

Several among them showed improved effects and were identified for follow-up studies, which could reveal critical binding motifs and further insights into nucleic acid-protein interactions. These results demonstrated that post-selection optimization is a valuable tool to improve aptamer properties. The chapter presented results from activity-based kinetic inhibitor screening and the demonstrated the customizable versatility of the analysis developed in chapter four. This allowed to shed light onto particular features of each experiment, depending on its rationale. The discussion included the limits of *in vitro* screening as a sole method to infer any information on structural or binding motif features and proposed respective follow-up assays. This could prove valuable in particular for aptamers to non-aptagenic targets, as it offers a time-effective way for optimization and revealing essential information without the need of a new *in vitro* selection. The motifs which emerged as potential (modest) inhibitors lend themselves to use in partially patterned libraries for future *in vitro* selections and to further development, as described in the chapters’ outlook. This could help innovate the still under-explored intersection of therapeutic nucleic acids and a key cancer drug target.

In summary, this thesis characterized a population of tubulin-binding aptamers by drug discovery screening *in vitro* and identified three ‘hit’ candidates for follow-up studies. A number of post-selection optimization strategies were pursued, like rapid prototyping and silencing binding motifs through hybridization. Some of these truncated sequences

showed significantly improved effects and would be excited to investigate further with complementary methods. It demonstrated that nucleic acid ligands are amenable to pharmaceutical drug discovery screening. This allows assessment of activity directly on the target, while providing analytical tools and assay design to maximize meaningful insights gained from a single experiment – to a certain extent. The work further demonstrated challenges and limitations of traditional nitrocellulose filter selection for the tubulin target, introduced a novel macrostructure-optimized miniature polymer as selection target and discussed future studies and applications comprehensively.

For future work, these findings could be advanced into varied directions, like structural and binding studies of the hit candidate aptamers and characterization of high-potential binding motifs, new selection approaches and use of patterned libraries and further post-selection development. This would shed much needed additional light into nucleic acid-protein interactions, especially for a selection target surrounded by only a vanishingly thin body of research. Studies involving non-aptagenic targets in particular may benefit from innovation in selection methodologies, such as CE, NGS and automation of routine steps. As the field progresses, a host of promising new targets for cancer research and therapy are likely to emerge, which could be probed for *in vitro* selection. Targeting aptamers for cancer-cell specific delivery could be pursued as a complementary approach to developing an active compound from scratch, as the former has brought successes to build upon (Liu *et al.*, 2012, Yu *et al.*, 2016) and benefits from a dynamic research field.

In the medium-term future, using a more established target for proof of concept work, like the anti-nucleolin aptamer, which destabilizes Bcl-2 mRNA (Girvan *et al.*, 2006, Rosenberg *et al.*, 2014,) or aptamers binding the HER2 receptor, which is overexpressed on HER2 breast cancer cells (Mahlknecht *et al.*, 2013, Gijs *et al.*, 2016, Moosavian *et al.*, 2015) could prove productive. Both targets have yielded promising aptamers from *in vitro* selection and have sparked intense research activity.

Appendix

Engineering synthetic aptamer switches for next-gen cancer therapeutics

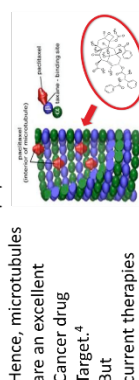
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Introduction

Cancer is an alarming global problem. Major cancer drugs, such as taxol (Paclitaxel) bind β -tubulin¹ and interrupt the mitotic spindle dynamics by hyperstabilizing microtubules. This mitotic block induces apoptosis.²



Hence, microtubules are an excellent cancer drug target.⁴ But current therapies lack cell specificity and attack healthy cells indiscriminately, causing severe adverse effects. New, effective and safe therapeutic agents are urgently needed to overcome this limitation.

Aptamers are single-stranded DNA or RNA oligonucleotides that can bind a rich variety of cellular targets with high specificity and affinity. These 'chemical antibodies' are a rising class of promising

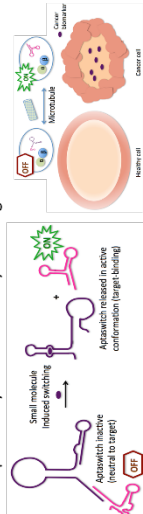
therapeutics³ recently, the first aptamer drugs were released to the market and more entered late-stage clinical trials.⁵

Aim

Building a anti-mitotic molecule that is activated by cancer specific signals for smart cancer therapeutics: It is only binding tubulin and killing cells, when a cancer biomarker is present (ON) and is otherwise switched OFF.

Experimental Approach

The principle is illustrated below. An antimitotic aptamer⁷ that can be switched on by small molecules and biomarkers would make therapies safer and more effective, because only cancer cells specifically are attacked by the drug.

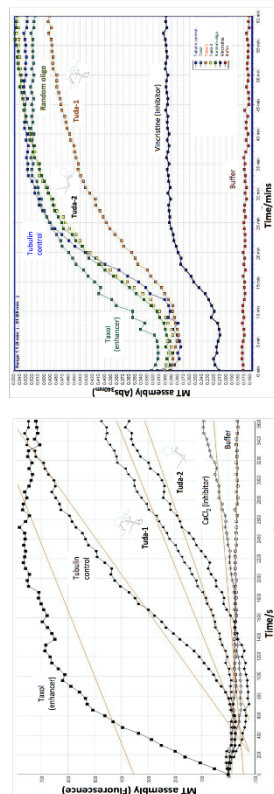


Proof of principle: Building an antimitotic aptamer switchable by small molecules.

Methods

In vitro tubulin polymerization assays were carried out according to manufacturer protocols for absorbance-based (OD₃₄₀) and fluorescence-based assays (λ_{ex} =340-360nm, λ_{em} =410-460nm) with kits from Cytoskeleton, Inc, USA. ssDNA aptamers were synthesized by Integrated DNA Technologies, UK.

Results



A) Fluorescence-based tubulin polymerization assay
 Decrease of polymerization level with both aptamers

B) Absorbance-based tubulin polymerization assay
 Decrease of polymerization level only for Tuda-1, not Tuda-2

Citations

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- 6) SELEX for Tubulin Affords Specific T-Rich DNA Aptamers
 Ei-ichiro Fukusaki et al *Bioorganic & Medicinal Chemistry Letters* **11** (2001) 2927-2930

Discussion

In the first assay (A), the apparent inhibition of Tuda-2 is possibly a combination of fluorescence quenching to some extent and inhibition, to another. **Quenching effects** were observed and quantified (data not shown) and are a common result of interaction between test compounds and fluorophore.

The results for Tuda-2 could not be replicated in the complementary assay B) so far. Further testing is required to...

For Tuda-1, which has a roughly half the quenching effect of Tuda-1, The decrease of polymerization levels in assay A is likely a combination of both inhibition and quenching and can not be explained by the latter alone. **The inhibitory effect to a significant extent was confirmed in the second assay (B).**

But a limitation here is the potential interaction between test compounds and glycerol, which may play a role in the effects observed. Further investigations are required for additional insights. An open question to address is, whether aptamer binding to tubulin affects polymerization dynamics at all and to what extent, as the binding site of the aptamers on tubulin remains yet to be determined.

Future Work

- Optimization of polymerization assays
- Testing of ON-OFF SWITCH designs
- Cancer biomarkers and bio-stabilization

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Identification of tubulin-binding aptamers with small, but significant inhibitory effects on tubulin assembly

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Tubulin dynamics are a major cancer target

The most effective cancer therapeutics interfere with polymerization of tubulin, a major oncology target,¹ but lack specificity and cause severe adverse effects. Aptamers are rising therapeutics,² often antagonists of protein function, such as for thrombin and blood coagulation,^{3,4} VEGF pathway inhibition,⁵ or cancer-relevant applications.⁶ Tubulin-binding aptamers (K_d (T1) 45 μ M, K_d (T2) 19.4 μ M) have previously been selected *in vitro*.⁷ Here, the inhibitory potential of an aptamer panel was assayed for the first time.

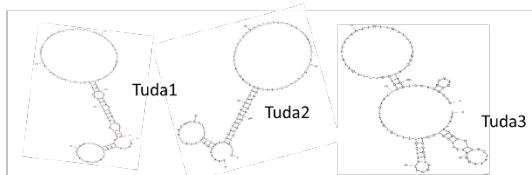


Fig. 1: Predicted secondary structures.⁸ The T-rich aptamers ³ from a panel of 16 were the most promising ones to inhibit tubulin assembly.

Dynamic stages of tubulin

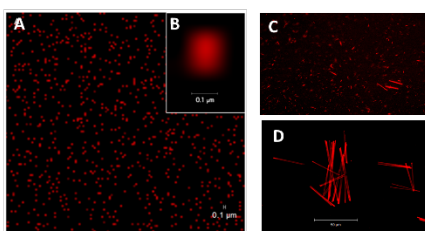


Fig. 2: Imaging tubulin in different dynamic states. Porcine brain tubulin (X-Rhodamine-labelled, from Cytoskeleton Inc.) was polymerized *in vitro* and imaged (Zeiss LSM 780). A, B) microseeds (Phase I) C) elongated seed (Phase II) D) MT (Microtubule) polymer (phase III)

Tubulin polymerizes in distinct stages⁹ and inhibitors interfere with these dynamics.¹⁰

Nucleation time more than doubles

Reaction checkpoints	Tub.	Rand.1	Rand.2	T1	T2	T3
1/2 time (min)	23	26	26	30	30	32
Ratio (1/2-time)	1.0	1.1	1.1	1.3	1.3	1.4
Nucleation (min)	8	11	14	14	19	19
Ratio (Nucleation)	1.0	1.4	1.8	1.8	2.4	2.4

Table 1: Kinetic reaction parameters of tubulin assembly. Obtained from O.D.-based *in vitro* polymerization.

Tudas 1-3 inhibited nucleation and half-time more effectively than the randomers. Half-time is the time it takes to polymerize half the maximal amount of available tubulin subunits.⁹

Overall effects on tubulin assembly

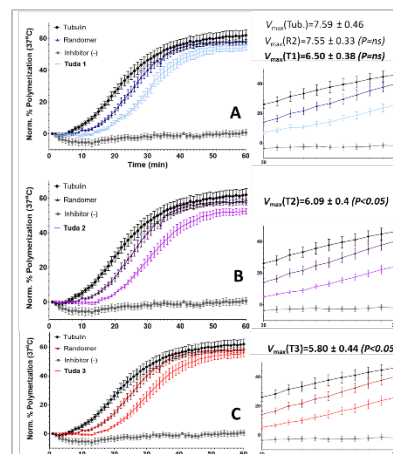


Fig. 3: Tubulin polymerization *in vitro*. Overall reaction (37°C) of A) Tuda1 B) Tuda2 and C) Tuda3 with V_{max} (n mol/min)

The aptamers appear to inhibit overall reaction dynamics to only a small extent, but beyond the randomer. The effect is strongest in phase I, clearly visible in phase II, but steady-state MT levels remain very close to tubulin.

Growth-phase impacted, but not steady-state

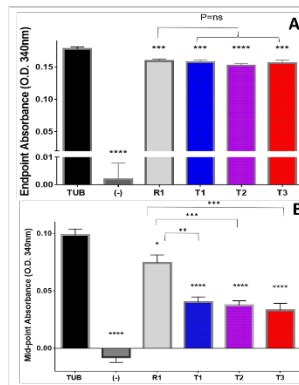


Fig. 4: MT Polymer levels. O.D. of Tuda1-3 at A) 60min (steady-state) and B) 25min (growth)

Steady-state MT levels (A) decrease with aptamers compared to tubulin alone (statistically significant), but the extent is only very minimal (see Fig. 3) and does not differ from the randomers (only one shown).

Growth-phase kinetics seem visibly (Fig.2) more affected. Aptamers decrease MT polymer mass further than the randomers.

Unpaired t-tests vs tubulin and vs randomer: * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$ **** $P < 0.0001$. See for a good critical discussion on using P values.¹¹

Discussion and future directions

T-rich aptamers emerged from the evaluated panel (not shown) and appear to inhibit the reaction stages to varying degrees. Given the only moderate binding affinities of Tuda 1 and 2 (the others unknown), only small effects are expected. However, these results seem encouraging to pursue development of more tightly binding aptamers to achieve stronger inhibition of tubulin.

Future studies will aim at measuring binding affinities and investigate potential presence of non-canonical secondary structure motifs (such as i-motifs). Insights might be valuable for future selection experiments.

Acknowledgements

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- Baker, M. *Nature* **531** 151 (2016)



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The aptamer clock: a rapid prototyping template for post-selection optimization of tubulin-binding aptamers

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香港大學
THE UNIVERSITY OF HONG KONG

The aptamer clock for optimizing tubulin inhibitors

The aptamer clock, in combination with HTS, is a novel approach for post-selection optimization of aptamers. A previously selected aptamer against tubulin,⁴ a prominent cancer drug target,² was optimized through rational truncations.^{3,5} Mutants were evaluated *in vitro* with tubulin assembly inhibition assays.⁶

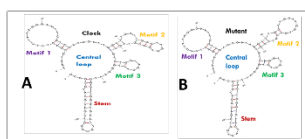


Fig. 1: Clock and mutant. From the parental clock sequence (A), a deletion mutant (B) is created to bear altered motifs.

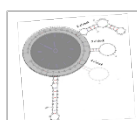


Fig. 2: The aptamer clock. Re-arranging motifs from 2 o'clock to 4 o'clock for rapid optimization of aptamer tubulin-inhibitors

Rapid prototyping of derivatives with novel features

Different truncation strategies were employed, based on secondary structure predictions.⁷ Some deletions in the motif 2 region resulted in completely altered structures, shifted motifs 'around the clock', or were barely noticeable (M10).

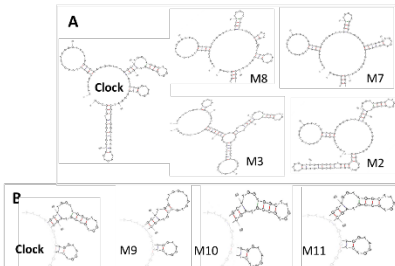


Fig. 3: The mutant groups 1 (A) and 2 (B).

Clock mutants improve nucleation inhibition

Half-time is the time, when half the total amount of tubulin is polymerized.⁸ This and other reaction parameters are commonly used to characterize tubulin inhibitors.⁶

Reaction Feature	Tub.	Parent.	M2	M3	M7	M8	M9	M10	M11
1/2 time (min)	29	28	33	33	35	31	32	35	35
% Inh. (1/2 time)	0	3.4	13.8	13.8	20.7	6.9	10.3	20.7	20.7
Nucleation (min)	12	18	21	21	23	22	20	24	26
% Inh. (Nucleation)	0	50	75	75	108.3	83.3	66.7	100	116.7

Table 1: Kinetic reaction parameters of tubulin assembly *in vitro*.

The mutants inhibit both parameters compared to the clock benchmark (parental). The stronger nucleation inhibition of mutants (first 0-30min) seems clearly visible on the overall reaction.

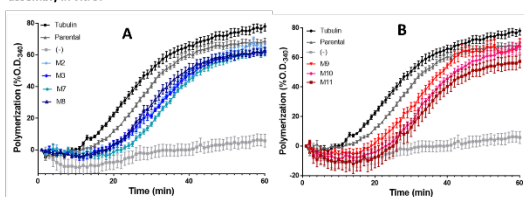


Fig. 4: Tubulin polymerization *in vitro*. Overall reaction (37°C) measured in O.D. units of mutant group 1 (A) and group 2 (B). Absorbance represents level of MT (microtubule) polymer.

The parental clock sequence appears to inhibit the reaction to a small extent. The mutants further enhance the effect during nucleation and growth, while endpoint MT levels remain similar to tubulin.

- Fukusaki, E. *et al.* *Bioorg. Med. Chem. Lett* **22** 11 2927-2930 (2001)
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- Rusconi, C. P. *et al.* *Nature* **419**, 90-94 (2002)
- Desai, A. & Mitchinson T. J. *Annu. Rev. Cell Dev. Biol.*
- Zuker, M. *Nucl. Acids Res* **31** 13 3406-3415 (2003)
- Keates, R. A. & Mason, G. B. *Can J Biochem* **59** 5 361-70 (1981)
- Baker, M. *Nature* **531** 151 (2016)

Significantly improved kinetic inhibition and V_{max}

The mutants M2, M3, M7 and M10 significantly improve assembly inhibition compared to both the clock (parental) and tubulin. M7 reaches 69.0±3.7% of V_{max} compared to the clock benchmark (94.4±2.2%), but far from clinically relevant ranges (inhibitor).

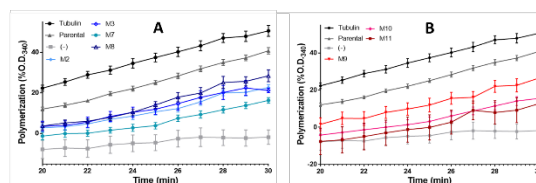


Fig. 5: Tubulin polymerization *in vitro*. Growth phase from 20-30min (at 37°C) of mutant group 1 (A) and group 2 (B) was measured in absorbance units compared to controls.

Samples	Tub.	Parent.	M2 **	M3 ***	M7 **	M8 *	M9	M10 *	M11
V_{max} (mOD/min)	7.3±0.2	6.9±0.2	5.8±0.2	5.5±0.2	5.0±0.3	5.9±0.3	6.3±0.4	5.9±0.4	5.7±0.9
% V_{max}	100	94.4±2.2	78.9±3.2	75.3±2.9	69.0±3.7	80.9±4.5	86.4±4.9	81.0±5.0	77.7±12.1

Table 2: V_{max} of growth phase. Kinetics were measured *in vitro* from growth-phase with linear regression. (Un-paired t-test vs parental sequence *P<0.05 **P<0.01 ***P<0.001)

Significantly improved growth-phase inhibition

Endpoint polymer levels (A) seem to match tubulin closely. Only M3, M7 and M8 significantly decrease MT mass, compared to the clock. In growth phase, however, the optimized reaction features clearly emerge for all mutants (B).

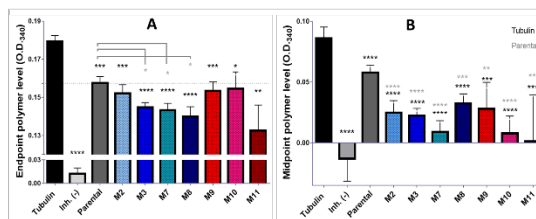


Fig. 6: MT Polymer levels. Microtubule (MT) levels were measured in O.D. units at (A) 60min (steady-state) and (B) 25min (growth), (P values denominated, see Fig.2). P values to be enjoyed responsibly.¹¹

Interestingly, the M10 predicted structure differs from the clock only minimally, (see Fig.2) yet the difference seems noteworthy. Results appear to correlate well with the change in V_{max} . Observing the reaction from phase I, II and III further suggests that the inhibitory effects might not persist. This would be required for developments towards the clinic.

Discussion

This study shows some promising improvements of tubulin inhibition parameters by mutants derived from a rapid prototyping aptamer clock. The effects appear strongest during nucleation, still significant during growth-phase, but remain only very small at end of the reaction. Post-selection optimizations are typically expected to offer only a moderate range of improvements. Follow-up studies on the mutants could shed additional light on these interactions. This points to the tremendous ongoing and need for tightly binding aptamers, as prerequisite for achieving strong effects and certainly for clinically relevant development.

The future

Future work will focus on structural and binding affinity measurements of the clock and a few mutants. This could yield unprecedented insights into aptamer-tubulin interactions.

Acknowledgements

Thanks is due to the International Office of Imperial College London for funding the Joint PhD scholarship, Prof. Jiaodong Huang for funding and discussion, Dr. Karen Polizzi and discussion and Dr. Travis Bayer for funding and the initial idea.

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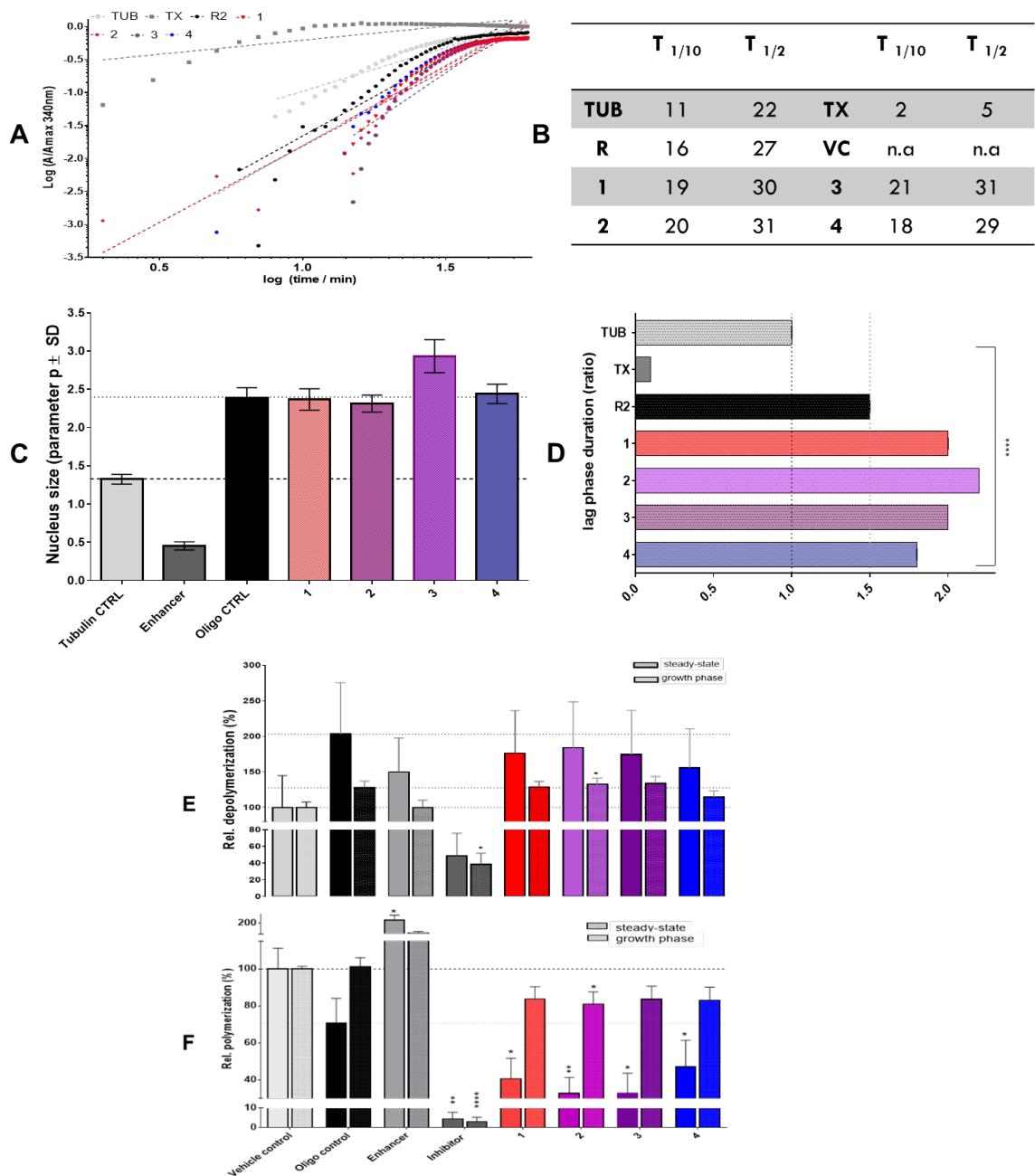
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Chapter 3 Appendix

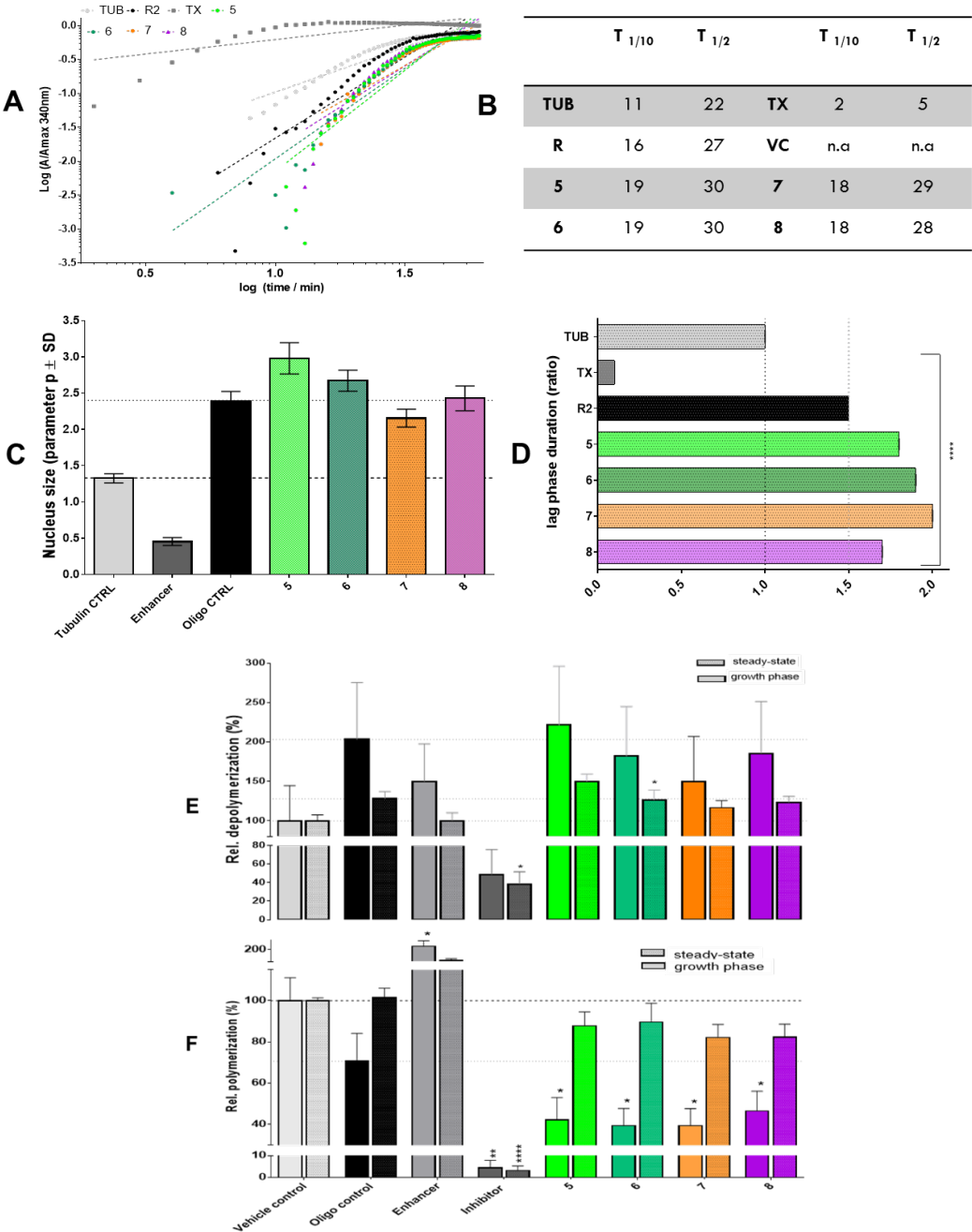
(Appendix) Table 3.5: Tuda aptamers

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T2	TAATACGACTCACTATAGGGAATTCGTCGACGGATGCGTTGGGCTACAGTACTTCCCCTTT TTTCTCTTCCTCCTTCTATCGCTCTCCCTACAGGTCGACGCATGCGCCG
T3	TAATACGACTCACTATAGGGAATTCGTCGACGGATAGGCTATTGTTTTGTGTTTCTTTTC CTGGTTTTGTTATTTTTTGTGTTGTTTTTGGCTACAGGTCGACGCATGCGCCG
T4	TAATACGACTCACTATAGGGAATTCGTCGACGGATGAATTCGTTTGTGTGCGGAGGTGG TTGTTTGTGTTTTTGTGTTCTTTGTTTGTGTTTGTCTACAGGTCGACGCATGCGCCG
T5	TAATACGACTCACTATAGGGAATTCGTCGACGGATAGATATGGTTTTGTTGTGCGTTATGT TGTGTTTTTGGGGTCTCTTTTTGGGTGTTTTGTCTACAGGTCGACGCATGCGCCG
T6	TAATACGACTCACTATAGGGAATTCGTCGACGGATTTAAGTTTGCTGTTAATTGGTGCGTA TCTCTGTAGTAGAGTCAGTTAGCGGTTTTGTGGTCTACAGGTCGACGCATGCGCCG
T7	TAATACGACTCACTATAGGGAATTCGTCGACGGATACGTTCCGCTGGTATAGTGAGTGAC CTTGGTCTGGTGGATGCGTTTATTCGTGGTCCTCCTACAGGTCGACGCATGCGCCG
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T9	TAATACGACTCACTATAGGGAATTCGTCGACGGATTTGGTGGGTTGTAGGCAGGCGTGG GCACTGTTTGAGAAGGACGTGTTTGGTCTATCTACAGGTCGACGCATGCGCCG
T10	TAATACGACTCACTATAGGGAATTCGTCGACGGATATGTATTACTAGGTGCTTGACGGAG AAGGGGGGTAGGTGCTTGGGTGTGTCTGTTTGGTCTACAGGTCGACGCATGCGCCG
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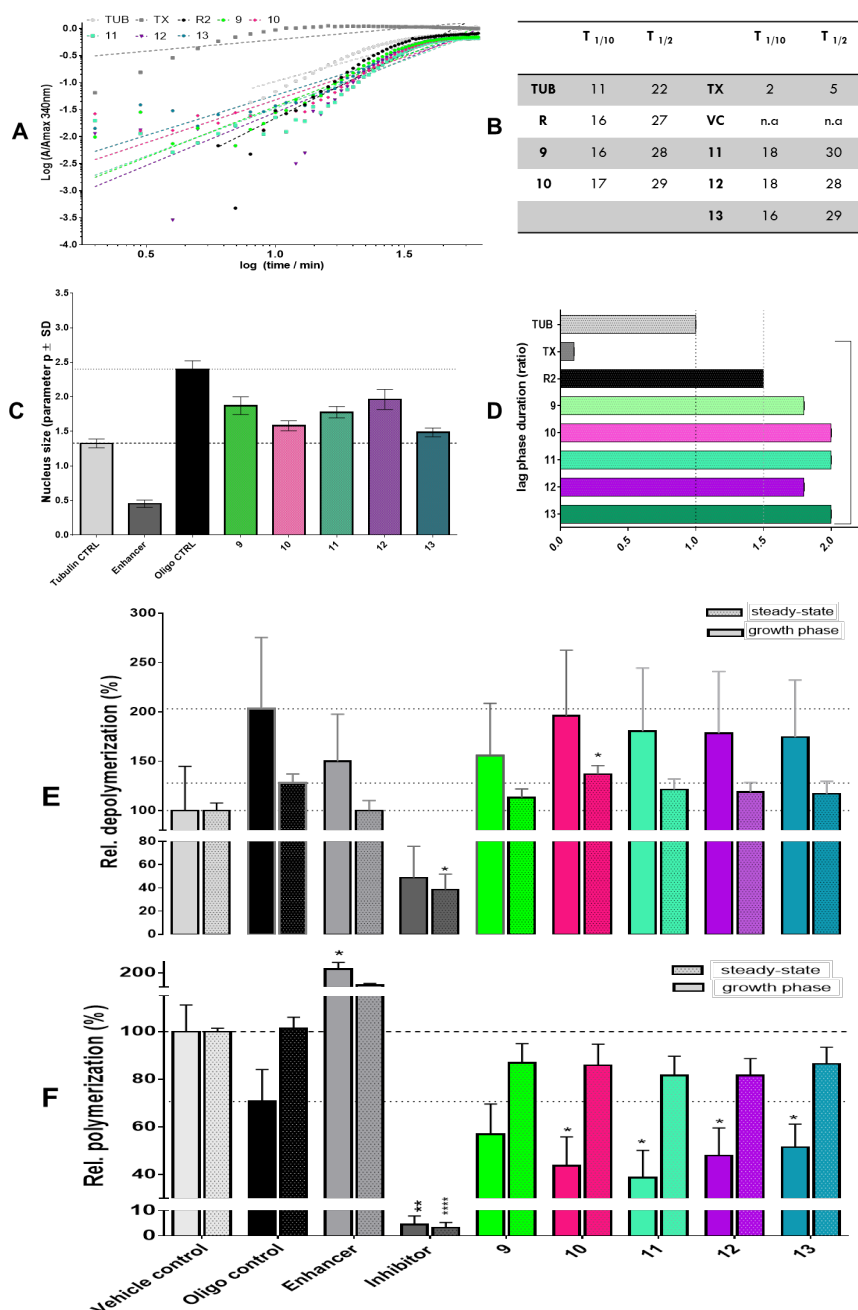
Cluster analysis: T-rich Tuda aptamers

A) Nucleus size estimation across reaction. By parameter p ($\log(t) / \log(\Delta A)$) **B) Key growth phase parameters.** Half-time and tenth-time (min) are the time to reach half and $1/10^{\text{th}}$ of the final absorbance ($O.D_{340}$) **C) Nucleus size at $t=15\text{min}$ (parameter p)** **D) Lag phase duration** (ratio of sample vs tubulin control) **E) Relative depolymerization levels (%)**. *In vitro* depolymerization levels at reaction endpoint (60min) of samples vs tubulin control **F) Relative polymerization levels (%)**. *In vitro* polymerization at endpoint (60min) of samples vs tubulin. All values were calculated based on averages from three independent experiments with triplicate samples.



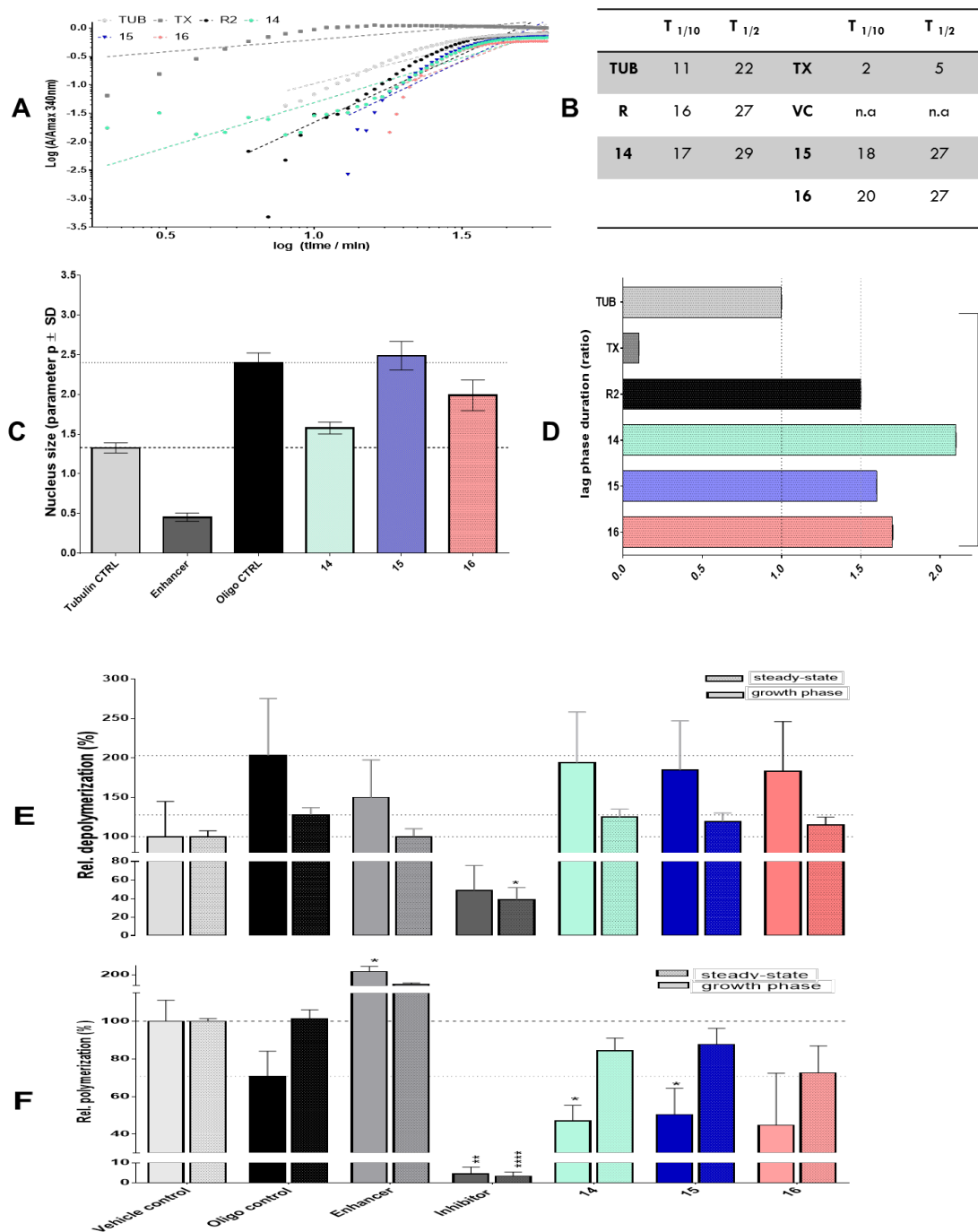
Cluster analysis: G/T-rich Tuda aptamers

By parameter p ($\log(t) / \log(\Delta A)$) **B) Key growth phase parameters.** Half-time and tenth-time (min) are the time to reach half and $1/10^{\text{th}}$ of the final absorbance ($O.D_{340}$) **C) Nucleus size at $t=15\text{min}$ (parameter p)** **D) Lag phase duration** (ratio of sample vs tubulin control) **E) Relative depolymerization levels (%).** *In vitro* depolymerization levels at reaction endpoint (60min) of samples vs tubulin control **F) Relative polymerization levels (%).** *In vitro* polymerization at endpoint (60min) of samples vs tubulin. All values were calculated based on averages from three independent experiments with triplicate samples.



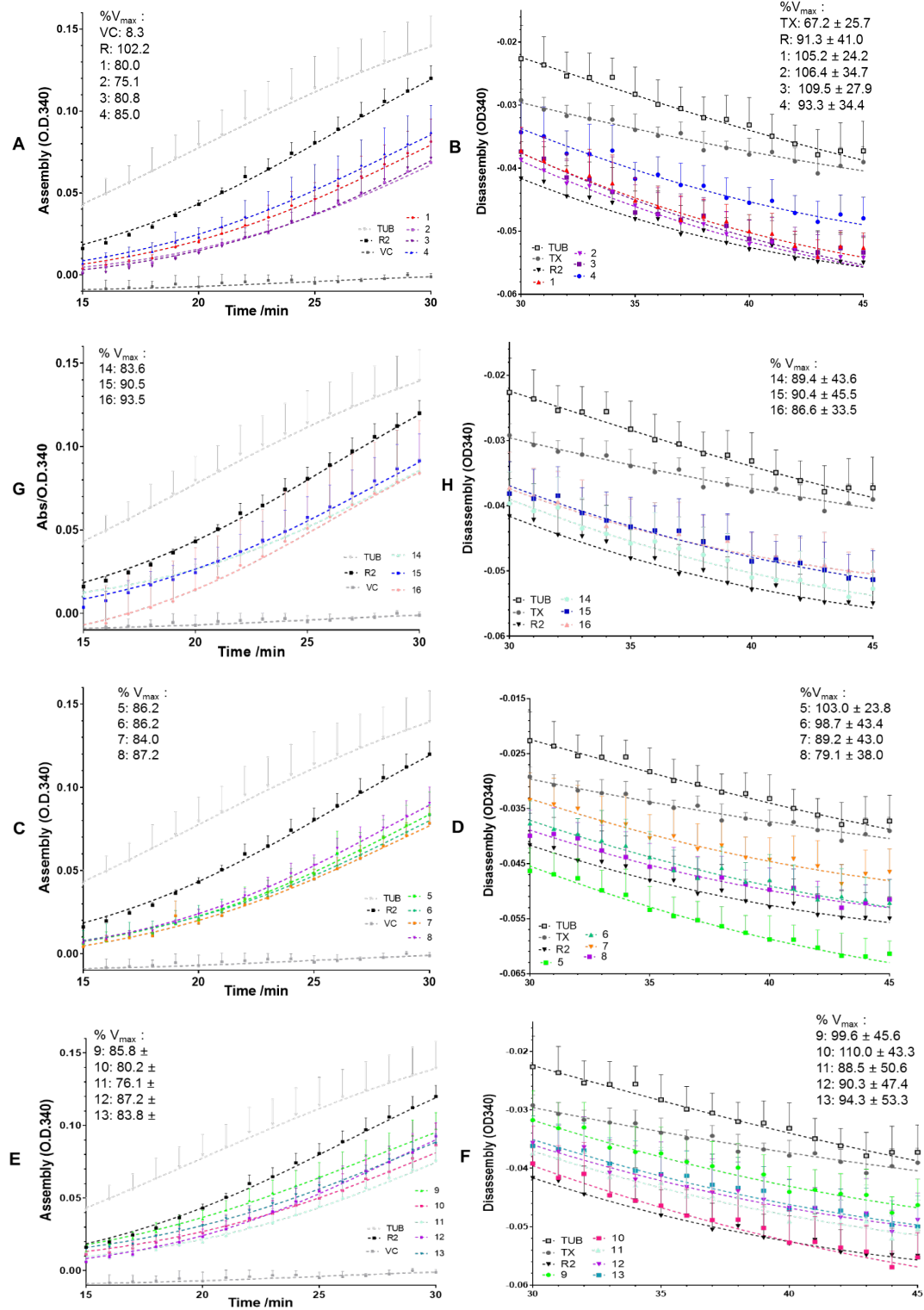
Cluster analysis: G-rich Tuda aptamers

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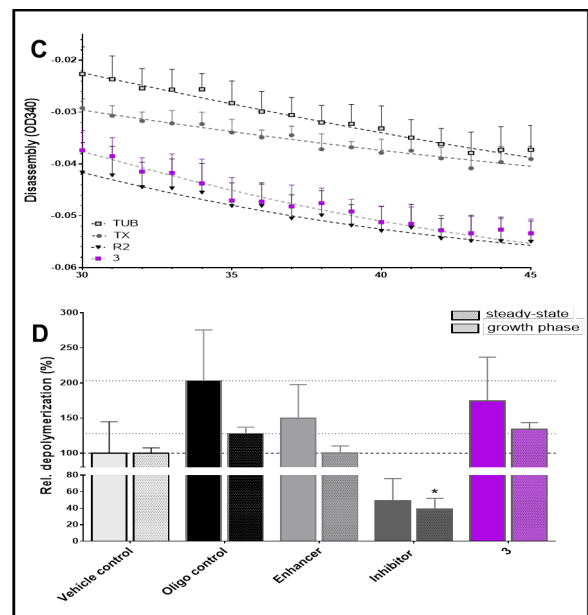
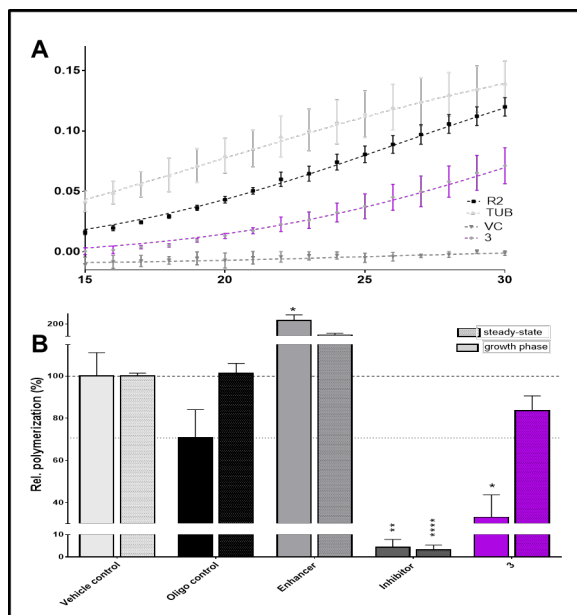
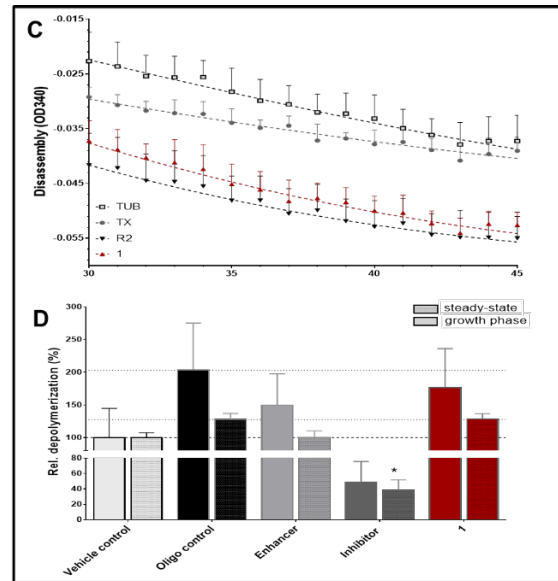
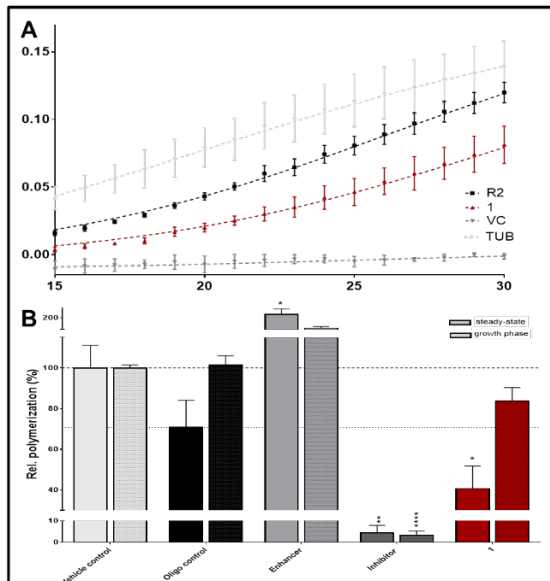
Cluster analysis: Tuda aptamers with un-skewed base composition

A) Nucleus size estimation across reaction. By parameter p ($\log(t) / \log(\Delta A)$) **B) Key growth phase parameters.** Half-time and tenth-time (min) are the time to reach half and $1/10^{\text{th}}$ of the final absorbance ($O.D._{340}$) **C) Nucleus size at $t=15\text{min}$ (parameter p)** **D) Lag phase duration** (ratio of sample vs tubulin control) **E) Relative depolymerization levels (%)**. *In vitro* depolymerization levels at reaction endpoint (60min) of samples vs tubulin control **F) Relative polymerization levels (%)**. *In vitro* polymerization at endpoint (60min) of samples vs tubulin. All values were calculated based on averages from three independent experiments with triplicate samples.



Tuda cluster analysis: Kinetics in vitro

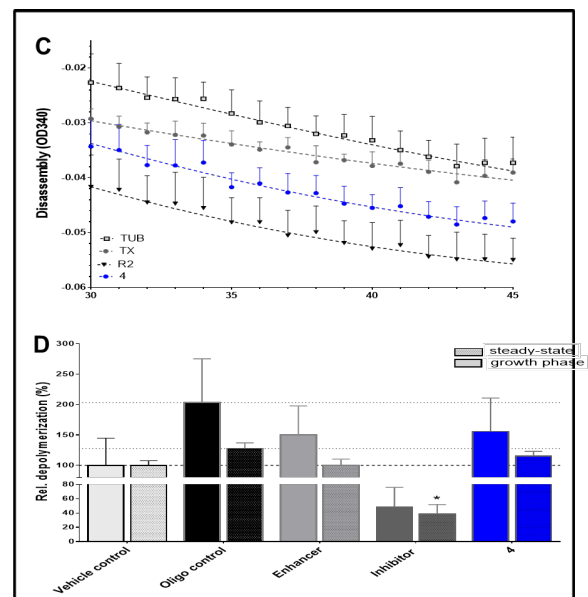
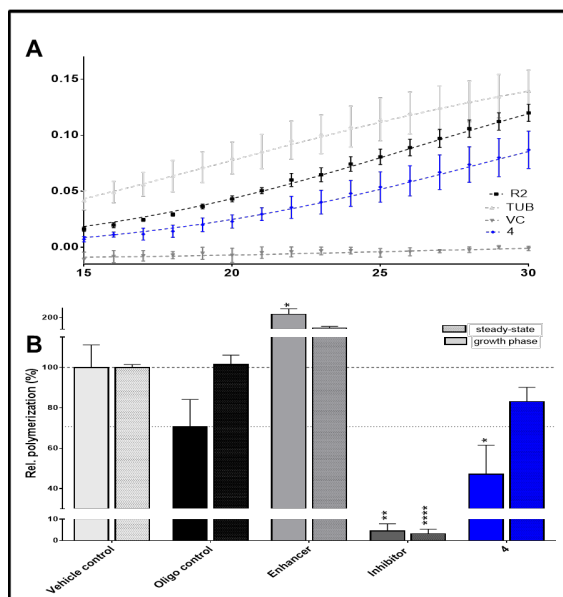
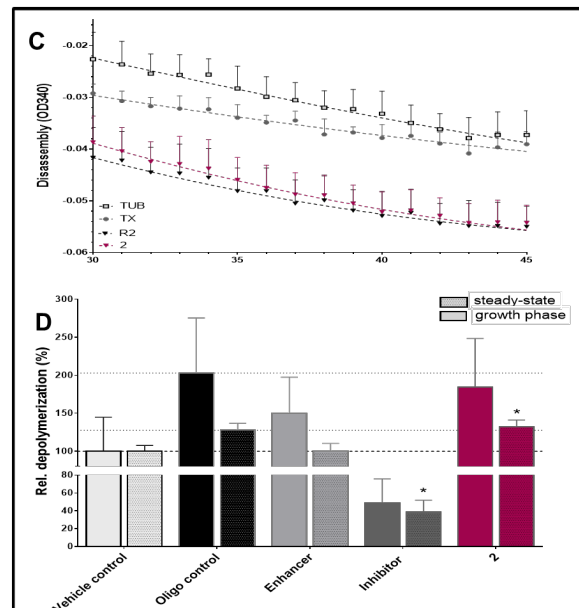
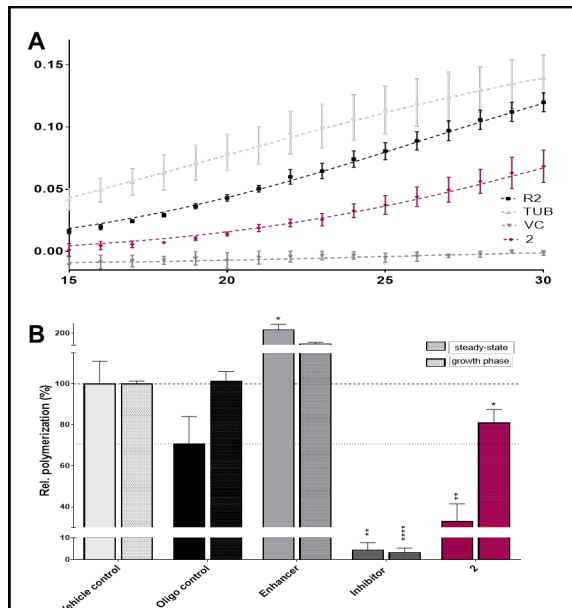
A), B) T-rich aptamers **C), D)** G/T-rich aptamers All values were calculated based on averages from three independent experiments with triplicate samples.



Single-aptamer analysis (Tuda population).

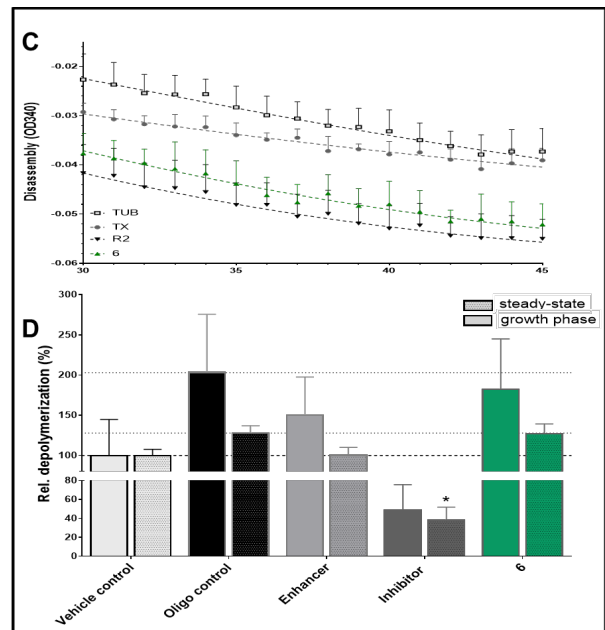
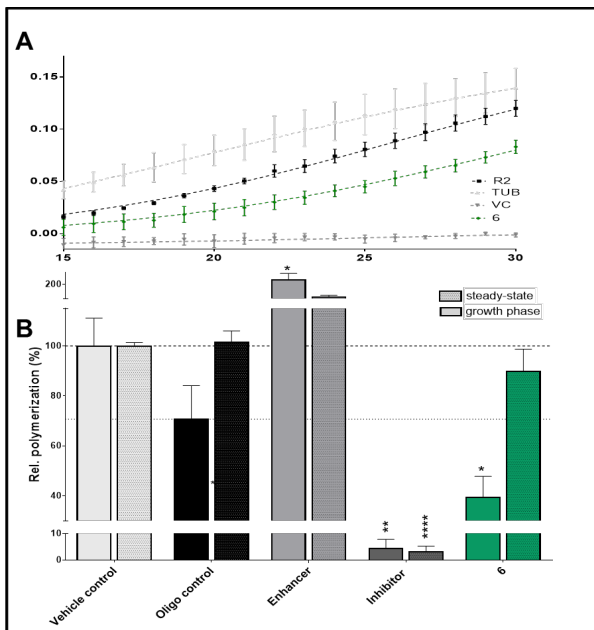
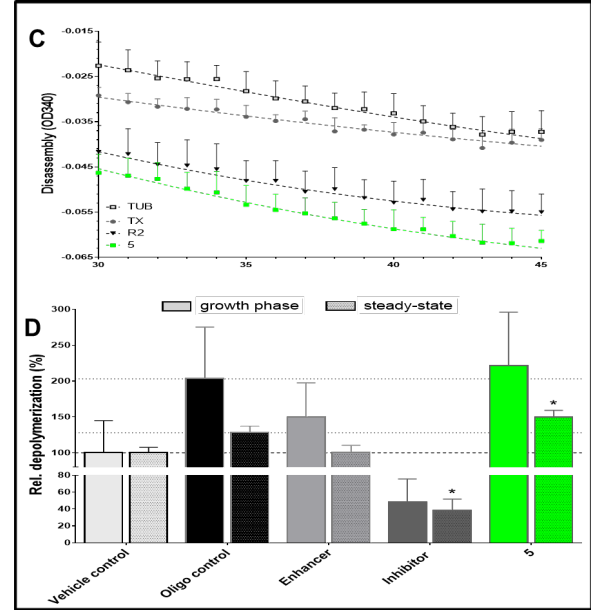
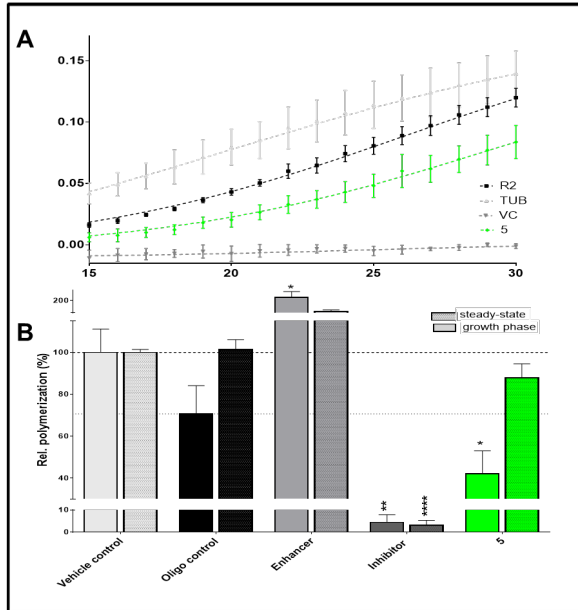
From 15-30min at 37°C **B**) **Relative polymerization levels (%)**. In growth-phase (15min) and steady-state (60min) from 30-45 min (O.D.₃₄₀) **C**) **Depolymerization curve (O.D.₃₄₀)**. From 15-30min at temperature decrease from 37-20°C **D**) **Relative de-polymerization (%) levels**. **n** growth-phase (15min) and steady-state (60min)

Results represent the average of three independent experiments with triplicate samples and error bars represent S.E.M. TUB= Tubulin control, R2= Randomer control VC= Inhibitor control (Vinecristine)



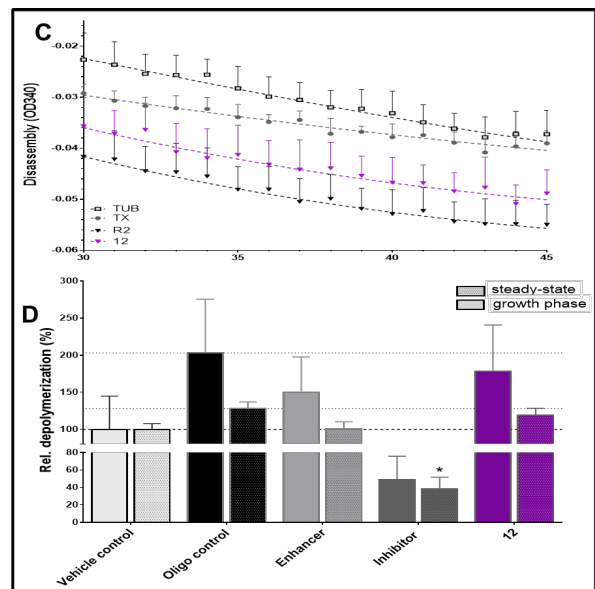
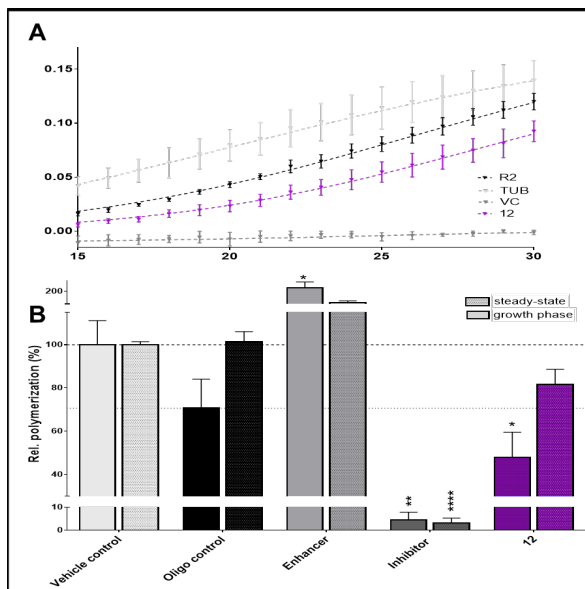
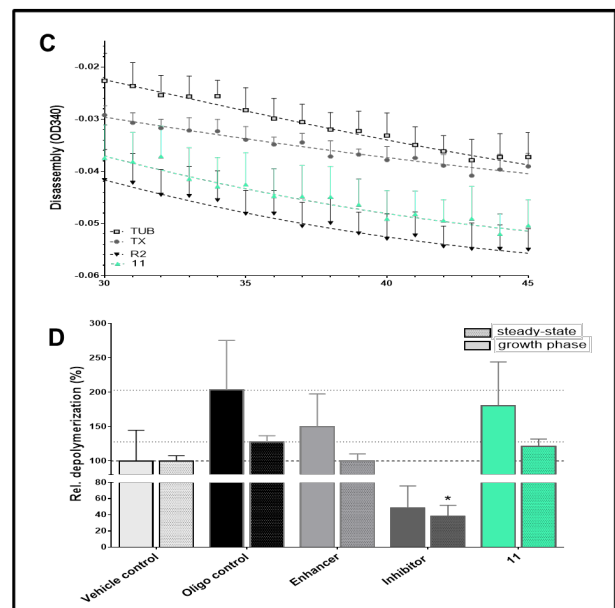
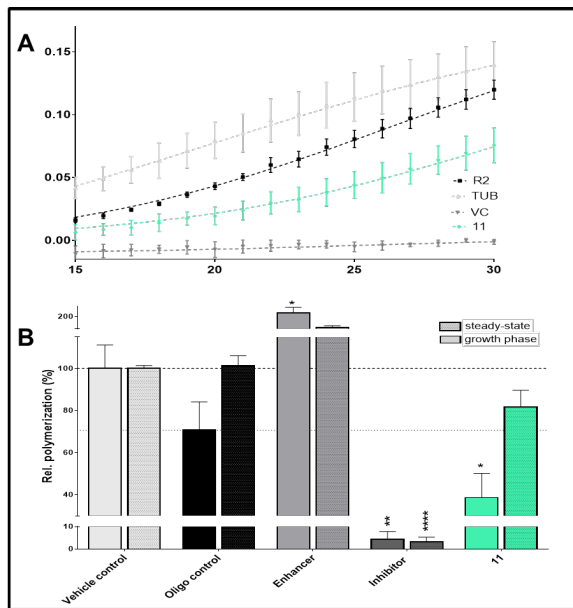
Single-apramer analysis (Tuda population).

From 15-30min at 37°C **B) Relative polymerization levels (%)**. In growth-phase (15min) and steady-state (60min) from 30-45 min (OD₃₄₀) **C) Depolymerization curve (O.D.₃₄₀)**. From 15-30min at temperature decrease from 37-20°C **D) Relative de-polymerization (%) levels**. n growth-phase (15min) and steady-state (60min)
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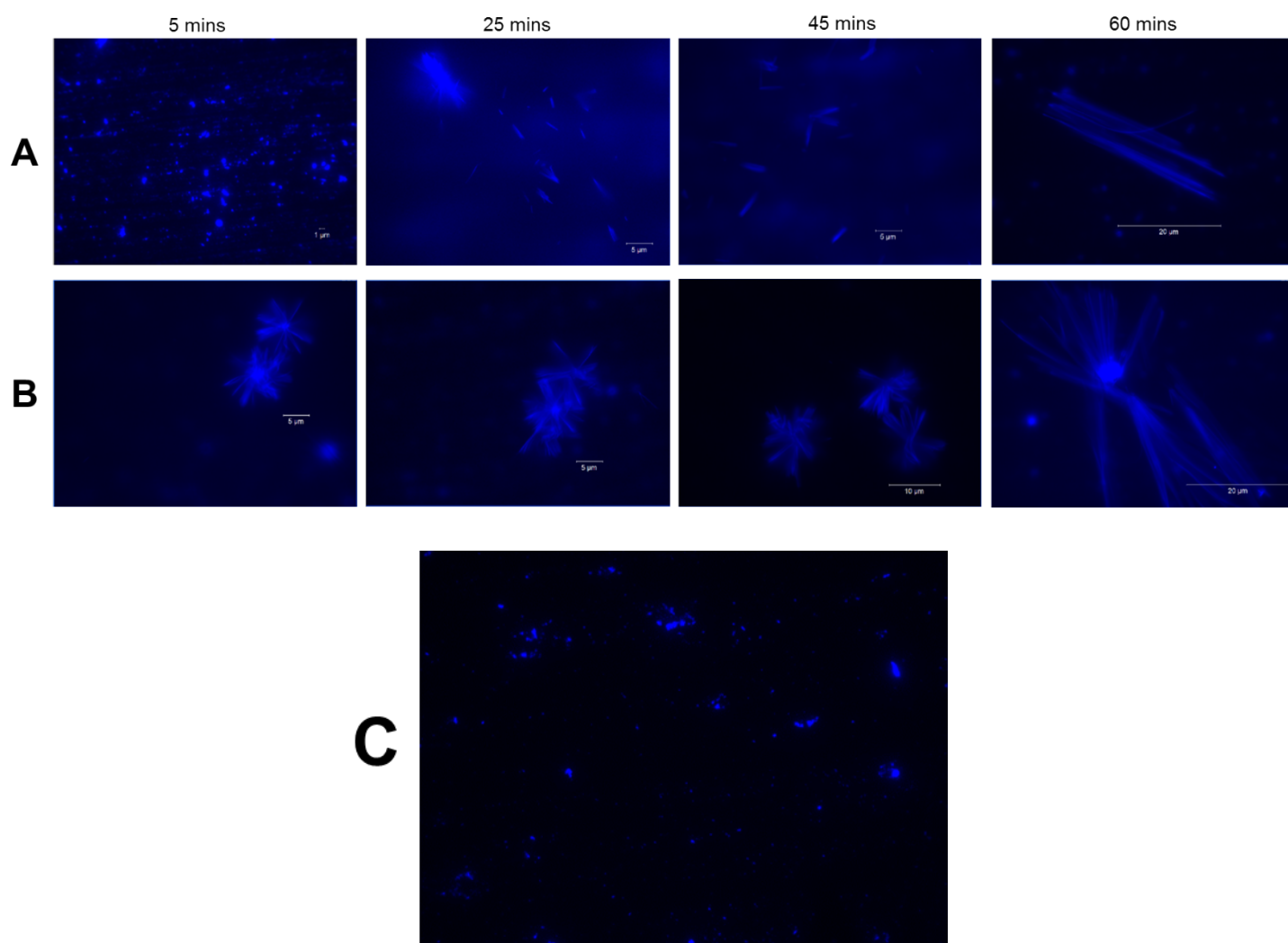
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Chapter 4 Appendix

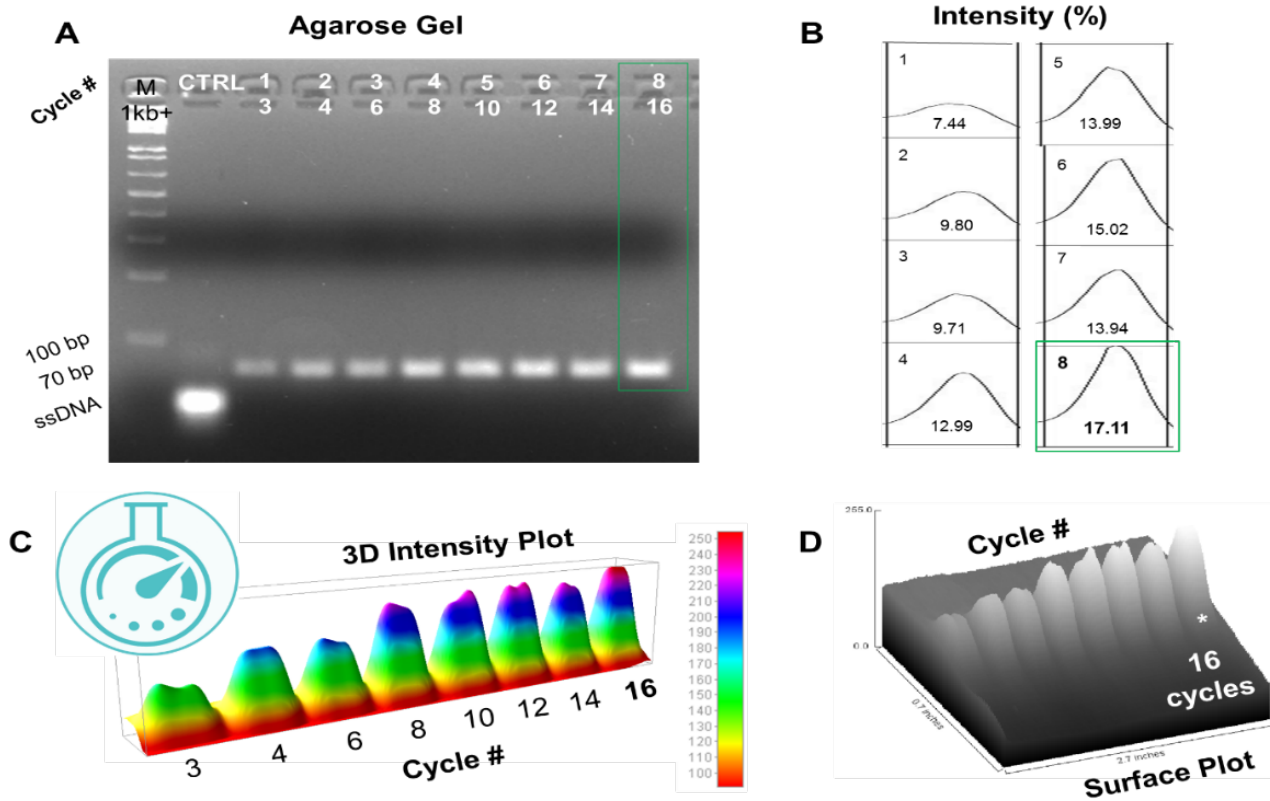
Here, a short independent assay was performed to evaluate feasibility of an imaging-based polymerization assay for anti-mitotic compounds.



Imaging-based growth assay of tubulin polymer

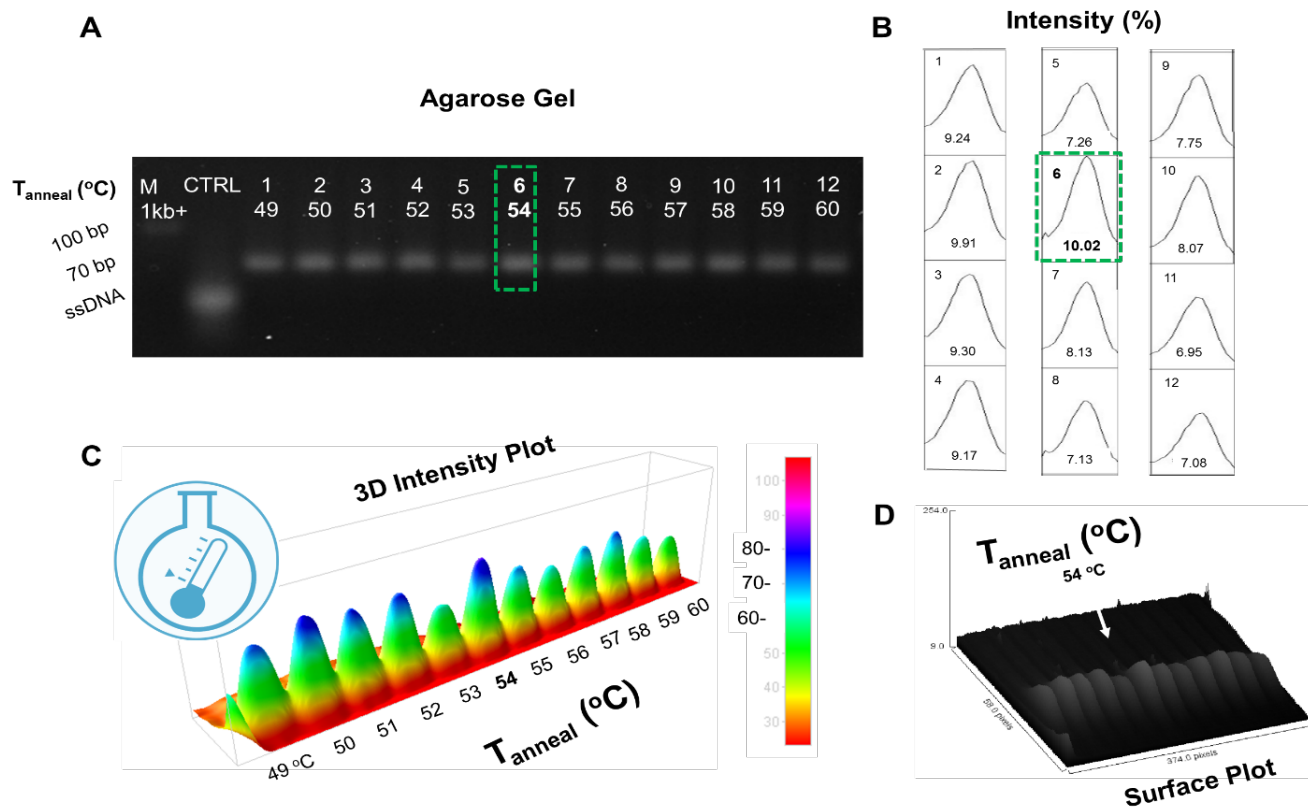
A) TRITC-labelled tubulin polymerized in reaction tube was aliquoted and imaged at different time-points (5mins, 25mins, 45mins and 60mins) **B)** TRITC-labelled tubulin asters polymerized and imaged at 4 reaction time-points. Scale bars = 20µm, unless otherwise indicated.

Chapter 5 Appendix



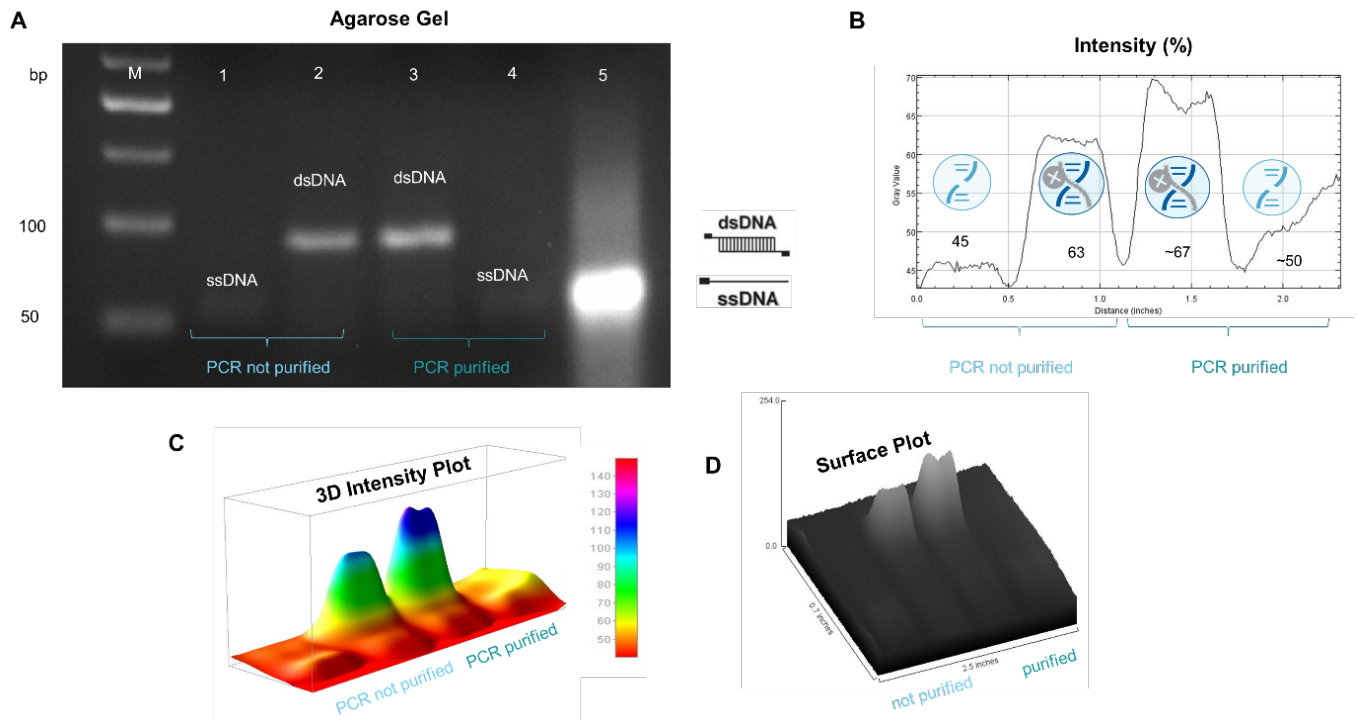
***In vitro* selection set up: Symmetric PCR (70mer).**

A) Agarose gel with PCR amplicons and ssDNA 70mer control **B)** Intensity Plots of the bands **C)** 3D intensity representation of bands **D)** Surface plot of bands. Experiments were repeated twice and yielded very similar results.



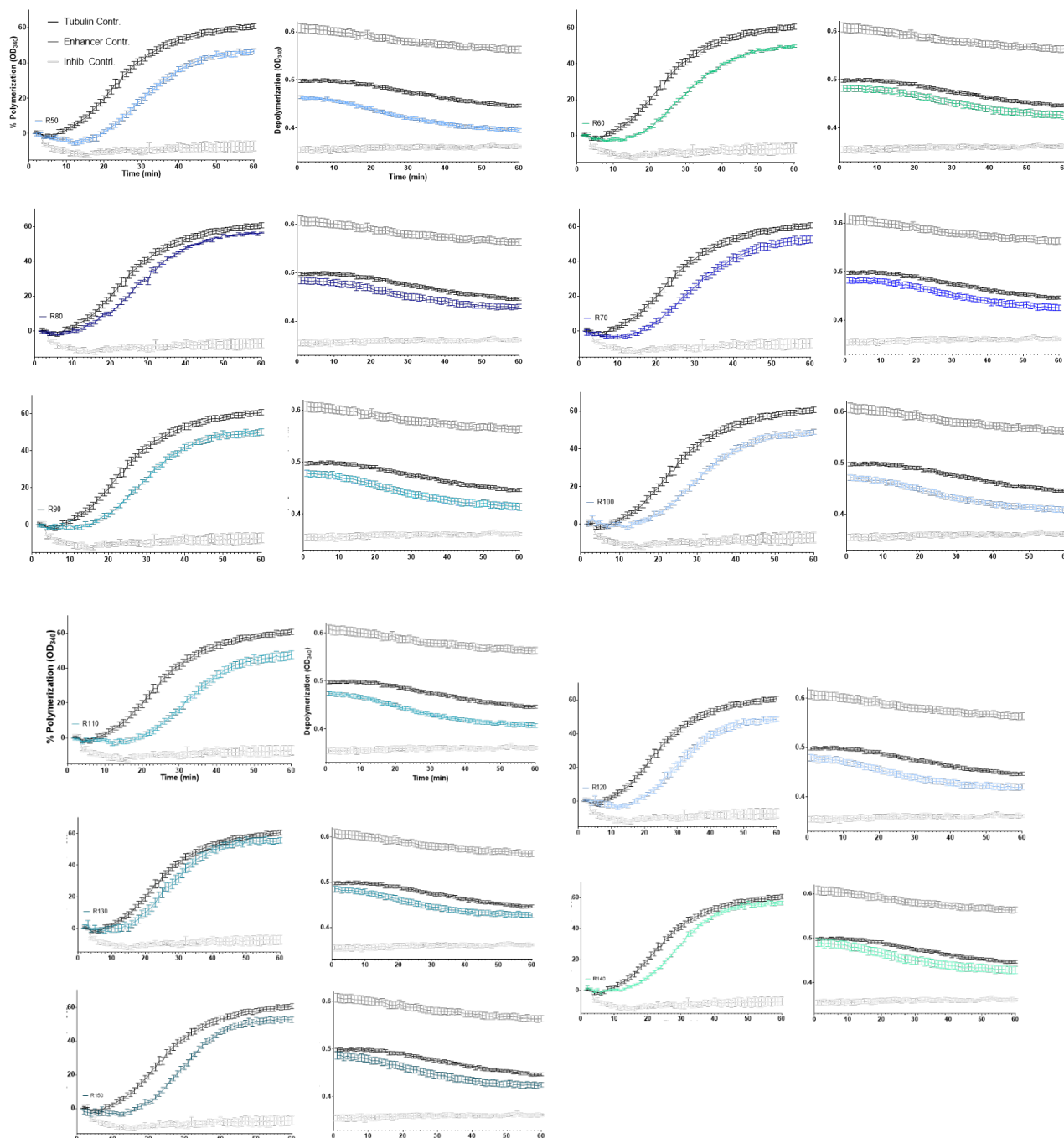
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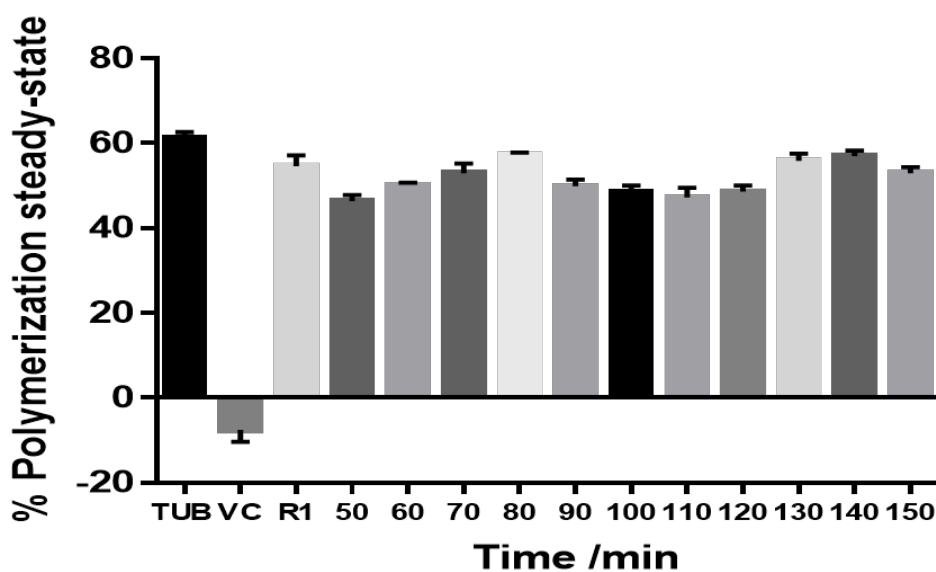
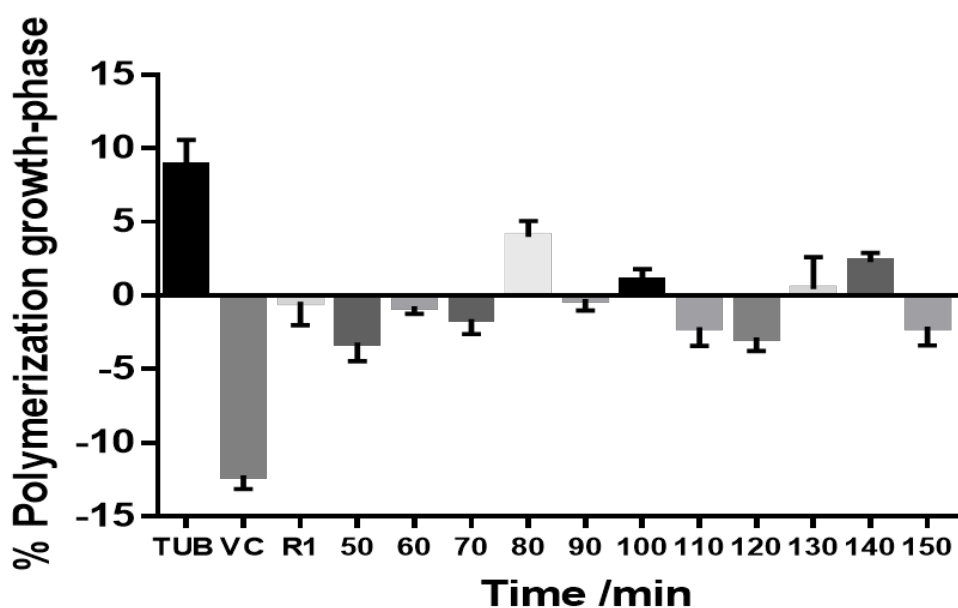
In vitro selection set up: Lambda digest optimization

A) 3% agarose gel with digest reaction product from purified and unpurified PCR **B)** quantitative intensity plots **C)** 3D band intensity plot **D)** intensity surface plot. Experiments were repeated twice with very similar results.



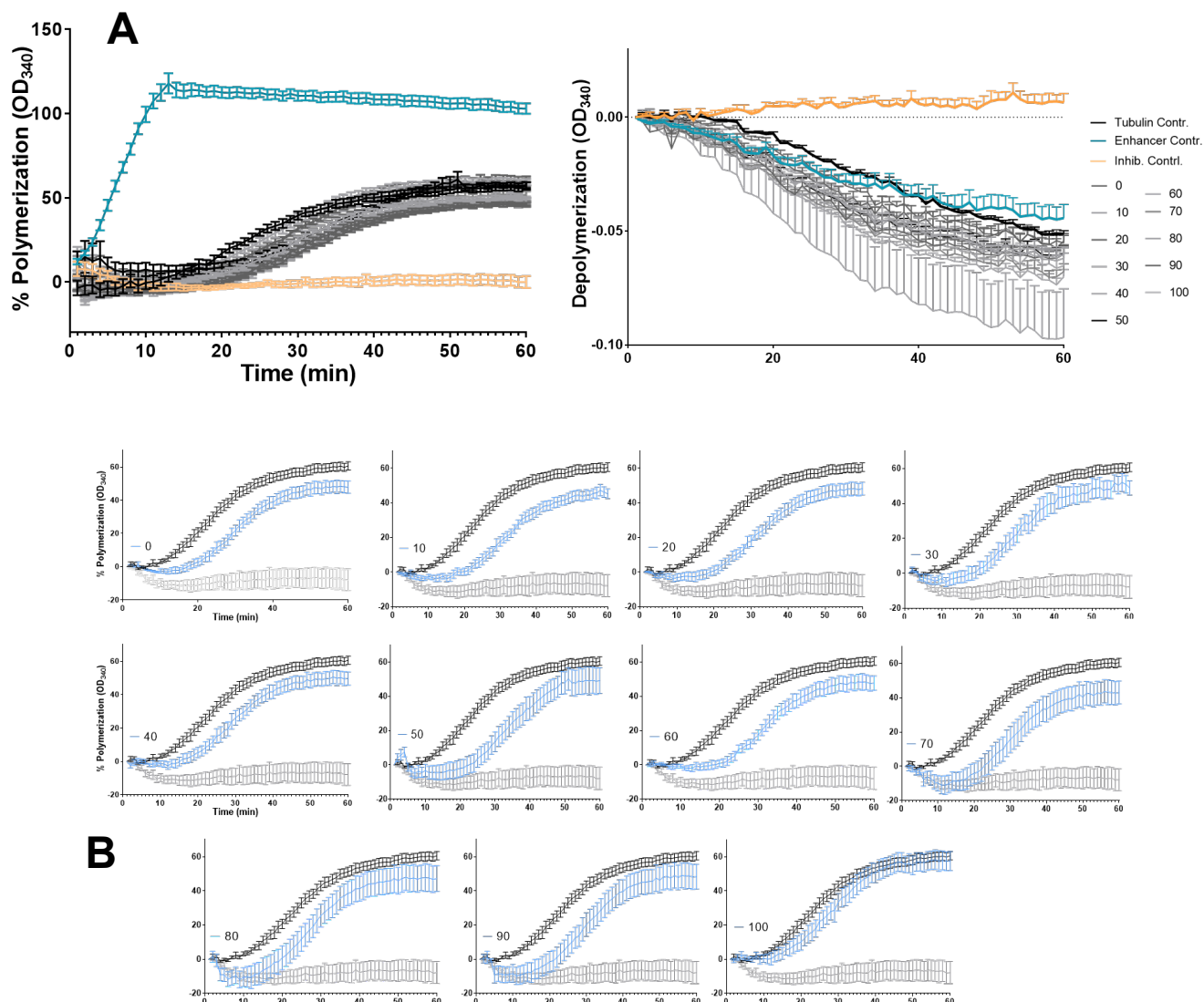
Random region prototyping.

Microtubule mass with randomers across size of 50-150nts are compared in growth-phase (25min) and steady-state (60mins) in the top and bottom graph, respectively. Values represent normalized %O.D and the average of three independent experiments performed in triplicate and error bars represent SEM.



Library random region prototyping.

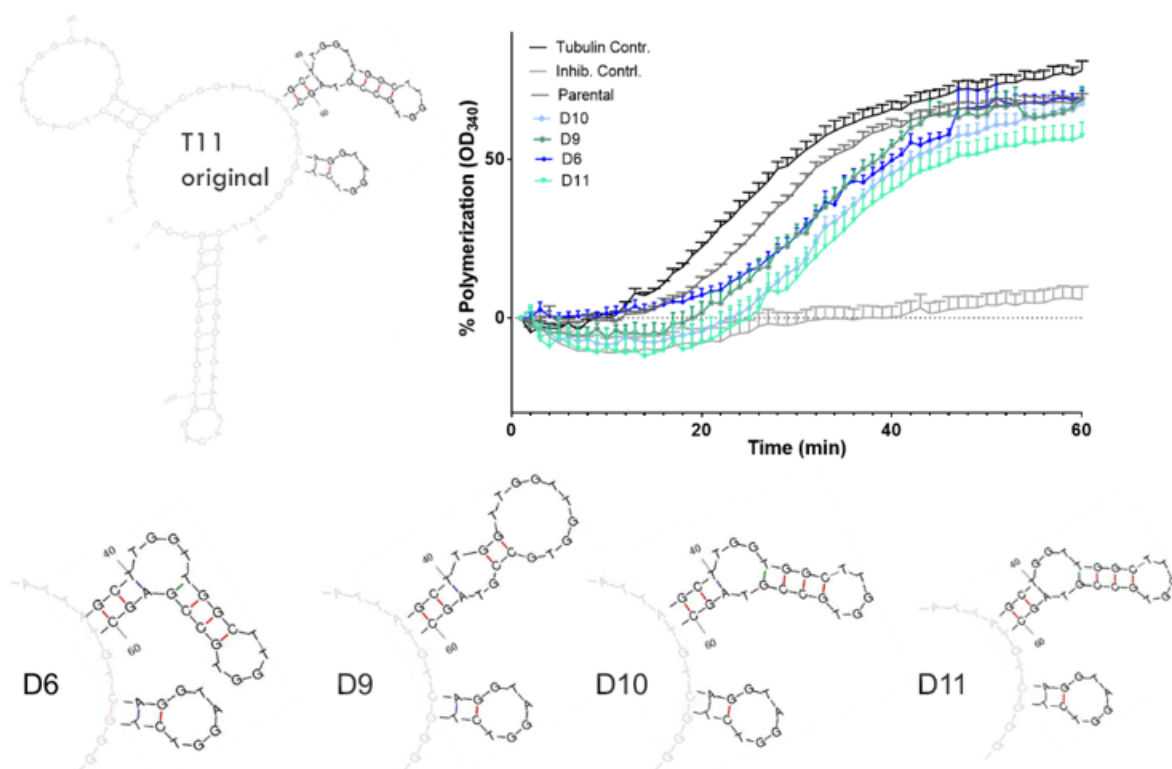
In vitro absorbance-based polymerization curves for randomers of different length from 50-150nts to assess potential patterns in non-specific effects of nucleic acids. Values represent the average of three independent experiments performed in triplicate and error bars represent SEM.



Library random region prototyping: finetuning nucleobase composition

In vitro absorbance-based polymerization curves for randomers of different nucleobase composition (numbers represent %GC content) from 0-100% are compared to assess potential patterns in non-specific effects of nucleic acids. **A)** absorbance-based tubulin slopes for assembly (left) and disassembly (right). **B)** assembly slopes per randomer. Values represent the average of three independent experiments performed in triplicate and error bars represent SEM.

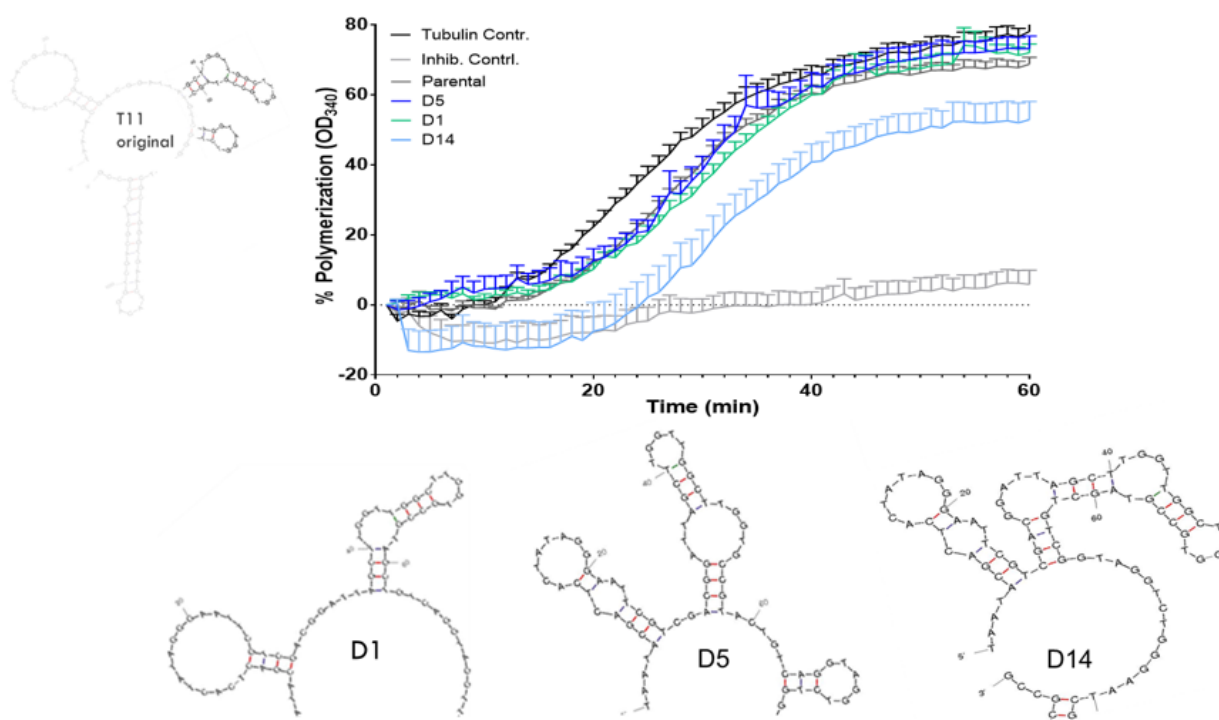
Chapter 6 Appendix



Group-wise reaction profile analysis of aptamer 'clock truncates.

D6, D9, D19 and D11 represent truncated motifs 2 of the parental sequence.

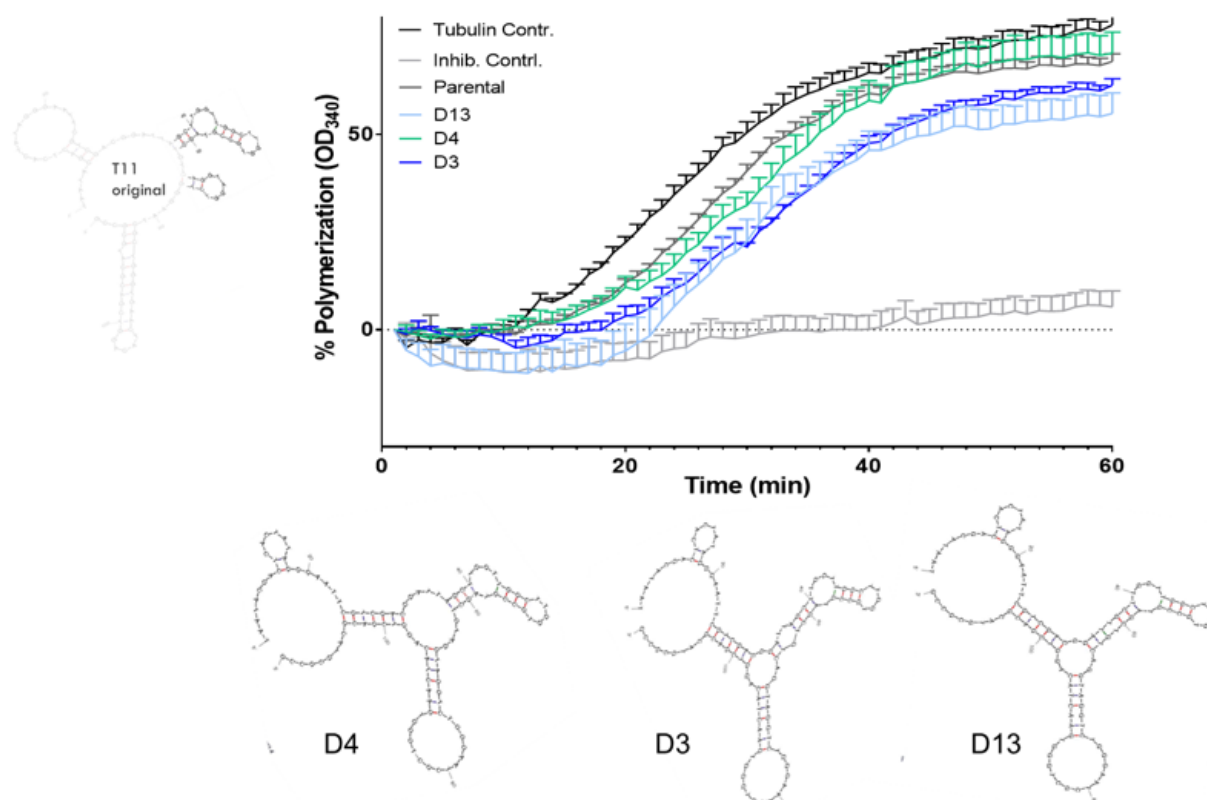
Absorbance was measured at 37°C in 1 minute-intervals for 60mins values represent the average of three independent experiments performed in triplicates and error bars express SEM. Secondary structures were predicted with the mfold webserver based on energy minimization. Approximate folding conditions and ionic strength in tubulin buffer were used for the prediction.



Group-wise reaction profile analysis of aptamer 'clock truncates.

D1, D5 and D14 represent truncations of all motifs 1-3 of the parental sequence.

Absorbance was measured at 37°C in 1 minute-intervals for 60mins values represent the average of three independent experiments performed in triplicates and error bars express SEM. Secondary structures were predicted with the mfold webserver based on energy minimization. Approximate folding conditions and ionic strength in tubulin buffer were used for the prediction.



Group-wise reaction profile analysis of aptamer 'clock truncates.

D4, D3 and D13 represent completely altered structures compared to the parental sequence. Absorbance was measured at 37°C in 1 minute-intervals for 60mins values represent the average of three independent experiments performed in triplicates and error bars express SEM. Secondary structures were predicted with the mfold webserver based on energy minimization. Approximate folding conditions and ionic strength in tubulin buffer were used for the prediction.

(Appendix) Table 6.2: Minimized aptamers

M1	ATGCTCGCGCCTGCTGTGTTGTTGCTTGGTTTGGTCTTTTTTTGGTTTGTTTTGGGT
M2	GTTGGGCTACAGTACTTCCCCTTTTTTCTCTTCCTCCTCCTATCGCTCTCC
M3	AGGCTATTCGTTTTGTGTTTCTTTTCCTGGTTTTGTTATTTTTGTTGTTGTTTTTGG
M4	GAATTCGTTTGTGTGCGGAGGTGGTTGTTTGTGTTTTTGTCTTTGTTTTGTTTG
M5	AGATATGGTTTTGTTGTGCGTTATGTTGTGTTTTTGGGGTCTCTTTTTGGGTGTTTTGT
M6	TTAAGTTTGCTGTTAATTGGTGCGTATCTCTGTAGTAGAGTCAGTTAGCGGTTTTGTGGT
M7	ACGTTCCGCTGGTATAGTGAGTGACCTTGGTCTGGTGGATGCGTTTATTCGTGGTCCTC
M8	CTTAGTGATATCAGGTGTCGTTACTTCAGGGTGAGTTCATGTCTTGCTTAAAGTTATTG
M9	TTGGTGGGTTGTAGGCAGGCGTGGGCACTGTTTGAGAAGGACGTGTTTGGTCTAT
M10	ATGTATTACTAGGTCGTTGACGGAGAAGGGGGGTAGGTGCTTGGGTGTGTCTGTTTGGT
M11	TAGCTTGGTTGGCTTGGTGCCGTAGCTGTCAGGTAGGTCTGGGAATCGCTGGGTGAA
M12	TGGTGGGATGTCGTTTGAAGATTGTTTGC GTGTGTGCCGTAGATATGCTGGGGTTGCAT
M13	CGAATGGGGAGTGGGACCGAATCTTGGTGTGTGTTCTGAAGCAGGGGTTCATATGGTT
M14	TCAAGATCGGTTGCCGTGGGTACAGTGGCGATTGTTACAAGGTGCAATAGTCCCTCGC
M15	ATCGAAAGGTGGGTGCGATTAGTATGAGAGGTCTTCGCATTCCGGTGCTGGGATCCTTTA
M16	CGAGGCTTAAGGCGCAACTAATCTCGTTGGTGGTTCGCCACAGGTGTTCCAAC
R	TTGTTTTAACAAGTATTGAGTTGCTCGTGCGTGTTGGTTGTCTGGGTTAGAATTGTGACT

(Appendix) Table 6.3: Antidotes: Silencing motifs through hybridization

R50	GCGGGAAAATCACCGACGGCATTATATAGAAGCCTAGGGGAACAGATTGG
R50_ C	CTGGCGTCTTGGCTAATCCCCCTACATGTTGTTATAAATAATCAGTGGAA
T11	TAATACGACTCACTATAGGGAATTCGTCGACGGATTAGCTTGGTTGGCTTGGTGCCGTA GCTGTCAGGTAGGTCTGGGAATCGCTGGGTGAACTACAGGTCGACGCATGCGCCG
T11_ C	ATTATGCTGAGTGATATCCCTTAAGCAGCTGCCTAATCGAACCAACCGAACCCACGGCAT CGACAGTCCATCCAGACCCTTAGCGACCCACTTGATGTCCAGCTGCGTACGCGGC
T11_ AD	GTGATATCCCTTAAGCGA

(Appendix) Table 6.4: Structure-switching chimera

1	CGTCCAACGTGTCGCCTGGACGGTTGGGCTACAGTACTTCCCCTTTTTTCTCTTCCTCC TTCCTATGCTCC
2	CGTCCAACGTGTCGCCTGGACGGTTGGGCTACAGTACTTCCCCTTTTTTCTCTTCCTCC TTCCTATGTTCC
3	CGTTCAACGTGTCGCCTGGACGGTTGGGCTACAGTACTTCCCCTTTTTTCTCTTCCTCC TTCCTATGTTCC
4	GCATCGTCCAACGTGTCGCCTGGACGATGCGTTGGGCTACAGTACTTCCCCTTTTTTCT CTTCCTCCTTCCTATGCATGCTCC
R	GTGGCAATTACCTAAGGTTTCAGTGTCGTTAGGCACAGGTCCTCCCGTGAACCTAACCTA GATTCATGCTGA

(Appendix) Table 6.5: Aptamer Clock

Paren tal (T11)	TAATACGACTCACTATAGGGAATTCGTCGACGGATTAGCTTGGTTGGCTTGGTGCCGTA GCTGTCAGGTAGGTCTGGGAATCGCTGGGTGAACTACAGGTCGACGCATGCGCCG
D1	TAATACGACTCACTATAGGGAATTCGTCGACGGATTAGCTTGGTTGGCTTGGTGCCGTA GCTGTCAGGTAGGTCTGGGAATCGCTGGGTGAACTACAGGTCGACGCATGCGCCG
D2	TAATACGACTCACTATAGGGAATTCGTCGACGGATTAGCTTGGTTGGCTTGGTGCCGTA GCTGTCAGGTAGGTCTGGGAATCGCTGGGTGAACTACAGGTCGACGCATGCGCCG
D3	TAATACGACTCACTATAGGGAATTCGTCGACGGATTAGCTTGGTTGGCTTGGTGCCGTA GCTGTCAGGTAGGTCTGGGAATCGCTGGGTGAACTACAGGTCGACGCATGCGCCG
D4	TAATACGACTCACTATAGGGAATTCGTCGACGGATTAGCTTGGTTGGCTTGGTGCCGTA GCTGTCAAGGTAGGTCTGGGAATCGCTGGGTGAACTACAGGTCGACGCATGCGCCG
D5	TAATACGACTCACTATAGGGAATTCGTCGACGGATTAGCTTGGTTGGCTTGGTGCCGTA GCTGTCAGGTAGGTCTGGGAATCGCTGGGTGAACTACAGGTCGACGCATGCGCCG
D6	TAATACGACTCACTATAGGGAATTCGTCGACGGATTAGCTTGGTTGGCTTGGTGCCGTA GCTGTCAGGTAGGTCTGGGAATCGCTGGGTGAACTACAGGTCGACGCATGCGCCG
D7	TAATACGACTCACTATAGGGAATTCGTCGACGGATTAGCTTGGTTGGCTTGGTGCCGTA GCTGTCAGGTAGGTCTGGGAATCGCTGGGTGAACTACAGGTCGACGCATGCGCCG
D8	TAATACGACTCACTATAGGGAATTCGTCGACGGATTAGCTTGGTTGGCTTGGTGCCGTA GCTGTCAGGTAGGTCTGGGAATCGCTGGGTGAACTACAGGTCGACGCATGCGCCG
D9	TAATACGACTCACTATAGGGAATTCGTCGACGGATTAGCTTGGTTGGCTTGGTGCCGTA GCTGTCAGGTAGGTCTGGGAATCGCTGGGTGAACTACAGGTCGACGCATGCGCCG
D10	TAATACGACTCACTATAGGGAATTCGTCGACGGATTAGCTTGGTTGGCTTGGTGCCGTA GCTGTCAGGTAGGTCTGGGAATCGCTGGGTGAACTACAGGTCGACGCATGCGCCG
D11	TAATACGACTCACTATAGGGAATTCGTCGACGGATTAGCTTGGTTGGCTTGGTGCCGTA GCTGTCAGGTAGGTCTGGGAATCGCTGGGTGAACTACAGGTCGACGCATGCGCCG
D12	TAATACGACTCACTATAGGGAATTCGTCGACGGATTAGCTTGGTTGGCTTGGTGCCGTA GCTGTCAGGTAGGTCTGGGAATCGCTGGGTGAACTACAGGTCGACGCATGCGCCG
D13	TAATACGACTCACTATAGGGAATTCGTCGACGGATTAGCTTGGTTGGCTTGGTGCCGTA GCTGTCAGGTAGGTCTGGGAATCGCTGGGTGAACTACAGGTCGACGCATGCGCCG
D14	TAATACGACTCACTATAGGGAATTCGTCGACGGATTAGCTTGGTTGGCTTGGTGCCGTA GCTGTCAAGGTAGGTCTGGGAATCGCTGGGTGAACTACAGGTCGACGCATGCGCCG
R	GTACAACAAAAGTATCAAACCCGTATAAAGACAACATCCTCACTTTAGTGAATCGAAGCG CGGCATCAGAATTACTTTTTGGATACCTGAAACAAAACCCATCGTAATTTTTAGA

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