

UC San Diego

UC San Diego Electronic Theses and Dissertations

Title

Synthesis of novel polymeric nanoparticles for hydrophobic and hydrophilic drug delivery

Permalink

<https://escholarship.org/uc/item/76f2f5jv>

Author

Sartor, Marta

Publication Date

2010

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Synthesis of novel polymeric nanoparticles for hydrophobic and hydrophilic drug
delivery

A dissertation submitted in partial satisfaction of the requirements for the

degree Doctor of Philosophy

in

Materials Science and Engineering

by

Marta Sartor

Committee in charge:

Professor Sadik C. Esener, Chair
Professor Jennifer N. Cha
Professor Michael J. Heller
Professor Andrew C. Kummel
Professor Liangfang Zhang

2010

Copyright

Marta Sartor, 2010

All right reserved.

The dissertation of Marta Sartor is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2010

DEDICATION

This doctoral dissertation is dedicated to my family. My parents who left me free to choose my path, even if it led me to a country afar, and my husband who has been there to cheer me up during the hard times.

I would also like to dedicate this work to all the first generation college students, like me, who defied the odds and pursued higher education degree. With honesty, good attitude and hard work anything can be achieved despite the disadvantages.

TABLE OF CONTENTS

Signature Page	iii
Dedication	iv
Table of Contents	v
List of Abbreviation	vii
List of Figures	viii
List of Graphs	x
List of Tables	xiii
List of Scheme	xiv
Acknowledgements	xv
Vita	xvii
Abstract of the dissertation	xviii
Introduction	1
Building material	4
Internal structure	5
Material compatibility	7
Bibliography	9
Chapter 1 DNA as building material: DNA nanoparticles	11
1.1 Introduction: hydrophilic nanoparticles	11
1.2 DNA nanoparticles: engineering and production	13
1.3 Selection process	18
1.4 Identifying the selected sequences	27
1.5 Conclusions on DNA nanoparticles	32
1.6 Acknowledgments	34
1.7 Materials and methods	34
1.8 Bibliography	38
Chapter 2 Modified internal structure: gradient nanoparticles	40
2.1 Introduction: polymeric nanoparticles	40
2.2 Theory of porosity gradient	41

2.3 Porosity gradient results.....	45
2.4 Introducing the chemical gradient	50
2.5 Chemical gradient results.....	51
2.6 Conclusions on gradient nanoparticles	60
2.7 Acknowledgements	61
2.8 Materials and methods.....	62
2.9 Bibliography	68
 Chapter 3 Combining incompatible materials: amphiphilic hybrid nanoparticles	70
3.1 Introduction on amphiphilic delivery vehicles	70
3.2 Hybrid nanoparticles: overview	73
3.3 PMAL: a powerful zwitterionic Amphipol.....	75
3.4 Oligonucleotides-PMAL complex	77
3.4 Physical characterization of the nanoparticles	83
3.6 Oligonucleotides loading in the nanoparticles	89
3.7 Biofunctionality: oligonucleotides delivery	91
3.8 Conclusions on amphiphilic hybrid nanoparticles	97
3.9 Acknowledgments	98
3.10 Materials and methods.....	98
3.11 Bibliography	103
 Conclusions and future work	106
DNA nanoparticles	106
Gradient nanoparticles	108
Amphiphile assisted loading	110

LIST OF ABBREVIATION

CHAPTER 1

DC	Dendritic cells
PBS	Phosphate Buffered Solution
BSA	Bovine Serum Albumin
PCR	Polymerase Chain Reaction
FACS	Flow Assisted Cell Sorting

CHAPTER 2 and 3

PLGA	Poly(lactic-co-glycolic acid)
PCL	Polycaprolactone
PEG	Polyethylene glycol
DSPE	1,2-distearoyl-sn-glycero-3-phosphoethanolamine
¹ H-NMR	Proton Nuclear Magnetic Resonance
GPC	Gel Permeation Chromatography
SEM	Scanning Electron Microscope
DLS	Dynamic Light Scattering
HPLC	High Pressure Liquid Chromatography
FACS	Flow Assisted Cell Sorting

LIST OF FIGURES

Figure 1.1: DNA nanoparticles with Sybr Green I under a 100X microscope lens	16
Figure 1.2: The amplified sequences out of the nine rounds of selection are compared by gel electrophoresis. The control is the amplified unselected library (in analogy with the real-time PCR)	24
Figure 1.3: The microscope images show the binding to DC of free Alexa-labelled oligonucleotides, nonspecific DNA nanoparticles and clone 03. The images of clone 03 show bright positive signal compared to both the free nucleotides and the non specific particles	31
Figure 1.4: FACS results and microscope images of clone 03 binding. Clone 03 (green) unlike its reverse complement (blue) is specific for DC. No affinity was seen for P815 (mouse mastocytoma mast cell) and THP1 (Human acute monocytic leukemia monocytes). Untreated control is in red.....	32
Figure 2.1: Chemical reaction to conjugate PLGA to PEG, with ^1H -NMR of the PLGA (40kDa). The peaks are clearly visible. Similar results were seen for the lower molecular weight PLGA	46
Figure 2.2: Chemistry for the ring opening polymerization of PCL and ^1H -NMR of the product	52
Figure 2.3: SEM image of the 25:75 PLGA:PCL composition representative for the nanoparticles.....	53
Figure 3.1: Chemical structure of the commercially available PMAL-C8	76

Figure 3.2: Sybr Green I stained DNA mixed with different amounts of PMAL-C8: 0, 2, 4, 6, 8, and 10 nano moles (from the left)	78
Figure 3.3: Sybr Green I mixed with different amounts of PMAL-C8: 0, 2, 4, 6, 8, and 10 nano moles (from the left)	79
Figure 3.4: DNA-Sybr Green I in water with (right) and without (left) 6 nano moles of PMAL-C8	79
Figure 3.5: Sybr Green I after 40 min incubation at 4° C with (right) and without PMAL-C8.....	80
Figure 3.6: 6-FAM labelled DNA mixed with different amounts of PMAL-C8: 0, 2, 4, 6, 8, and 10 nano moles (from the left)	81
Figure 3.7: 6-FAM labelled DNA in water after 40 minutes at 4° C with (right) and without (left) PMAL-C8	81
Figure 3.8: 6-FAM labelled DNA in acetonitrile with (right) and without (left) PMAL-C8.....	82
Figure 3.9: SEM images of the nanoparticles loaded with (left) and without (right) the PMAL-C8/DNA complex	87
Figure 3.10: Microscope images of cells with targeted (top) and non-targeted (bottom) nanoparticles. The nuclei are stained with DAPI (blue), while the nanoparticles are labeled with DiD (red)	93
Figure 3.11: Microscope images of the cells after silencing with lipofectamine (left) and nanoparticles (center) compared to the untreated cells (right). The nuclei are DAPI stained (blue), while the eGFP protein is green	94

LIST OF GRAPHS

Graph 1.1: Dynamic light scattering size measurements of DNA nanoparticles made with the same sequence	16
Graph 1.2: The RCA reaction monitored through the increase of fluorescence of Oligreen.....	17
Graph 1.3: The different real-time PCR for each round of selection. In each set 2uL of 1nM library were used as a control. For the first round of selection the two halves were amplified in parallel. In all the other sets 2uL of the lysate only were used for the amplification	20
Graph 1.4: Control real-time PCR were run for possible amplification of the lysed cells genome (blue) and of possible plastic binders/super amplifiers (red). The graph scale is set to be comparable with Figures 1.3 and 1.5	21
Graph 1.5: The dissociation curves of the different cycles (blue) show the variation of the higher intensity peak relative to the control (red)	22
Graph 1.6: The ratio of the peak heigh of the selection to that of the control shows no significant variation between selection rounds for the lower temperature peak, but a significant change for the higher temperature peak.	23
Graph 1.7: FACS results of the binding of the ninth round of selection (enriched library) compared to a control sequence (CpG containing sequence) and the plain cells	26
Graph 1.8: FACS results for two positively selecting clones (3 and 4) and for two negative clones (7 and 11). Controls were: DCs only (gray), DCs with free Alexa nucleotides (blue) and Control sequence (pink)	29

Graph 2.1: Size and ζ -potential distribution of the five different combinations	47
Graph 2.2: Loading yield percent of nanoparticles loaded with 5% wt/wt paclitaxel.....	48
Graph 2.3: Drug release of the uniform core nanoparticles for short (100:0) and long (0:100) PLGA chain, compared to the gradient nanoparticles of equal molar percent of the two different length PLGA (50:50)	49
Graph 2.4: Size and ζ -potential distribution of the five different combinations	54
Graph 2.5: A) Intensity maxima and B) calculated Net polarization value for the different nanoparticles combinations.....	56
Graph 2.6: A) Intensity maxima and B) calculated Net polarization value for polymer mix and nanoparticle mix of 75:25 PLGA:PCL combination.....	57
Graph 2.7: Drug loading yield of paclitaxel (hydrophobic) and doxorubicin (hydrophilic) for the five different PLGA:PCL combinations	58
Graph 2.8: Drug release for the gradient nanoparticle (25:75 PLGA:PCL) and the individual polymer nanoparticles PLGA (100:0) and PCL (0:100)	59
Graph 3.1: Intensity at 520nm of 6-FAM dye in acetonitrile with and without PMAL	82
Graph 3.2: Size and ζ -potential of the hybrid nanoparticles with different amounts of PMAL-C8.....	84
Graph 3.3: Size and ζ -potential of the hybrid nanoparticles loaded with DNA (40bp) combined with different amounts of PMAL-C8	85
Graph 3.4: Size and ζ -potential of the hybrid nanoparticles loaded with DNA (20bp) combined with different amounts of PMAL-C8	86

Graph 3.5 : Molarity of the DNA (40bp) nanoparticles with different amounts of PMAL-C8.....	90
Graph 3.6: Molarity of the DNA (20bp) nanoparticles with different amounts of PMAL-C8.....	91
Graph 3.7: Quantification of siRNA in the loaded nanoparticles with and without PMAL-C8	92
Graph 3.8: FACS results for eGFP plasmid DNA transfection. The cells transfected with the nanoparticles (blue) are compared to the cells transfected with lipofectamine (red) and the non transfected cells (gray)	96

LIST OF TABLES

Table 1.1: Dynamic light scattering size measurements results represented in Graph 1.1	17
Table 1.2: The different sequences obtained with cloning. The letters in RED had to be edited by analysis of the sequence output of the two primers T7 and SP6	28
Table 2.1: GPC results for PCL-PEG and PLGA-PEG	53

LIST OF SCHEME

Scheme 1.1: The padlock hybridizes on the two extremes of the template, the loop is sealed by the T4 ligase	13
Scheme 1.2: Template and padlock (left) will combine in a ring-structure (center) which will be amplified by Phi29 creating a DNA nanoparticle (NP) (center)	15
Scheme 1.3: Scheme of the biopanning used to select for DC binding sequences	25
Scheme 2.1: Representation of the nanoprecipitation process. The PLGA-PEG polymer dissolved in the organic solvent is added drop-by-drop into water. The nanoparticles will form by self-assembly in the aqueous solution .	42
Scheme 2.2: Polymer used in a porosity gradient and expected distribution at the water/polymer interface	44
Scheme 2.3: Polymer used in the chemical gradient and expected distribution at the water/polymer interface	51
Scheme 3.1: Structure of the hybrid nanoparticles with PMAL-C8/Oligonucleotides complex loaded.....	88

ACKNOWLEDGEMENTS

I would like to thank all the professors I have had the chance to meet and work with during my Ph.D. program. I appreciate all their suggestions and their encouragements. I have learned a lot from them, as they've all mentored me to become a creative scientist and a dependable leader. I am glad that Professor Sadik C. Esener took me in his group and gave me the chance to work with an extremely diverse and dynamic team at the Moores Cancer Center. In particular I am glad that I have had the chance to work in very fruitful collaborations with Professor Andrew Kummel and Professor Liangfang Zhang.

I am extremely grateful to all the students of the Nano Tumor Group, as they have made my Ph.D. experience unforgettable, with good healthy fun and always welcomed scientific discussions.

I would also like to thank Dr. Bradley Messmer, Dr. Ana B. Sanchez and Dr. Jennifer Y. Marciniak for sharing my same interest on the DNA-nanoparticles. With them the DNA project can keep growing even now that my thesis is done.

The work involving PLGA-based nanoparticles received the great contribution of all the group members of Professor Zhang. In particular I would like to thank Jack Hu for his feedbacks and help with grammar. A special thanks goes to Christoph Burkart and David Dangoor who have unveiled the mysteries of siRNA to me and shared their experience. I would also like to thank two of the most brilliant undergraduate I had the pleasure to train in the lab: Zeling Xu and Sorena Ramanian.

Finally I would like to thank the San Diego Fellowship for supporting part of my studies.

Chapter 1 is mostly based on the material as it appears in Journal of Biotechnology 2009. Sartor, Marta; Steiner, Jason M.; Sanchez, Ana B.; Messmer, Davorka; Freed, Ana; Esener, Sadik; Messmer, Bradley T.. The dissertation author was the primary investigator and co-author of this paper.

Chapter 2, in part, has been submitted for publication of the material as it may appear in ACS Nano, 2010, Sartor, Marta; Xu, Zeling; Aryal, Santosh; Hu, Che-Ming J., Pornpattananangkul, Dissaya, Clawson, Corbin; Esener, Sadik; and Zhang Liangfang. The dissertation author was the primary investigator and author of this paper.

Chapter 3, in part is currently being prepared for submission for publication of the material. Sartor, Marta; Burkart, Christoph; Dangoor, David; Zhang Dong-Er; Esener, Sadik; Zhang, Liangfang. The dissertation author was the primary investigator and author of this material.

VITA

- 2005 Laurea in Fisica. Università degli studi di Padova. (Padua, Italy)
Equivalent to a Master in Physics
- 2005-2010 Research Assistant. University of California, San Diego
- 2010 Doctor of Philosophy. University of California, San Diego

PUBLICATIONS

C. Pacholski, M. Sartor, M. J. Sailor, F. Cunin, G. M. Miskelly, "Biosensing using porous silicon double-layer interferometers: Reflective interferometric Fourier transform spectroscopy". *Journal of the American Chemical Society* 2005, 127, 11636.

M. Sartor, J. M. Steiner, A. B. Sanchez, D. Messmer, A. Freed, S. Esener, B. T. Messmer, "DeNAo: Selectable deoxyribonucleic acid nanoparticle libraries". *Journal of Biotechnology* 2009, 145, 330.

D. Pornpattananankul, S. Olson, S. Aryal, M. Sartor, C. M. Huang, K. Vecchio, L. Zhang, "Stimuli-Responsive Liposome Fusion Mediated by Gold Nanoparticles". *ACS Nano* 2010, 4, 1935.

C. M. J. Hu, S. Kaushal, H. S. T. Cao, S. Aryal, M. Sartor, S. Esener, M. Bouvet, L. Zhang, "Half-Antibody Functionalized Lipid-Polymer Hybrid Nanoparticles for Targeted Drug Delivery to Carcinoembryonic Antigen Presenting Pancreatic Cancer Cells". *Molecular Pharmaceutics* 2010, 7, 914.

M. Sartor, Z. Xu, S. Aryal, C. M. J. Hu, D. Pornpattananankul, C. Clawson, S. Esener, L. Zhang. "Synthesis of Polymeric Nanoparticles with Hydrophobicity Gradient Interior Structures". Submitted to *Advanced Functional Materials*.

M. Sartor, C. Burkart, D. Dangoor, D. E. Zhang; S. Esener, L. Zhang, "Amphiphile-assisted oligonucleotides delivery by lipid-PLGA hybrid nanoparticles". In preparation for submission to *Biomaterials*.

S. D. C. Kong, M. Sartor, L. Zhang, S. Jin, "Magnetically controlled drug release from polymeric nanoparticles". In preparation for submission to *Journal of Controlled Release*.

FIELDS OF STUDY

Major Field: Drug delivery and Nanoengineering

Studies in Cancer therapeutics
Professor Sadik Esener

Studies in Nanoparticles and drug delivery
Professor Liangfang Zhang

ABSTRACT OF THE DISSERTATION

Synthesis of novel polymeric nanoparticles for hydrophobic and hydrophilic drug delivery

by

Marta Sartor

Doctor of Philosophy in Materials Science and Engineering

University of California, San Diego, 2010

Professor Sadik Esener, Chair

Modern medicine has achieved extraordinary results with the use of nanotechnologies. The combination of the two disciplines created the modern field of nanomedicine, in which drug delivery is one of the most prominent branches. Several aspects are involved in drug delivery; this work will focus on the drug delivery vehicle. In particular three aspects will be investigated: building material, internal structure and material compatibility.

In a first project DNA was proposed as an innovative building material. DNA nanoparticles were made from self-folding of long concatameric repeats of a single strand sequence. Nanoparticles with different sequences created a library that was bio-panned against dendritic cells (DC). Particles from the enriched library were sequenced

and individually tested for affinity towards DC. The use of DNA as building material offers several advantages. For instance DNA binding drugs (such as Doxorubicin) can be easily incorporated, and immunostimulatory sequences (such as GpC) and any other encoding sequence can be integrated within the concatamers. In addition, any other molecule or small particle of interest can be conjugated to a short complementary sequence and hybridized on the outer layer of the DNA nanoparticle.

DNA nanoparticles' payloads are limited to hydrophilic drugs. In addition to an hydrophobic payload, some therapies require a high loading and steady release. To achieve such results a gradient structure was created within the core of a polymeric nanoparticle. Physical and chemical gradient were considered. A chemical gradient was created by combining a low molecular weight polycaprolactone (PCL) to a higher molecular weight poly(lactic-co-glycolic acid) (PLGA). PCL and PLGA have different degradation rate and hydrophobicity. The particles created by combining the two polymers showed properties (such as loading) dependent on the two polymers' proportion into the composition. The chemical gradient nanoparticles are characterized by high loading and steady release of hydrophobic drugs.

A new technique was examined to encapsulate hydrophilic oligonucleotides into hydrophobic nanoparticles. PMAL-C8, a zwitterionic polymer, interacts with oligonucleotides of different length and improves their loading into the hydrophobic core. The nanoparticles created were successfully used in transfection experiments, showing the bioavailability of the loaded oligonucleotides.

INTRODUCTION

Cancer is one of the biggest challenges that modern medicine is facing. It is not a single disease, but a group of more than 100 different diseases characterized by uncontrolled growth, invasion and sometimes metastasis (National cancer institute: www.cancer.gov). According to the American Cancer Society “half of all men and one-third of all women in the US will develop cancer during their lifetimes. Today, millions of people are living with cancer or have had cancer.” (www.cancer.org) Not surprising cancer is the second leading cause of death in the US.

Cancer is not a new condition, as the origin of the name is credited to Hippocrates (460–370 B.C.) and the oldest description of cancer can be found in an ancient Egyptian text book on trauma surgery (1600 B.C.). Moreover, there are archeological evidences of cancer among fossilized bone and Egyptian mummies. Despite its older origin in history, the significant increase of life expectancy achieved with modern medicine created an older and healthier population in which the dramatic effects of cancer are more evident.

The modern clinic can offer a high variety of therapies against cancer, while for many years in the past, the only available treatment involved surgical removal of the advanced malignant mass. Since not all cancers form a mass, serious conditions such as leukemia didn't have for a long time a cure. Nowadays, the therapies are more targeted, personalized and less toxic; but above all, they are part of a multidisciplinary effort. Since Marie Curie proposed the use of radiation to treat cancer, doctors have begun to work in close collaboration with other scientists. The modern technological advances played a significant role in the progress achieved on prevention and early

stage detection of cancer. High precision instrumentations of today allows us to visualize small tumor masses without the need of invasive exploratory surgery. The importance of multidisciplinary approach is reflected on the birth of the contemporary combined disciplines such as biophysics, nanomedicine, bioengineering and computational biology. The results of this new approach can be seen on the Human Genome Project. According to the National Institute of Health (NIH), thanks to the Human Genome Project “over 70 common genetic variants have been discovered that are associated with risk for 15 cancers to date, including cancers of the breast, prostate, colon, pancreas, brain, bladder, testis, and lung, as well as chronic lymphocytic leukemia and non-Hodgkin lymphoma.” (nih.gov) “In 2007—for the first time in history—the absolute number of cancer deaths in the United States went down.”

It can be concluded that the most effective approach against cancer is the result of team efforts. Along with the increased medical knowledge, it is important to keep an open dialogue and interface with other disciplines to pursue the most efficient therapy against cancer. It is from this multidisciplinary dialog that nanomedicine was born to offer the most impressive tools to work and look inside the body from the outside. Advances in nanoengineering has made it possible to create small, targeted vehicles which can be successfully used in many stages of the cancer therapy such as imaging, early stage detection and aimed drug delivery[1].

Drug delivery is one of the more important fields in nanomedicine. Targeted nanoparticles are often used as delivery vehicles, because they can deliver highly toxic drugs to the malignancy without affecting the healthy cells[2-6]. Three main intertwined aspects involved in creation of the perfect nanoengineered therapy are payload, targeting and delivery vehicle. The research for the most effective small molecule

therapeutics has brought a wide range of payloads, while the studies on cancer cells have provided a big array of possible targets and therapeutical approaches. For this reason, it is important to complement this variety by offering a range of efficient delivery vehicles. Some of the challenges encountered in delivering part of the newly discovered drug can be overcome by the opportune nanoparticle. For instance, a highly hydrophobic drug can be delivered by using particles with a hydrophobic core.

Despite the effort on creating the perfect delivery vehicle, only a small number of nanoparticles are available to the clinic. Only few materials have been approved by the Food and Drug Administration (FDA). Any new proposed vehicle would need to undergo extensive studies to understand its possible immunogenicity and toxicity[7, 8].

Considering all the requirements for a fast adoption into the clinic, this work will address three different aspects of a nanoparticle:

- building material
- internal structure
- material compatibility

All three aspects present significant challenges that are important to address. During this work we have examined these aspects in detail and attempted to put forward simple and practical approaches. To be more suitable for a clinical study this work has mostly focus on two different types of nanoparticles: DNA nanoparticles and poly(lactic-co-glycolic acid) (PLGA) based polymeric nanoparticles. Both DNA and PLGA are biocompatible and biodegradable, in addition both materials are already in use in the clinic.

Our contribution is essentially based on the utilization of the unique properties of known and well characterized materials in a new way to generate smarter and more flexible drug delivery vehicles. We believe these new vehicles will pave the way for new research and significantly improved capabilities. The goal of this work therefore goes beyond creating a novel nanoparticle, as it aims to offer a novel approach to the creation of a nanoparticle. From the results presented here new applications and ideas can be developed.

BUILDING MATERIAL

DNA has been extensively used and studied, but it has not been considered as a building block of nanoparticle. Oligonucleotides have received a lot of attention because they can have therapeutical (gene therapy) and targeting abilities. DNA (or RNA) small targeting units are called aptamers. The potentials of these small units have been thoroughly described in a recent review[9]. Aptamers were initially discovered and used because they could be selected to bind with high specificity to a protein or part of a protein. Today aptamers can be selected to bind to cells and can be used to do more than just targeting. For example, aptamers can be used as extracellular therapeutics to block the coagulation cascade[10]. One of the more important limitation of aptamers is their size. They are so small that they have to be conjugate to a long polymer (such as polyethylene glycol (PEG)) to increase their circulation half-life from 5-10 minutes to 12 or 24 hours[11].

The DNA nanoparticles proposed in this work offers more than a higher molecular weight targeting unit. They are made of concatameric repeats from a

randomized library, hence their bigger size. The DNA nanoparticles present a complex DNA structure at their surface, this structure can account for a higher avidity of a repeating epitope. Alternatively, a DNA nanoparticle can offer the possibility of selecting not for a single protein or location, but for a specific combination of proteins. This new aspect can lead to a higher degree of specificity respect to the aptamers. The flexibility of the DNA allows to hybridize on the surface small units (e.g. peptides, nano-golds) as well as the integration within the sequence of small immunostimulatory sequences (CpG)[12]. Moreover the DNA nanoparticles can potentially find future application as delivery vehicles. In such application DNA nanoparticle could be loaded with DNA-binding chemotherapy drugs like doxorubicin[13] and offer a highly specific delivery vehicle.

INTERNAL STRUCTURE

Not all the drugs available are hydrophilic or DNA-binding and most therapies require a steady release within the “effective window” of the considered drug. It is therefore important to have a slow release, targeted nanoparticle with a hydrophobic core. Poly(lactic-co-glycolic acid) (PLGA), often as block-polymer with polyethylene glycol (PEG), is one of the most commonly used materials to deliver hydrophobic drugs such as paclitaxel.

PLGA nanoparticles can offer a good loading and a slow release of paclitaxel[14], although the loading depends on method of fabrication, and often a higher loading is associated to a bigger size. In addition the release profile cannot be easily modified. A

slower release can be achieved with the use of a shell made by a blend of polymer[15] or with the use of a lipid layer[16].

In this work we have modified the internal structure of the PLGA nanoparticle. The nanoparticles are created by nanoprecipitation of PLGA-PEG block-polymer. This one-step scalable process allows to achieve a small size (80-100nm) of fairly uniform population[17]. The modification of the internal structure is achieved by using two different length of hydrophobic PLGA tails. The densely populated interface with water decreases towards the core, because of the presence of two different length PLGA. A porosity gradient core structure can load less drug in the shell and more into the core. While the shell is contemplated to be densely packed with small pores, the core is characterized by a bigger porosity and can easily trap more drug during precipitation. The gradient structure affects the release profile as well. The drug in the core has to move towards increasingly dense layers; the encountered difficulty of migration slows the diffusion.

A further degree of control on loading and release can be achieved by combining both the use of two different length of hydrophobic tails and two different materials. Polycaprolactone (PCL) is another FDA approved hydrophobic polymers that has been used in combination with PLGA[15]. PCL is more hydrophobic and has a slower degradation rate than PLGA. In this new structure a loading increase with the use of a more hydrophobic polymer can be envisioned, along with a slower release due to the slow degrading PCL. Such a structure offers a chemical gradient along with a porosity gradient and it has a significant role on possible future applications in combined drug therapy. Researchers has shown the positive effect of combined drug therapy[18]; a

chemical gradient structure will be an excellent candidate to use for future work to combine and possibly modulate the release of two drugs with different hydrophobicity.

MATERIAL COMPATIBILITY

Both vehicles described so far can only load drugs compatible with the material comprising the core; thus, DNA nanoparticles can only load DNA-binding drugs or hydrophilic drugs hybridized onto them, while PLGA-based nanoparticles can only load hydrophobic drugs.

Double stranded oligonucleotides (DNA and RNA) have been chosen in this work as hydrophilic units to load inside a PLGA-based nanoparticle. This has been a high risk high return undertaking. The proposed method differs from the conventionally used techniques because it doesn't recur to highly charged lipids or peptides to load the oligonucleotides[19]. Our approach allowed us to load the oligonucleotides with the use of an amphiphilic polymer which will act as interface between the two incompatible materials. In this technique the hydrophilic oligonucleotides are made hydrophobic with a short polymer which is both hydrophilic and hydrophobic. PMAL-C8[20] was chosen to act as interface between different oligonucleotides and PLGA. The new method was thoroughly analyzed and it gave new insight for further improvements and applications. It will be a starting point to create an even better tailored amphiphilic polymer and it may offer the basis for an even higher loading core. What's more important we hope we may be able to offer a solid approach for future work aimed to combine different payloads otherwise incompatible with the polymeric core.

In each of the above described approaches all the materials have been combined or used in a new way, pushing the limits of the state of the art structures. DNA was used as a building block material in a DNA nanoparticle, where it maintained its targeting properties. Improved loading and release profiles were achieved by modifying the internal structure of polymeric nanoparticles. The uniform core was substituted with a gradient core characterized by a physical and a chemical gradient. Hydrophilic and hydrophobic materials were combined with the use of a chaperon polymer, PMAL-C8. PMAL-C8 act as transition between the two environments.

BIBLIOGRAPHY

- [1] S. Sandhiya, S. A. Dkhar, A. Surendiran, *Fundamental & Clinical Pharmacology* **2009**, 23, 263.
- [2] R. Singh, J. W. Lillard, *Experimental and Molecular Pathology* **2009**, 86, 215.
- [3] H. Cabral, K. Kataoka, *Science and Technology of Advanced Materials* **2010**, 11.
- [4] Y. Malam, M. Loizidou, A. M. Seifalian, *Trends in Pharmacological Sciences* **2009**, 30, 592.
- [5] M. Ferrari, *Trends in Biotechnology* **2010**, 28, 181.
- [6] M. Wang, M. Thanou, *Pharmacol Res*, 62, 90.
- [7] B. S. Zolnik, A. Gonzalez-Fernandez, N. Sadrieh, M. A. Dobrovolskaia, *Endocrinology* **2010**, 151, 458.
- [8] M. A. Dobrovolskaia, D. R. Germolec, J. L. Weaver, *Nature Nanotechnology* **2009**, 4, 411.
- [9] A. D. Keefe, S. Pai, A. Ellington, *Nat Rev Drug Discov*, 9, 537.
- [10] R. C. Becker, T. Povsic, M. G. Cohen, C. P. Rusconi, B. Sullenger, *Thrombosis and Haemostasis* **2010**, 103, 586.
- [11] J. M. Healy, S. D. Lewis, M. Kurz, R. M. Boomer, K. M. Thompson, C. Wilson, T. G. McCauley, *Pharm Res* **2004**, 21, 2234.
- [12] J. Kindrachuk, J. Potter, H. L. Wilson, P. Griebel, L. A. Babiuk, S. Napper, *Mini-Reviews in Medicinal Chemistry* **2008**, 8, 590.
- [13] V. Bagalkot, O. C. Farokhzad, R. Langer, S. Jon, *Angewandte Chemie-International Edition* **2006**, 45, 8149.
- [14] F. Danhier, N. Lecouturier, B. Vroman, C. Jerome, J. Marchand-Brynaert, O. Feron, V. Preat, *J Control Release* **2009**, 133, 11.
- [15] J. K. Jackson, T. Hung, K. Letchford, H. M. Burt, *Int J Pharm* **2007**, 342, 6.
- [16] J. M. Chan, L. F. Zhang, K. P. Yuet, G. Liao, J. W. Rhee, R. Langer, O. C. Farokhzad, *Biomaterials* **2009**, 30, 1627.
- [17] J. Ren, H. Y. Hong, J. X. Song, T. B. Ren, *Journal of Applied Polymer Science* **2005**, 98, 1884.
- [18] L. D. Mayer, A. S. Janoff, *Molecular Interventions* **2007**, 7, 216.

- [19] C. Qiu Zhong, D.M.D., Pamujula, S., Haifan Wang, Xin Yao, Mandal, T.K., Luftig, R.B., *Journla of Nanobiotechnology* **2010**, 8, 10.
- [20] J. L. Popot, E. A. Berry, D. Charvolin, C. Creuzenet, C. Ebel, D. M. Engelman, M. Flotenmeyer, F. Giusti, Y. Gohon, P. Herve, Q. Hong, J. H. Lakey, K. Leonard, H. A. Shuman, P. Timmins, D. E. Warschawski, F. Zito, M. Zoonens, B. Pucci, C. Tribet, *Cellular and Molecular Life Sciences* **2003**, 60, 1559.

CHAPTER 1 DNA AS BUILDING MATERIAL: DNA NANOPARTICLES

1.1 INTRODUCTION: HYDROPHILIC NANOPARTICLES

Researchers have created a broad spectrum of hydrophilic drug delivery vehicles. The most popular among them are nanogels and liposomes. Liposomes have been extensively studied in the last decade and some of their formulations are available in the clinic for drug delivery[1-3]; Doxil® and Daunoxome® are examples of the most successful liposome assisted drug delivery systems. Despite the wide use and well studied structure, applications of liposomes are hindered by stability issues. Nanogels were first created as oligonucleotide delivery systems, but their ability to deliver drug and their stability have made them excellent competitor against liposomes[4, 5]. Nanogels are mostly hydrogel, protein or chitosan based nano size gel particles. The elements are cross-linked in a gel and they can load proteins or drugs. In general, nanogels can offer an additional property of an environmental response (temperature or pH). Particularly, hydrogel based nanogel offers a high degree of different responsiveness based on type and strength of the cross-linker used.

Along with nanogel and liposome, inorganic nanoparticles such as iron oxide[6] and silica[7] have found applications in drug delivery. These platforms can load drug and release it with or without the presence of an environmental stimulus, but their targeting ability entirely depends on the type and quality of the targeting ligands that can be conjugated onto them. Often antibodies are used as surface targeting moiety, and they need to be used in a well balanced number since they can compromise the stealth

properties of the particle. It is indeed extremely important that the targeting moiety won't trigger an immune response, decreasing the effectiveness of its function.

In this highly competitive scenario of co-presence of drug delivery and targeting, DNA offers significant advantages over other building materials: encoding. DNA can be seen both as a building block and a code; it can create organized structures and load DNA binding drugs (such as doxorubicin[8]), as well as it can be translated into a protein or an RNA sequence. DNA nanoparticles have by default an immediate advantage over other types of nanoparticles on the quantity and type of information they can deliver.

Extensive research has been focusing on the use of oligonucleotides as therapeutics[9-11], and targeting oligonucleotides (aptamers) are already used in the clinic. Macugen® is the most successful example. It is the first FDA approved (2004) anti-VEGF aptamer–poly ethylene glycol conjugate in the treatment of age-related macular degeneration therapy[12].

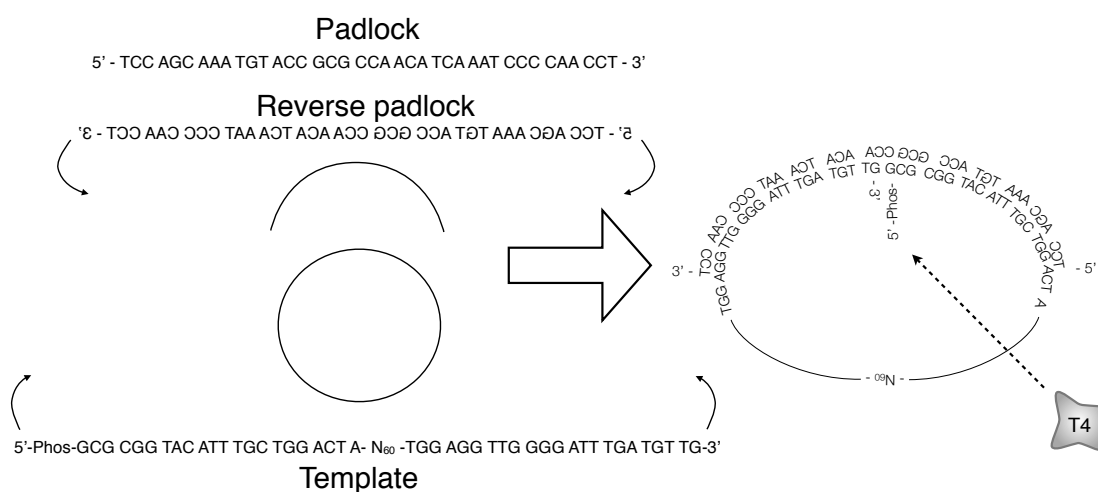
DNA nanoparticles can be created by rolling circle amplification (RCA)[13]. One of their first application was to amplify the detection signal from microarrays[14], but as today they've been used on a high variety of detection assays[15-21], transformed into high efficiency fluorescent tags[22] or attomolar-sensitive detection systems[23], but more strikingly, into tools for genome sequencing[24, 25].

In this work, DNA nanoparticles of about 250nm have been created with a 30min RCA reaction and are bio-panned against a target. Dendritic Cells (DC) were chosen as a target with consideration of possible future applications such as vaccine[26] or adjuvant[27, 28]. The bio-panning process used for the DNA nanoparticles, named DeNA_{no}, is somewhat similar to the method used for aptamer selection, but it differs in one important aspect. While the latter targets one element, the former can bind to a

combination of different targets. Consequent to this multiple target ability and avidity, it is expected that the DNA nanoparticles can carry a higher specificity for the target than a regular aptamer.

1.2 DNA NANOPARTICLES: ENGINEERING AND PRODUCTION

The nanoparticles are created from a DNA loop obtained by the hybridization of a padlock sequence on a template sequence (Scheme 1.1). The template is the working sequence, about 100 bases long, and at its ends there are two priming sites of 22-24 bases. The core of the template sequence is made of 60 random bases, creating a stock library with approximately 10^{36} different combinations. The padlock sequence is about 39 base pairs, and it is based on the primers of the template. First half of the sequence contains part of the complement of the 3' end and remaining part is a fragment of the reverse complement of the 5' end.

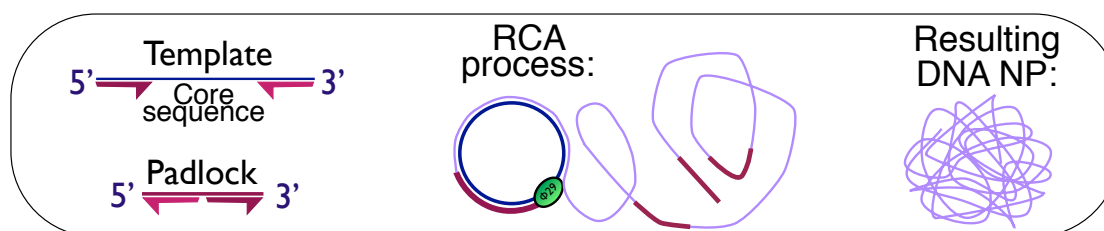


Scheme 1.1: The padlock hybridizes on the two extremes of the template, the loop is sealed by the T4 ligase.

Padlock and template are annealed together in presence of T4 ligase in a reaction with a three times molar excess of padlock. The reaction is performed at room temperature and sometimes, for better results, left overnight at 4°C. The ligase enzyme needs the phosphorylation of the 5' end of the template. It requires a 5'-phosphate and a 3'-OH to link two oligonucleotides (phosphodiester bond is formed).

Considering the hybridization process, the 5' and the 3' ends of the template are locked into a circle by the padlock (Scheme 1.1). The DNA nanoparticles are created in an isothermal reaction with the DNA polymerase ϕ 29. This polymerase has an inherent 3'→5' proofreading exonuclease activity for a more reliable replication, although the use of a less precise enzyme can introduce random changes that can potentially lead to “evolving particles”.

Phi29 will bind to the 3' terminus (writing 5'→3') of the padlock and will start adding to it the complementary sequence of the template. Once the 5' terminus of the padlock is reached, it will displace the sequence and continue with the duplication until either there are no more nucleotides available or it is heat/EDTA inactivated. In this reaction, EDTA (EthyleneDiamineTetraAcetic acid) removes the Mg^{2+} and Ca^{2+} that Phi29 needs to be functional. During this research, 50mM EDTA was used to inactivate the Phi29 enzyme. The created sequence is made by concatamers of the complementary sequence of the template. It will tend to self-hybridize based on the sequence affinity and the electrostatic interactions (Scheme 1.2). According to the scheme Phi29 polymerase will bind to the padlock and start creating concatameric copies of the template sequence. The resulting DNA single strand will self-hybridize in a ball-like structure[13, 22, 24].



Scheme 1.2: Template and padlock (left) will combine in a ring-structure (center) which will be amplified by Phi29 creating a DNA nanoparticle (NP) (center).

The DNA nanoparticles created by RCA were incubated with Sybr Green I and imaged with a fluorescent microscope under 100x magnification (Figure 1.1), the figure shows the presence of discrete particles. The particles size was characterized by dynamic light scattering measurements (Graph 1.1), and its diameter was determined to be of about 280nm, for a 30 minutes RCA reaction (Scheme 1.1). Independent experiments were in agreement with this size measurement and showed that the particles size was dependent with the reaction time of the Phi29, corroborating the hypothesis of a growing particle.

During the selection process, the RCA reaction was qualitatively evaluated by monitoring the signal of OliGreen (Invitrogen) incubated in the reaction mix. OliGreen is a dye used to quantify oligonucleotides and ssDNA. Monitoring the dye intensity change at 525nm allows to observe the progress of the RCA reaction (Graph 1.2). Little to no increase in intensity can be associated with poor or low ligation of the padlock to the template. This evaluation step offers good clues for quality control of the RCA products. As it can be seen from the graphs in Graph 1.2, the selection rounds 6 and 7 showed saturation on the intensity signal before the 30 minutes reaction time. It can be assumed that the concentration of the loops padlock/template for these selection rounds are

higher than for the other, and that the saturation of the signal can be related to either interference by nearby fluorophores or to insufficient amount of free fluorophores.

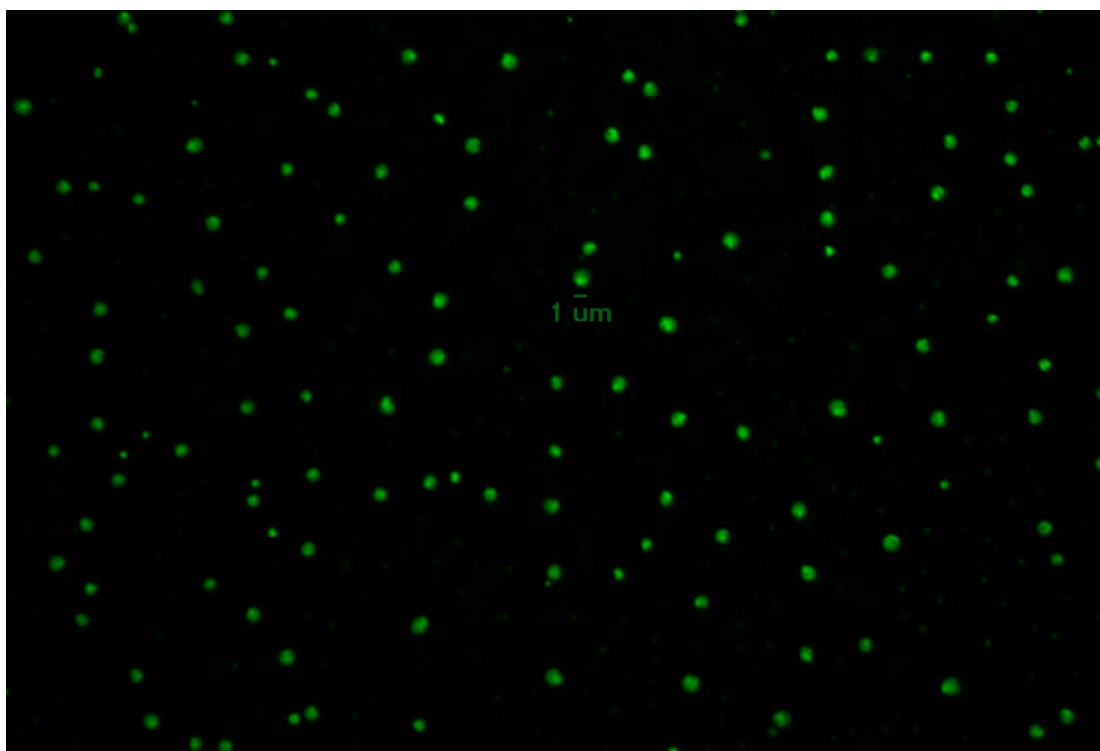


Figure 1.1: DNA nanoparticles with Sybr Green I under a 100X microscope lens.

Graph 1.1: Dynamic light scattering size measurements of DNA nanoparticles made with the same sequence.

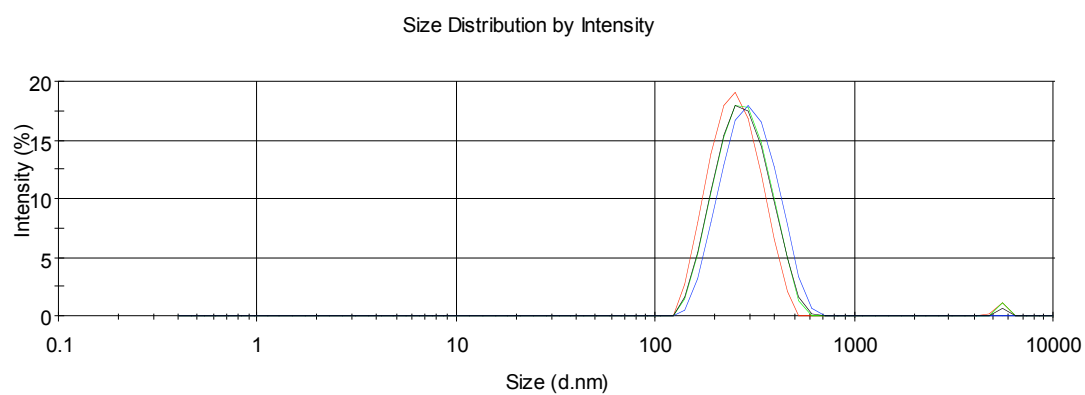
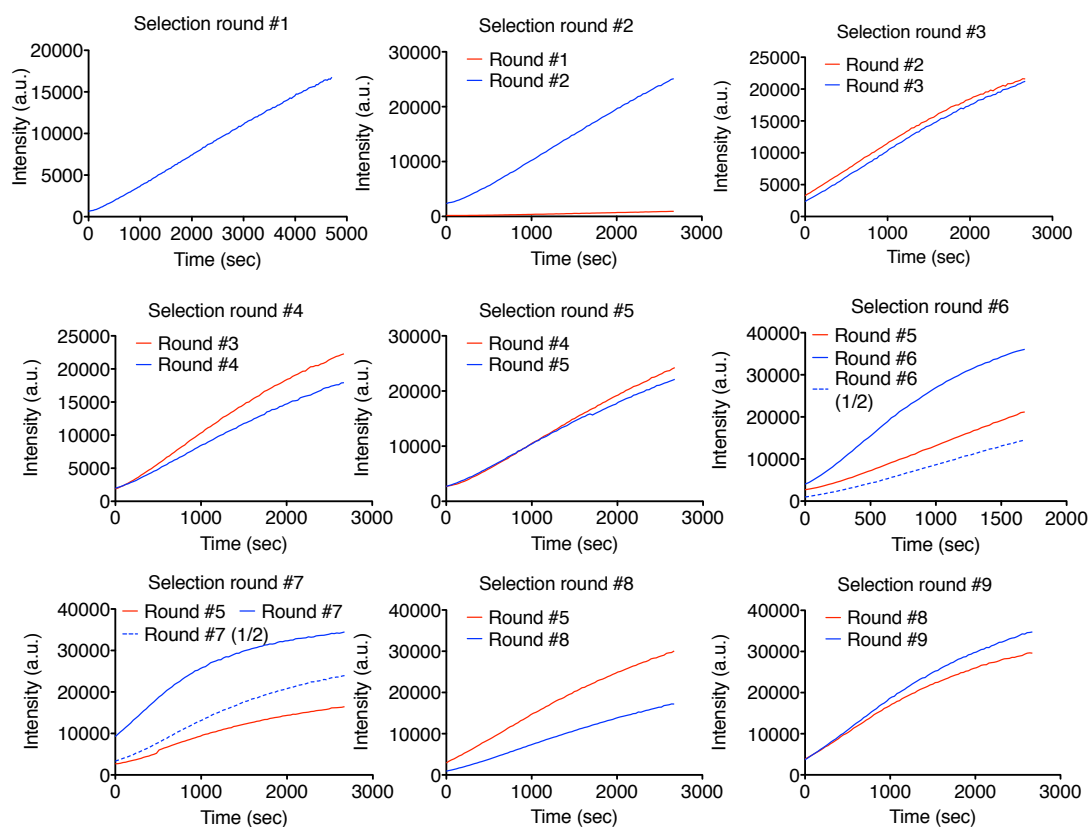


Table 1.1: Dynamic light scattering size measurements results represented in Graph 1.1.

SAMPLE NUMBER	Z-AVERAGE (nm)	PDI
1	267.1	0.274
2	283.3	0.349
3	288.9	0.257

Graph 1.2: The RCA reaction monitored through the increase of fluorescence of Oligreen.



1.3 SELECTION PROCESS

The RCA products created from the template library formed a final pool of about 3×10^{10} different sequences. The pool was incubated for one hour with 10^5 DC extracted from the blood of a donor. The cells were harvested and kept on ice for about 20 minutes before being combined with the DNA nanoparticles. The temperature was maintained during the entire incubation process. Low temperature is important to put the DC on a dormant state to prevent their activation and the consequent engulfment of the DNA nanoparticles during incubation. Unbound DNA nanoparticles were removed by washing the cells. In the first two cycles, only three washes were done, but from the third selection round on, the cells were washed five times. The washes were done by centrifuging the test tube with the cells for 3 minutes at 3000 r.p.m.. Then, supernatant was removed, and the DC were resuspended in PBS 5% BSA (Bovine Serum Albumin). The resuspended cells were transferred to a new tube for further wash. To prevent nonspecific binding, the test tube used for the incubation and the following washes were pre-blocked with a 5% BSA in PBS solution.

After this first incubation only some of the sequences from the initial pool have shown affinity to the DC and were left in the solution after the washes. In this first selection round, it is expected that every DNA nanoparticle is unique. Therefore, the rinses were gentle and the whole collected material was used. With the progress of the selectivity, it is expected to have an increasing number of targeting particles. When the number of DC-specific particles is high, it is normal to notice some agglutination effect on the cells during the washes. In particular, the pelleted cells will be resuspended with a stronger effort. For example, in the fifth round of selection, part of the material was

accidentally spilled in attempt to re-suspend the cells, by pipetting up and down to break the pellet.

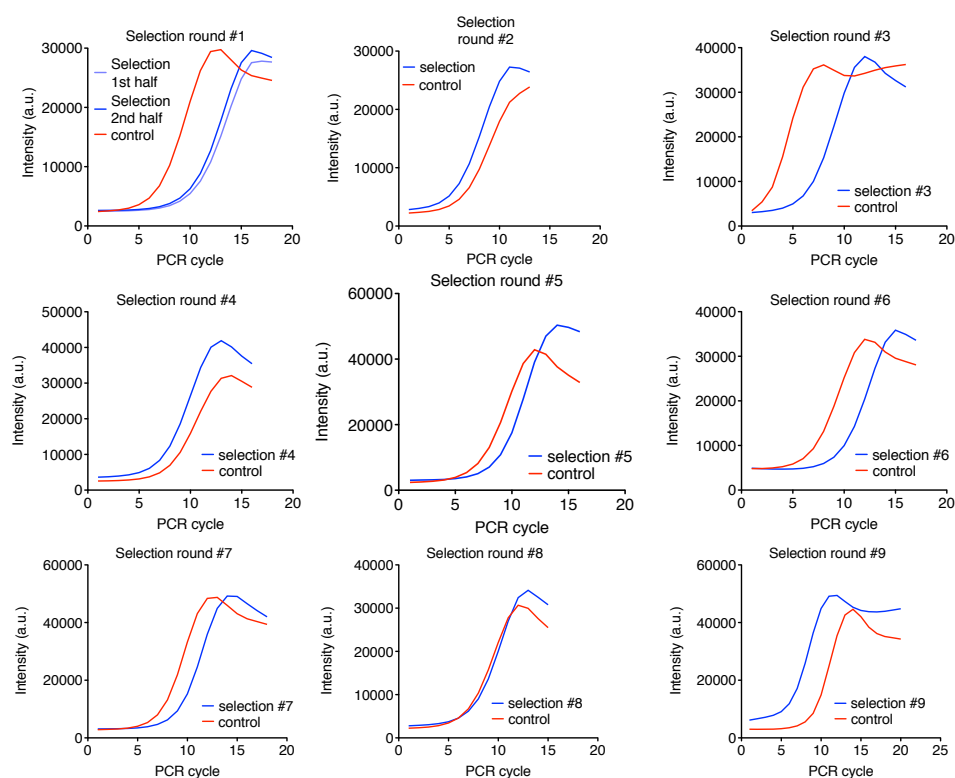
To reconstruct an enriched pool of templates, it is necessary to amplify the fragments obtained on the selection (Graph 1.3). The template has, by design, two priming sites at the 3' and 5' ends, and a simple PCR can be performed to create multiple copies of the DC-targeted DNA nanoparticles. Before the amplification step, the DC were lysed to avoid the interference by the heat-degraded proteins during the PCR heating cycles. Proteinase K was used to lyse the DC in a hypotonic buffer. The enzyme was heat inactivated and the entire lysate was used for PCR. A DC lysate without any nanoparticle at all was amplified as a negative control to make sure that the set of primers used won't amplify any fragment of the nucleic DNA (Graph 1.4).

In order to better visualize and interpret the progress, a real-time PCR was performed. A dissociation curve was obtained after the last amplification cycle. During the first cycle of selection, the material was divided into two aliquots to be amplified in the proper volume for real-time PCR. The two aliquots were recombined during ligation in the following round. It is expected that from the second selection cycle, several copies of the same sequence are available in the enriched pool, and therefore only a small aliquot was used for real-time PCR.

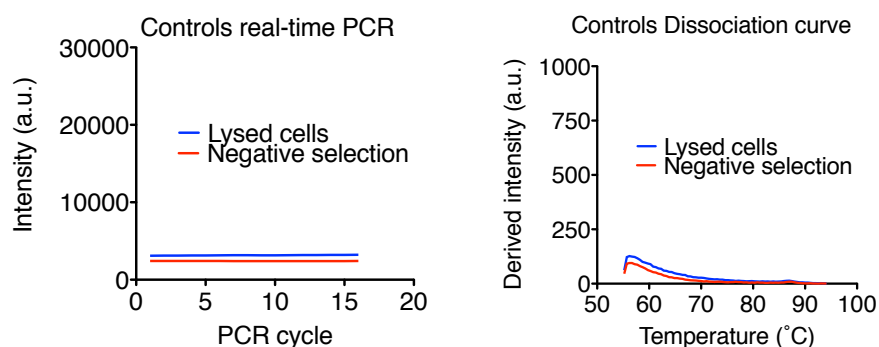
Real-time PCR can reveal the variation in concentration between different selection cycles. The number of PCR cycles necessary to amplify the sample are proportional to the initial DNA concentration. An enriched library will present more sequences with affinity for DC, therefore more DNA will remain in the sample after removal of the nonspecific sequences. The dilution of the initial library was amplified along with the sample to account for enzymatic activity fluctuations and to provide a

reference between different selection rounds. Additional evaluation were performed after a few cycles of bio-panning. To quantify the nonspecific binding, a selection was done in an empty tube and amplified in real-time PCR. Little to no signal was seen (Graph 1.4), indicating that no selection was done for the sticky particles.

Graph 1.3: The different real-time PCR for each round of selection. In each set 2uL of 1nM library were used as a control. For the first round of selection the two halves were amplified in parallel. In all the other sets 2uL of the lysate only were used for the amplification.



Graph 1.4: Control real-time PCR were run for possible amplification of the lysed cells genome (blue) and of possible plastic binders/super amplifiers (red). The graph scale is set to be comparable with Figures 1.3 and 1.5.

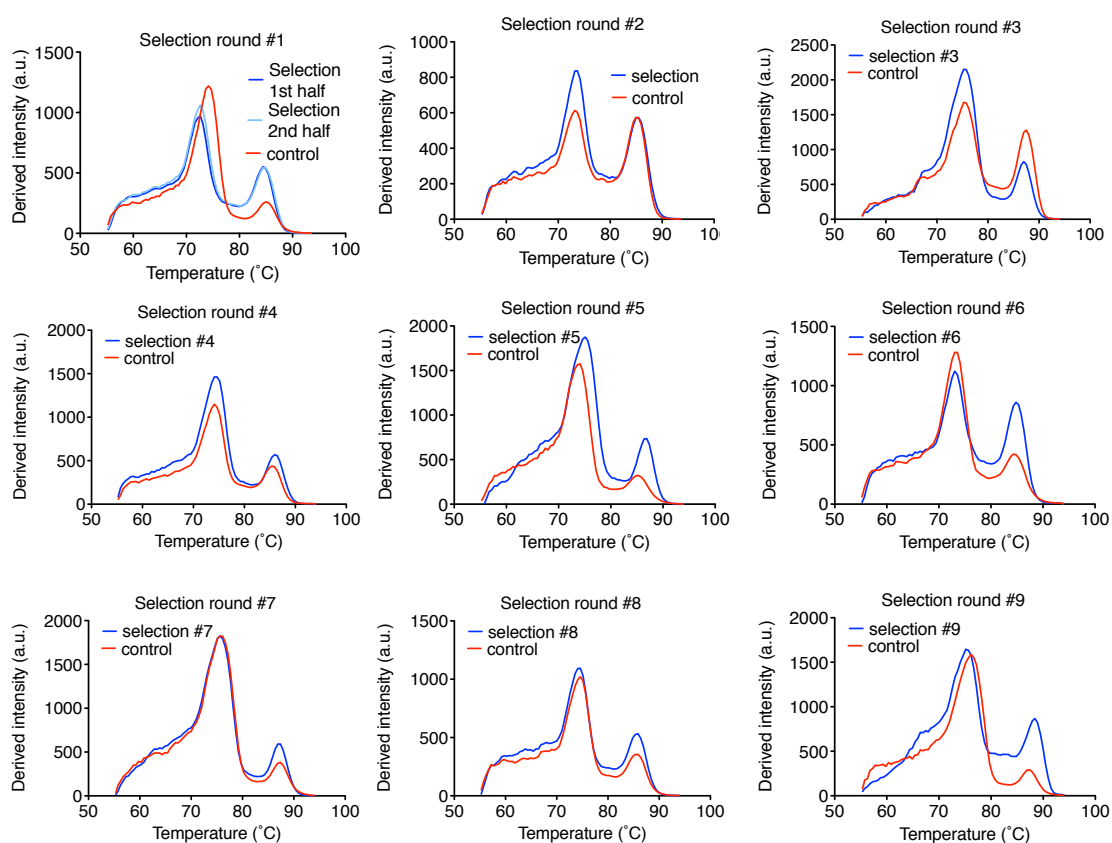


Further insight into the selection were given by observing the dissociation curves (Graph 1.5). In the initial library, several copies are present. Some of them might differ within a few bases. The dissociation temperature of such slightly mismatched sequences is lower than the ones shown by the perfectly matching ones. It is expected that the enrichment of the library can be associated with a peak at high dissociation temperatures. The entity and magnitude of this higher temperature peak is strictly dependent on the sequences types and composition. Therefore, it cannot be defined as a universal number for every selection or library. Despite not being universal, dissociation curves provide a valuable tool as quality control to monitor for contaminations.

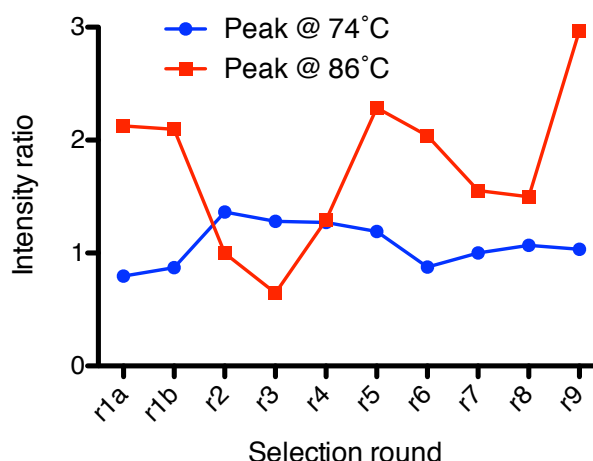
The two peaks of the dissociation curve are around the same value throughout the selection, but their height changed. To compare the different selection cycles, the intensity was related to the control (Graph 1.6). This gave a value lower than 1 when the selection peak was lower than the control. Comparing the different cycles in parallel, it is possible to see that, while the height of the lower temperature peak is the same for the different cycles, one of the peaks at higher temperature changes. The change is not

random. In fact, the value decreases when less material was used or harsher washes were done. Similar behavior was observed on a parallel experiment aimed to select for neutravidin. In this parallel experiment, the increase of the higher temperature peak was more evident. Despite the possible association of the higher intensity peak and the successful enrichment, extreme care needs to be taken when drawing conclusions. Further selection studies needs to be done to have a sufficient number of experiments to formulate statistically valid conclusions.

Graph 1.5: The dissociation curves of the different cycles (blue) show the variation of the higher intensity peak relative to the control (red).



Graph 1.6: The ratio of the peak height of the selection to that of the control shows no significant variation between selection rounds for the lower temperature peak, but a significant change for the higher temperature peak.



At each selection cycle, a small aliquot of the real-time PCR reaction was run on a 2.5% Agarose (Sigma) gel with RedGel dye (Biotium). The gel electrophoresis results were necessary to determine the possible presence of artifacts. For example, primer dimers and contaminations would have been seen easily in the electrophoresis gel because of their different size compared to the working template. The gel, in Figure 1.2 compares all the PCR products from different selection rounds. Analogous to the real-time PCR, the gel contains the unselected library as a control. It can be seen that the size is initially higher than the actual template size, and it gets closer to the actual values with the increasing selection rounds. The effect is not a gel artifact, as it shows the control positioned right next to the band of the last selection cycle. The variation of the position of the band can be explained as a higher number of perfectly matching sequences, according to the hypothesis made for the dissociation curve. A sequence hybridized to a better match will give less resistance while traveling through the electrophoresis gel, showing a lower band, closer to the actual template value. In

general, each template will have in common both priming sites at the extreme (for a total of 45 oligonucleotides), increasing the strength of hybridization for two mismatching sequences.

Once the enriched pool has been amplified, it is possible to re-create a template pool by performing a few cycles of an asymmetric PCR on a small aliquot of the real-time PCR products. The primer used for this PCR reaction had a phosphate on the 5' end. In this way, the reconstructed pool of template could have the same T4 ligation efficiency of the initial library. Unreacted primers and nucleotides can be removed from the asymmetric PCR by PCR cleaning. The enriched pool of template is now ready to be ligated into a circular sequence by the padlock, and the selection process can be repeated. The whole selection cycle is depicted in Scheme 1.3. The loop is repeated several times, after the first few cycles the enriched library is screened for significant enrichment against the initial library by flow cytometry. Once the peak shift recorded by Fluorescence-Activated Cell Sorter (FACS) is big enough, the selection process is stopped, and the enriched pool is analyzed by cloning.

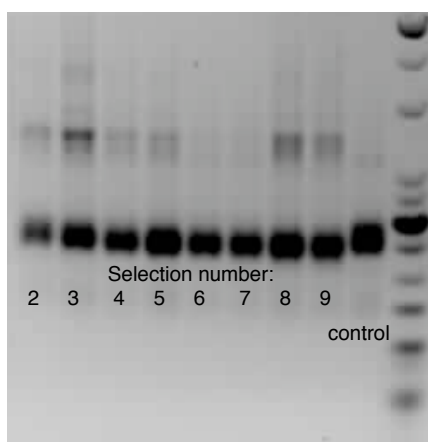
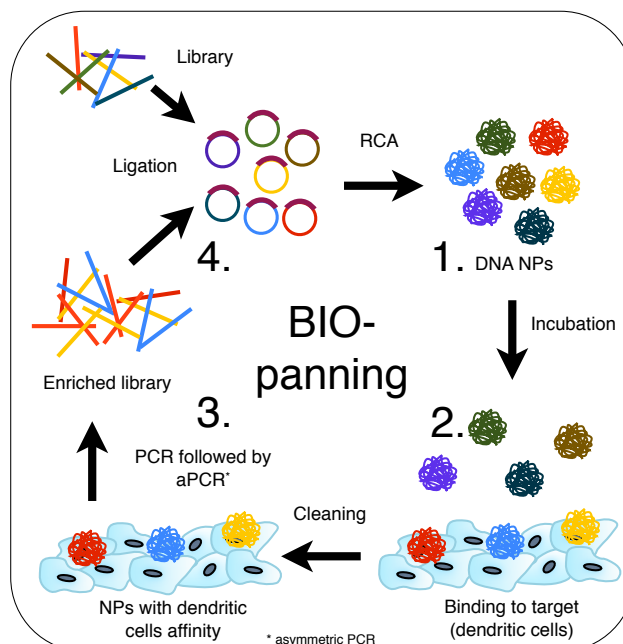


Figure 1.2: The amplified sequences out of the nine rounds of selection are compared by gel electrophoresis. The control is the amplified unselected library (in analogy with the real-time PCR).

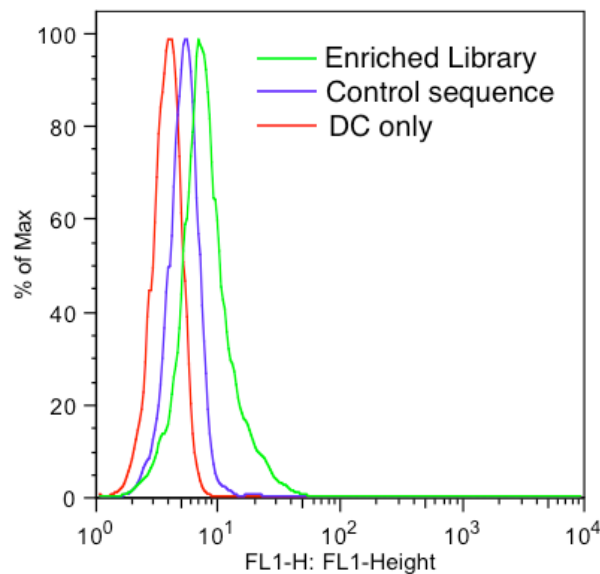


Scheme 1.3: Scheme of the biopanning used to select for DC binding sequences.

This selection procedure aims to enrich particles that strongly bind to DC and that amplify the best on PCR. At the ninth cycle, the degree of selection was tested by Flow Assisted Cells Sorting (FACS) (Graph 1.7). In brief, the particles were made in presence of Alexa-488 labelled nucleotides. The number of incorporated fluorescent nucleotides was sufficient to see the nanoparticles by FACS. The fluorescent DNA particles were incubated with the DC, and the cells were washed three times with a PBS based wash solution (FACS WASH) and then fixed with a PBS solution containing 3.7% formaldehyde (FACS fix). The unselected library defined our negative control for nonspecific binding. Additional controls were run to differentiate the nonspecific binding of free nucleotides and the baseline from untreated DC. After nine selection cycles, it was clear that there was a positive selection signal from the enriched pool (Graph 1.4).

At this point it was necessary to identify which of the sequences out of the pool was the best candidate.

Graph 1.7: FACS results of the binding of the ninth round of selection (enriched library) compared to a control sequence (CpG containing sequence) and the plain cells.



It is important to highlight that the bio-panning applied to a cell line or a defined cell population should always be accompanied by a negative selection against a non specific or similar cell line. For instance a selection on cancer should have a negative selection against healthy cells. In this experiment, the DC were harvested from the blood of different donors at different stages of their life-cycle, and this aspect might have induced a selection stress even without a negative selection.

1.4 IDENTIFYING THE SELECTED SEQUENCES

The templates from the pool recovered out of the ninth round of selection were cloned. The plasmids extracted from each of the 19 selected colonies were sequenced using both T7 and SP16 primers (Table 1.2).

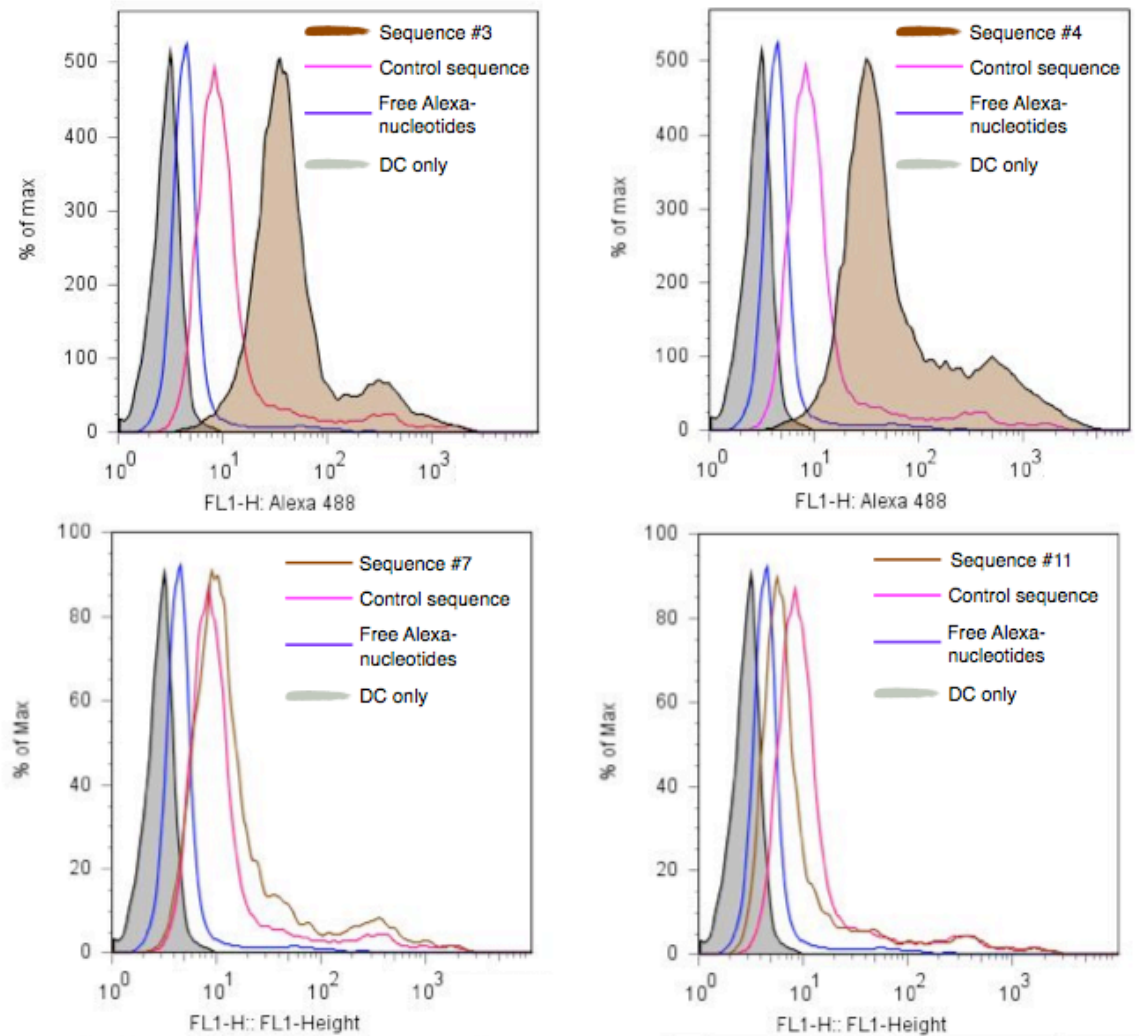
Out of all the sequences analyzed, only 15 gave results that were satisfying, and among them two gave the same sequence. When the selection process is done to find the appropriate aptamer, the expected enriched library will show either common sections in the sequence or a sequence that is common to more than one of the selected colony. In our DeNA process, the common key might be hidden behind the far more complex interactions within the sequence itself. It is possible that sequences that seem completely different will self-fold on a more complex structure with common surface moieties. In addition, it is possible that what the DNA nanoparticles have to offer is not a higher avidity for a single target but for a combination of targets that is specific for that cell line. For this reason, the plasmids of the discovered sequences were used to create a pool of identical templates to individually test for affinity towards DC.

Table 1.2: The different sequences obtained with cloning. The letters in RED had to be edited by analysis of the sequence output of the two primers T7 and SP6.

NAME	SEQUENCE
MS013 Clone 01	<u>GCNCGGTACATTTGCTGGACTA</u> ACAGGGGAAAGTGAAATAGTAGGCAGGAAT CAAATTAAAATGCGTAGGTCTTTTGGCAT <u>TGGAGGTTGGGGATTTGATGTTG</u>
MS014 Clone 02	<u>GCGCGGTACATTTGCTGGACTA</u> AGCAAGATCTCAAGGGGCGCAATAACTGAGA AGTCTACGATAAAATTACGTTATCAGGTT <u>TGGAGGTTGGGGATTTGATGTTG</u>
MS015 Clone 03	<u>GCGCGGTACATTTGCTGGACTA</u> TGCATGTTTCGTAGTTATATAGGGGGATTGTT TGATAGTCGGAACCGCTGTGCTCAAAGTT <u>TGGAGGTTGGGGATTTGATGTTG</u>
MS016 Clone 03	<u>GCGCGGTACATTTGCTGGACTA</u> TGCATGTTTCGTAGTTATATAGGGGGATTGTT TGATAGTCGGAACCGCTGTGCTCAAAGTT <u>TGGAGGTTGGGGATTTGATGTTG</u>
MS024 Clone 04	<u>GCGCGGTACATTTGCTGGACTA</u> TCGGATAACATCGGGGGCCGAGACCTAGTA AGGATGCAAGTAGAAAAGCT <u>TGGAGGTTGGGGATTTGATGTTG</u>
MS025 Clone 05	<u>GCGCGGTACATTTGCTGGACTA</u> GTACCCGCAGGGCGATGGTGCGCACGTGTGT GCATAGTATCATTATTCATGTGACGCT <u>TGGAGGTTGGGGATTTGATGTTG</u>
MS026 Clone 06	<u>GCGCGGTACATTTGCTGGACTA</u> GGGTCAATGGGCGGAGTCAGTGCATCTCATG TAAAGTTAGTAGACCGCTTTTTTCGAAGT <u>TGGAGGTTGGGGATTTGATGTTG</u>
MS027 Clone 07	<u>GCGCGGTACATTTGCTGGACTA</u> TCTTAGTTAAAGTCCCCAGAAGTTAGTACGC AACGCCAATACGTGTATGAGAACGATCAC <u>TGGAGGTTGGGGATTTGATGTTG</u>
MS028 Discarded	<u>GCGCGGTACATTTGCTGGACTA</u> GGTTCGAATTTGCTGGTGCGCTATGGTTTA GGAGTATGCTGAGAGAAATCATC <u>TGGAGGTTGGGGATTTGATGTTG</u>
MS029 Clone 08	<u>GCGCGGTACATTTGCTGGACTA</u> ACCGGCGGTATGTGATAGTGCGGAGTGATGG TGAGCGGGACTGGCGCGGCGATTGAGTGAT <u>TGGAGGTTGGGGATTTGATGTTA</u>
MS030 Clone 09	<u>GCGCGGTACATTTGCTGGACTA</u> TTTCGTTGGTTGGTTTAGTCCAGAGTGTTGA AGAGCGTCAGTTTGTAGGTCTTCTAGGTAT <u>TGGAGGTTGGGGATTTGATGTAA</u>
MS031 Discarded	<u>GCGCGGTACATTTGCTGGACTA</u> ATAGCCCTGAGGTCGAGTGTTTTGATGCT ATCCAAGAAATAATTTAGATGTTACACATG <u>TGGGGGGGGGAGATGAGGTGG</u>
MS032 Clone 10	<u>GCGCGGTACATTTGCTGGACTA</u> GTGTTGTGAGGGTGCGTATCAGGTCGTAGAA TGCAAAACAGCGGTGAGTAAATTTGTGTC <u>TGGAGGTTGGGGATTTGATGTTG</u>
MS033 Clone 11	<u>GCGCGGTACATTTGCTGGACTA</u> TTGGTGAATTCTGTAAAGTACGTGGTCAAACC ATTTGGGTCGGCTGTTGAGTTCTGGACTC <u>TGGAGGTTGGGGATTTGATGTTG</u>
MS034 Clone 12	<u>GCGCGGTACATTTGCTGGACTA</u> AAACACTTAATTAATCAACGGTACGCCTGCC TATTCTGACGCTTTCCTGATGTAGGTGA <u>TGGAGGTTGGGGATTTGATGTTG</u>
MS035 Discarded	<u>GCGCGGTACATTTGCTGGACTA</u> GACTGAACCTGAGATTTGTTAGAGCATTCGA GGATGCCCCGTTGGTGTGTAAGGAAGAGG <u>TGGAGGTTGGGGATTTGATGTTG</u>
MS036 Clone 13	<u>GCGCGGTACATTTGCTGGACTA</u> GTATGCTGTGTAGATTGGCGGCGTTGATAAA GTGCGGTAAGAAGTGGAAGGCTAAT <u>TGGAGGTTGGGGATTTGATGTTG</u>
MS037 Clone 14	<u>GCGCGGTACATTTGCTGGACTA</u> CGCCAAAAAATGCTAGACGAGAGGATGAAGA AGGATGCAATAGTTCAAGTGTCCGAAT <u>TGGAGGTTGGGGATTTGATGTTG</u>
MS038 Discarded	<u>GCGCGGTACATTTGCTGGACTA</u> GGATAACCGGAAGGAATGGCGGCCAGCTGTA TGGATGATTAACAATCGCAGCTGTAGCGAT <u>TGGAGGTTGGGGATTTGATGTTG</u>

FACS was used to quantify the binding affinities of the DNA nanoparticles (Graph 1.8). For the preparation of the nanoparticles, 20% of the dCTP were substituted with Alexa-488 labelled dCTP and incorporated during RCA. The 14 different DNA nanoparticles were individually incubated with 4×10^4 DC for 30 min on ice (similarly to the selection), washed 3 times with a PBS-based wash solution (FACS WASH) and resuspended in 200 μ L of a 3.7% formaldehyde solution (FACS FIX).

Graph 1.8: FACS results for two positively selected clones (3 and 4) and for two negative clones (7 and 11). Controls were: DCs only (gray), DCs with free Alexa nucleotides (blue) and Control sequence (pink).



To fully appreciate the binding due to the unique sequence, the signal from the selected clones was compared to the signal from a nonspecific sequence which didn't have any affinity for DC (Control Sequence). This set the base line for "sticky particles", rather than "specific particles".

The DNA nanoparticles weren't cleaned before incubation with DC. Therefore, unreacted Alexa labeled nucleotides were still present in solution. As a control for nonspecific binding of the free Alexa-labelled oligonucleotides, DC were incubated with the same oligonucleotides at the same concentration as used in the RCA. An additional negative control was DC without any DNA at all. This latter control was used to calibrate the FL-1 channel on FACS to its best zero value. Both controls were washed and fixed just like the sample to test the individual clones and the control sequence.

From FACS it was possible to notice that some of the sequences had a good affinity for DC (Graph 1.8). The experiment was repeated several other times with DC, as each time collected from the blood of a different donor. Among the different results, two of the sequences seemed to be strong candidates for DC affinity: clone 03 and clone 04. Clone 03 was chosen for the subsequent tests because its sequence appeared twice among the cloned sequences.

Along with FACS analysis, microscope images were taken for the labeled DNA nanoparticles targeting a DC (Figure 1.3). Both techniques were used also to compare the affinity of the selected clone 03 for DC and not for another similar cell line. First test against a breast cancer line (MDA-MB-231) gave a good indication of specificity for DC. Dr. Sanchez did extensive repeated tests over different cell lines (THP1, CLL, K562 and MDA-MB-231) and proved that the clone 03 has a high affinity for DC, but no affinity for

any of the other cell lines tested. The results were proved both by FACS and microscope imaging (Figure 1.4).

Additional experiments conducted by Dr. Sanchez showed that the reverse complement of the sequence clone 03 does not show any specificity for DC, as well as a loss of specificity if the sequence is blocked with its complementary.

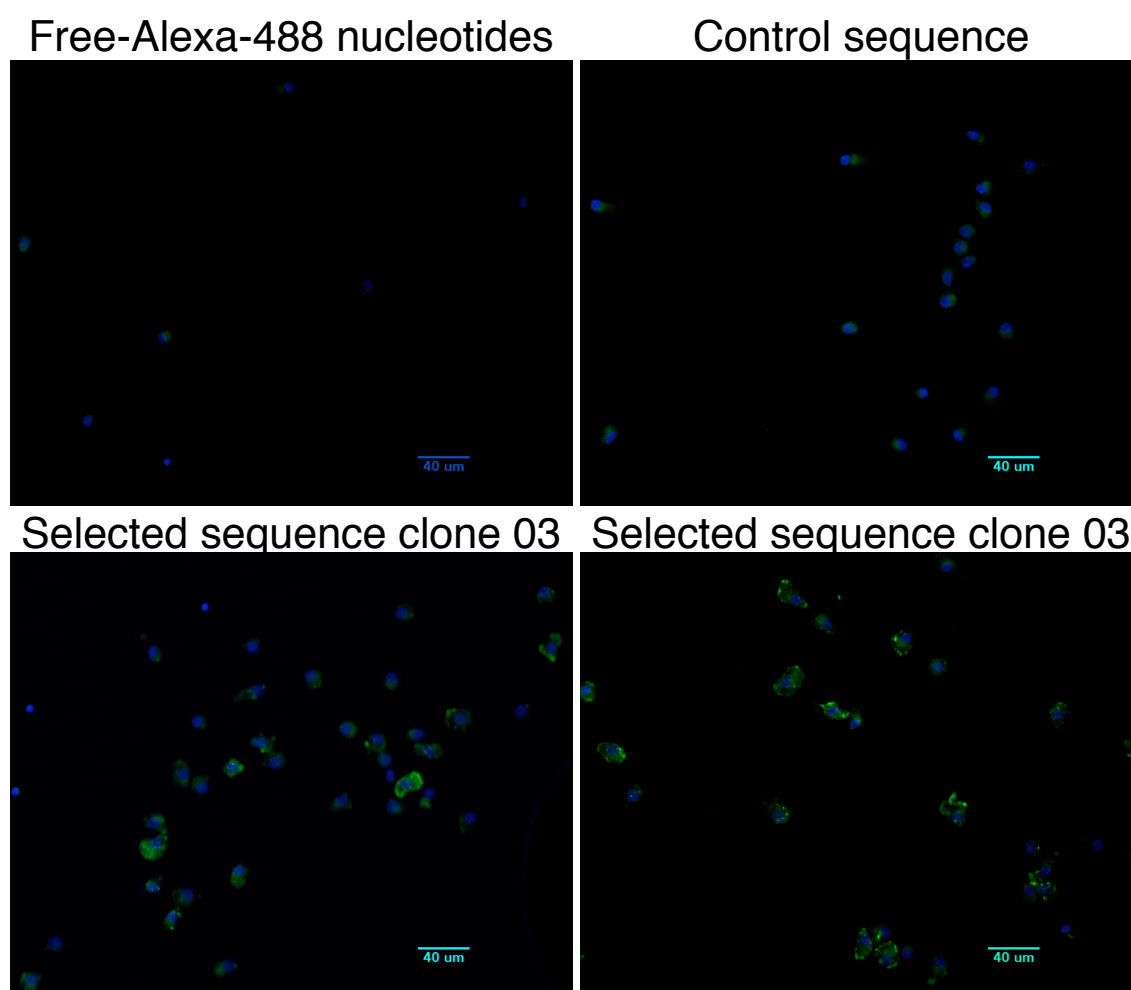


Figure 1.3: The microscope images show the binding to DC of free Alexa-labelled oligonucleotides, nonspecific DNA nanoparticles and clone 03. The images of clone 03 show bright positive signal compared to both the free nucleotides and the nonspecific particles.

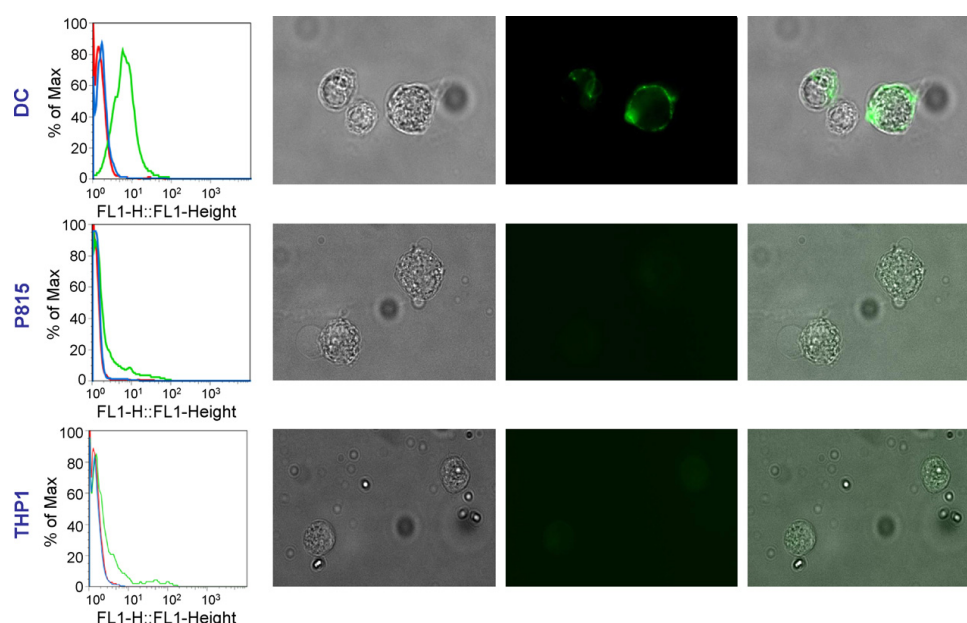


Figure 1.4: FACS results and microscope images of clone 03 binding. Clone 03 (green) unlike its reverse complement (blue) is specific for DC. No affinity was seen for P815 (mouse mastocytoma mast cell) and THP1 (Human acute monocytic leukemia monocytes). Untreated control is in red.

1.5 CONCLUSIONS ON DNA NANOPARTICLES

The DNA nanoparticles created in this work target exclusively DC. Additional studies need to be done at this point to identify which receptor or receptors are involved. The results can give further insight on how the DNA nanoparticles targets and why they can be so specific for DC. Along with the excellent targeting to DC, there could be additional benefits encoded in this sequence or on the effect it can have by binding to the DC. Unfortunately, it is also possible that the DNA nanoparticles can themselves be immunogenic and trigger some form of response on the DC. Overall, this aspect is something very complicated, and it might only be understood with the proper in-vivo studies. The power of DNA as a material and a code at the same time, encloses the

possible problematic of sending the wrong message, and particular attention needs to be made on fully understand the effect of the chosen working sequence before considering it as material on a clinical trial or application.

Once its effect on DC has been evaluated, several perspectives are now open for the selected clone 03, above all if it can show a sufficiently long circulation life in blood (separate preliminary tests conducted by A. Freed and J. Steiner, indicates good stability in serum and blood). The DNA nanoparticles might be used as a vaccine adjuvant by adding an unmethylated cytosine-phosphate-guanine sequence (CpG sequence) either integrated in the template or hybridized onto the surface. CpG sequences are known to trigger the innate immune response[29, 30]. Alternatively an antigen can be hybridized on the surface of the DNA nanoparticles, which binding to a DC might trick the body into generating an immune response against the exposed antigen. These two scenarios are possible applications, but the proper experiments will need to be conducted to prove the actual feasibility of the idea.

It can be concluded that DNA nanoparticles created by RCA are a powerful targeting vector. The level of targeting that it can achieve is extremely high, allowing the concept to be extended to a more complex level of differentiation such as healthy versus cancer cells, or even malignant versus benign cancer. While extending the idea on a less variable population of cells, it is important to keep in mind that a negative selection needs to be carried along with a positive one. DC, because primary cells, offer an extremely high variability compared to an established cell line. For this reason, when bio-panning against an established cell line, special measurements needs to be taken to eliminate from the pool those receptors that widely diffused on all the cells of that type or organ. A good method can be screening for DNA nanoparticles that targets, for instance,

a pancreatic cancer, but eliminate all those that are binding to healthy pancreas cells. In this aspect DNA nanoparticles can become the ultimate tool for a pathologist as alternative to antibody staining.

1.6 ACKNOWLEDGMENTS

Part of this chapter was published on Journal of Biotechnology. The author wants to thank Dr. Davorka Messmer and her whole research group for providing fresh DC for this experiment. Special thanks also goes to Dr. Sanchez for some of the microscope and FACS images.

1.7 MATERIALS AND METHODS

LIGATION REACTION

T4 ligase (New England BioLabs), DTT (Pierce), PCR water, DNA sequences were all purchased from IDT. The reaction was done in a final volume of 50 μ L. T4 ligase was used with the addition of DTT to assure the efficiency of the enzyme. 1.5 μ L of 10 μ M Padlock were used with 0.5 μ L of a 10 μ M Library template.

Procedure: Place the vial of the reaction in a beaker with water, bring the water to boil, remove from heat and let the water cool down to $\sim 30^{\circ}\text{C}$. Add the enzyme only then and let react 1hr. Store at 4°C .

RCA REACTION

Phi29 polymerase (New England BioLabs), dNTPs Mix (New England BioLabs).

The reaction was done in a final volume of 50 μ L, DTT was used to ensure the efficiency of the enzyme. In the reaction were used 0.5 μ L of the Ligated library. For RCA monitoring 0.13 μ L of Oligreen (invitrogen) were added to the reaction.

Procedure: Incubate the reaction 30min at 30°C, stop with 5 μ L of 500mM EDTA (Teknova).

Real-time PCR was done with:

DC CULTURE

Immature DC were cultured in RPMI 1640 medium supplemented with 2mM L-glutamine, 50 mM 2-mercaptoethanol, 10 mM HEPES, penicillin (100 U/mL), streptomycin (100 mg/mL), 5% human AB serum, 1000 U GM-CSF/mL and 200 U IL-4/mL and harvested in days 5–7.

LYSIS OF THE CELLS

For the lysis 50 μ L of hypotonic buffer were used with 3 μ L Proteinase-K (10 mg/mL). The hypotonic buffer was made by: 10 mM Tris (pH 8.0), 1 mM EDTA and 0.001% Triton X-100 with 1% SDS.

Procedure: Cells were heated @ 56°C for 1hr, then the enzyme was blocked @ 95°C for 15 min.

PCR REACTION

Stoffel (Invitrogen), SybrGreen I (Invitrogen). The reaction was done in a final volume of 50 μ L with 1mM of dNPTs and 5mM of $MgCl_2$. Sybr Green I was used to a final concentration of 2x, while 0.2 μ M of each primers were used for the reaction. Stoffel fragment was used because it lacks intrinsic 5' to 3' exonuclease activity. In the first round of selection ~25 μ L of lysate were used for each of the two 50 μ L reaction, while for the following selection rounds one PCR reaction only was made and just 2 μ L of lysate were used.

Procedure: The reaction had an initial heating time of 2 minutes @ 95°C, cycles between 30 sec @ 95°C, 1 min @ 61°C, 20 sec @ 72°C. Dissociation curve was done at each selection round: 30 sec @ 95°C, 1 min @ 55°C, slowly heating to reach 95°C. For the first cycle the sample used was 26.5uL, in this instance no PCR water was added.

ASYMMETRIC PCR REACTION

The reaction was done in a final volume of 50 μ L with 0.8mM of dNPTs and 4mM of $MgCl_2$. Unlike the PCR, asymmetric PCR used only the reverse primer with protected phosphate 0.4 μ M. The asymmetric PCR was done using 10 μ L of PCR product.

Procedure: The reaction had an initial heating time of 2 minutes @ 95°C, cycles between 30 sec @ 95°C, 1 min @ 61°C, 20 sec @ 72°C.

FLOW CYTOMETRY (FACS)

Flow cytometry was performed using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). The acquired data were analyzed using the FLOW-JO software (Tree Star Inc, Ashland, OR).

FACS WASH: PBS supplemented with 5% Fetal Bovine Serum and 10% sodium
azyde

FACS FIX: PBS supplemented with 3.7% formaldehyde

1.8 BIBLIOGRAPHY

- [1] C. J. Chen, D. D. Han, C. F. Cai, X. Tang, *Journal of Controlled Release* **2010**, 142, 299.
- [2] M. L. Immordino, F. Dosio, L. Cattel, *International Journal of Nanomedicine* **2006**, 1, 297.
- [3] R. Fanciullino, J. Ciccolini, *Current Medicinal Chemistry* **2009**, 16, 4361.
- [4] A. V. Kabanov, S. V. Vinogradov, *Angewandte Chemie-International Edition* **2009**, 48, 5418.
- [5] J. K. Oh, R. Drumright, D. J. Siegwart, K. Matyjaszewski, *Progress in Polymer Science* **2008**, 33, 448.
- [6] O. Veisheh, J. W. Gunn, M. Q. Zhang, *Advanced Drug Delivery Reviews* **2010**, 62, 284.
- [7] J. Liu, F. Liu, K. Gao, J. S. Wu, D. F. Xue, *Journal of Materials Chemistry* **2009**, 19, 6073.
- [8] V. Bagalkot, O. C. Farokhzad, R. Langer, S. Jon, *Angewandte Chemie-International Edition* **2006**, 45, 8149.
- [9] L. M. Alvarez-Salas, *Current Topics in Medicinal Chemistry* **2008**, 8, 1379.
- [10] S. M. Nimjee, C. P. Rusconi, B. A. Sullenger, *Annual Review of Medicine* **2005**, 56, 555.
- [11] A. D. Keefe, R. G. Schaub, *Current Opinion in Pharmacology* **2008**, 8, 147.
- [12] E. W. Ng, D. T. Shima, P. Calias, E. T. Cunningham, Jr., D. R. Guyer, A. P. Adamis, *Nat Rev Drug Discov* **2006**, 5, 123.
- [13] J. Jarvius, J. Melin, J. Goransson, J. Stenberg, S. Fredriksson, C. Gonzalez-Rey, S. Bertilsson, M. Nilsson, *Nature Methods* **2006**, 3, 725.
- [14] G. Nallur, C. H. Luo, L. H. Fang, S. Cooley, V. Dave, J. Lambert, K. Kukanskis, S. Kingsmore, R. Lasken, B. Schweitzer, *Nucleic Acids Research* **2001**, 29, art. no.
- [15] L. W. Wu, Q. J. Liu, Z. W. Wu, Z. H. Lu, *Molecules* **2010**, 15, 619.
- [16] Z. S. Wu, H. Zhou, S. B. Zhang, G. L. Shen, R. Q. Yu, *Analytical Chemistry* **2010**, 82, 2282.
- [17] T. Z. G. de la Torre, M. Stromberg, C. Russell, J. Goransson, M. Nilsson, P. Svedlindh, M. Stromme, *Journal of Physical Chemistry B* **2010**, 114, 3707.
- [18] Q. Su, D. Xing, X. M. Zhou, *Biosensors & Bioelectronics* **2010**, 25, 1615.

- [19] J. S. Li, T. Deng, X. Chu, R. H. Yang, J. H. Jiang, G. L. Shen, R. Q. Yu, *Analytical Chemistry* **2010**, *82*, 2811.
- [20] Z. S. Wu, S. B. Zhang, H. Zhou, G. L. Shen, R. Q. Yu, *Analytical Chemistry* **2010**, *82*, 2221.
- [21] S. Beyer, P. Nickels, F. C. Simmel, *Nano Lett* **2005**, *5*, 719.
- [22] J. P. Anderson, B. L. Reynolds, K. Baum, J. G. Williams, *Nano Letters* **2010**, *10*, 788.
- [23] E. Schopf, Y. Chen, *Analytical Biochemistry* **2010**, *397*, 115.
- [24] G. J. Porreca, *Nature Biotechnology* **2010**, *28*, 43.
- [25] R. Drmanac, A. B. Sparks, M. J. Callow, A. L. Halpern, N. L. Burns, B. G. Kermani, P. Carnevali, I. Nazarenko, G. B. Nilsen, G. Yeung, F. Dahl, A. Fernandez, B. Staker, K. P. Pant, J. Baccash, A. P. Borcharding, A. Brownley, R. Cedeno, L. S. Chen, D. Chernikoff, A. Cheung, R. Chirita, B. Curson, J. C. Ebert, C. R. Hacker, R. Hartlage, B. Hauser, S. Huang, Y. Jiang, V. Karpinchyk, M. Koenig, C. Kong, T. Landers, C. Le, J. Liu, C. E. McBride, M. Morenzoni, R. E. Morey, K. Mutch, H. Perazich, K. Perry, B. A. Peters, J. Peterson, C. L. Pethiyagoda, K. Pothuraju, C. Richter, A. M. Rosenbaum, S. Roy, J. Shaffo, U. Sharanhovich, K. W. Shannon, C. G. Sheppy, M. Sun, J. V. Thakuria, A. Tran, D. Vu, A. W. Zaranek, X. D. Wu, S. Drmanac, A. R. Oliphant, W. C. Banyai, B. Martin, D. G. Ballinger, G. M. Church, C. A. Reid, *Science* **2010**, *327*, 78.
- [26] L. Fong, E. G. Engleman, *Annual Review of Immunology* **2000**, *18*, 245.
- [27] N. C. Robson, S. Hoves, E. Maraskovsky, M. Schnurr, *Current Opinion in Immunology* **2010**, *22*, 137.
- [28] K. Shortman, M. H. Lahoud, I. Caminschi, *Experimental and Molecular Medicine* **2009**, *41*, 61.
- [29] J. Kindrachuk, J. Potter, H. L. Wilson, P. Griebel, L. A. Babiuk, S. Napper, *Mini-Reviews in Medicinal Chemistry* **2008**, *8*, 590.
- [30] P. Malyala, D. T. O'Hagan, M. Singh, *Advanced Drug Delivery Reviews* **2009**, *61*, 218.

CHAPTER 2 MODIFIED INTERNAL STRUCTURE: GRADIENT NANOPARTICLES

2.1 INTRODUCTION: POLYMERIC NANOPARTICLES

Liposome and polymeric nanoparticles are widely used as drug delivery vehicles [1, 2]. Liposomes are a well established platform, already present in the clinic, and they offer the possibility to load, even though to a different extent, both hydrophilic and hydrophobic small molecule drugs. Despite their valuable qualities, liposomes need some further improvements. Although it is possible to load hydrophobic small molecules among the lipid tails of the membrane, the volume offered is small compared to a particle of the same size that can load the same drug into its core. In addition, liposomes still have some stability issues that need to be addressed. On the other hand, high stability and large hydrophobic core can be easily achieved with polymeric nanoparticles. Despite the improvements done in making liposomes more stable [3-5], their use is still limited by their short shelf-life and they cannot be compared to the sturdier polymeric nanoparticles.

Among the different hydrophobic polymers used for drug delivery vehicles, poly(lactic-co-glycolic acid) (PLGA) and polylactic acid (PLA) have been used for more than 20 years with high success [6, 7] and several formulations are available in the clinic: Lupron Depot® and Arestin® are some examples.

Bare PLGA nanoparticles are not stable, and to make them both stable and stealth they are often used as block-polymer with polyethylene glycol (PEG). PEG is widely used in several products, because of its low toxicity. PEG is used in toothpastes, creams and eye drops. In pharmaceutical applications, it is the basic excipient of

laxatives, but probably the most common application of PEG is as a conjugate for drug delivery. In fact PEG is known to increase the circulation time of drug delivery vehicles and small molecules[8-10].

Different methods are available to prepare PLGA-based nanoparticles with PLGA-PEG block polymer [11], with nanoprecipitation [12, 13] (also defined as self-assembly) being one of the most popular. Nanoprecipitation leads to small size nanoparticles with a low polydispersity [14]. In addition, it is a highly scalable method which is a crucial characteristic for future commercialization and clinical studies.

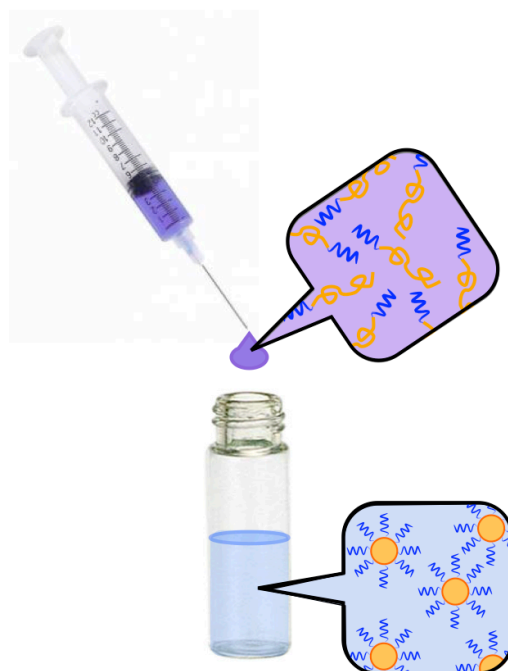
Almost all the PLGA-based nanoparticles available in the literature, have a uniform hydrophobic core [12], including formulations that possess a core/shell structure [15, 16]. A physical or chemical gradient structure within the hydrophobic core can lead to significant improvements on drug loading and release profile of the nanoparticle. In particular, it is expected that a gradient structure will directly affect the release profile by changing the diffusion rate across the nanoparticle's core.

In this work, both porosity and chemical gradient structures are evaluated. The resulting two configurations have been characterized and examined in depth, and detailed conclusions have been drawn to highlight the changes that induce the most significant improvements.

2.2 THEORY OF POROSITY GRADIENT

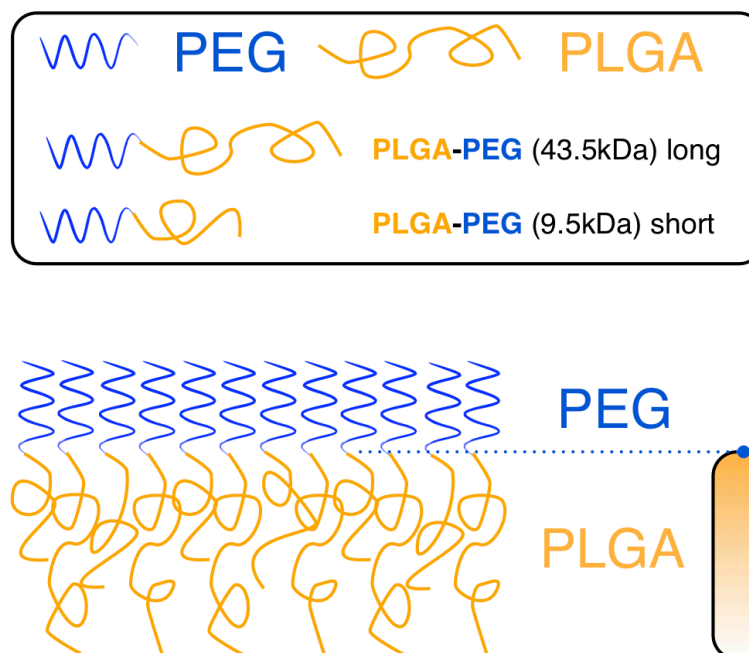
The nanoparticles herein described are made by nano-precipitation, in which self-assembly is the driving force. Self-assembly is a valuable tool to achieve a fairly uniform population in a single step without the need of a cast or a mold[17]. During the

nano-precipitation process, the PLGA-PEG polymer units are dissolved in an organic solvent (acetonitrile), and the solution is drop wise precipitated into constantly stirred water. The hydrophobic chains of the PLGA will organize close to each other and far from the aqueous side (Scheme 2.1). On the other hand, the hydrophilic PEG will distribute towards the water “separating” the PLGA from the aqueous environment. The organic solvent is water-soluble, and it will easily mix with water during the precipitation. The final core structure of the PLGA-PEG nanoparticles is a solid polymeric core. Small molecules added into the organic solvent solution can be entrapped inside the PLGA-PEG nanoparticles during nanoprecipitation. For best results, the small molecules need to be soluble in the organic solvent used to precipitate the PLGA-PEG.



Scheme 2.1: Representation of the nanoprecipitation process. The PLGA-PEG polymer dissolved in the organic solvent is added drop-by-drop into water. The nanoparticles will form by self-assembly in the aqueous solution.

Considering this self-assembly principle, a gradient structure can be created by using two PLGA-PEG units with a different PLGA tail length. During precipitation, it can be expected that the PEG chains will distribute uniformly on the surface, while the hydrophobic tails will occupy with the same density the outer shell of the core (Scheme 2.2). The random interaction between the two different lengths of PLGA chains will contribute to create a gradient towards a lower density core. The density of the interactions between the PLGA tails are going to be different between the core and the shell. Areas closer to the shell, where there density of PLGA is higher, will have a tightly packed matrix. Because a PLGA tail can interact both between itself and different units, it is not possible to picture all the shorter tails completely extended. For this reason a uniform density shell of the thickness of the shorter tails is highly unlikely. In addition, the two polymers' molecular weight (MW) is not an exact value but an average over a broad Gaussian distribution (the lower MW's polymer has the broadest distribution). The PLGA tail interactions will decrease with the depth, along with their density. Consequent to this gradual variation, the thick and complex polymeric net at the water interface will slowly fade into a looser matrix inside the core.



Scheme 2.2: Polymer used in a porosity gradient and expected distribution at the water/polymer interface.

A big hollow core is not contemplated as a possible scenario because it is energetically unfavorable. A hollow core will cause strong strains on the surface interface, which will lead to a collapse or rupture of the particle. At this point, it is important to highlight that the organic solvent used in nanoprecipitation is water miscible. The organic solvent will diffuse out of the particle and mix with water during nanoprecipitation, leaving the hydrophobic polymers as the only component of the particle's core. In addition, water won't penetrate inside the hydrophobic layer. This decreases the chances of a liquid filled core that might favor a hollow core structure.

A gradient porosity core is expected to give a higher loading on the areas with the higher porosity, where more drug can be located. The gradient porosity nanoparticle here described is expected to have its bigger pores into the core, where the drug is most likely to locate. Hence, the gradient porosity particle is expected to have a higher loading

than a uniform core. In addition, the release profile is expected to be more sustained in a gradient structure, since the drug in the core constitutes some sort of reservoir for the loaded drug.

2.3 POROSITY GRADIENT RESULTS

The two carboxylate-functionalized PLGA-PEG used had roughly a 4 fold molecular weight difference. The block copolymer was created by covalently conjugating 3.5kDa COOH-PEG-NH₂ (JenKem) and PLGA-COOH (Lactel) of either 6kDa or 40kDa molecular weight through N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) chemistry based on a published protocol[12]. The quality of the conjugation was evaluated by ¹H-NMR (Figure 2.1). All the characteristic proton resonance of PLGA-COOH and NH₂-PEG-COOH were intact after conjugation indicating the co-presence of PLGA-PEG-COOH copolymer. Most importantly, the characteristic methyl proton (labeled as d in Figure 2.1) of lactide moiety and methylene of PEG moiety (labeled as c in Figure 2.1) were intact after conjugation without altering its resonance at δ 1.5 and 3.6 ppm, respectively. In addition, the methylene protons corresponding to lactide and glycolide moiety showed the resonance at δ 5.25 and 4.85 ppm. The ¹H-NMR proved the co-presence of both polymers in the final solution, after cleaning the unreacted material by solvent precipitation.

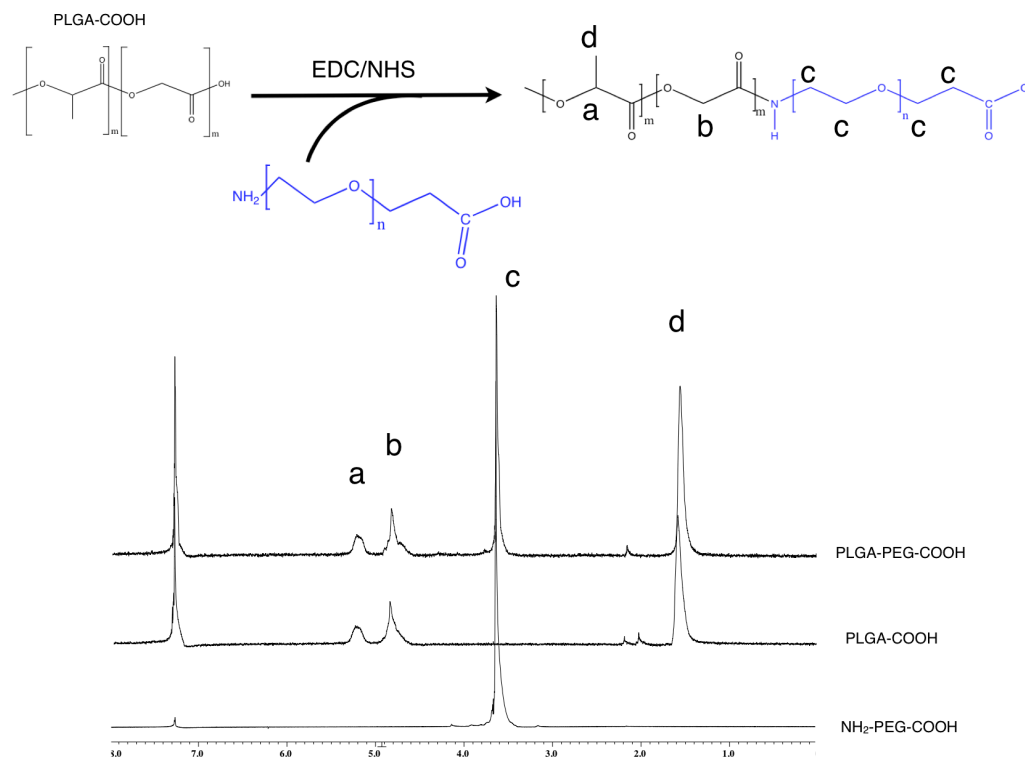
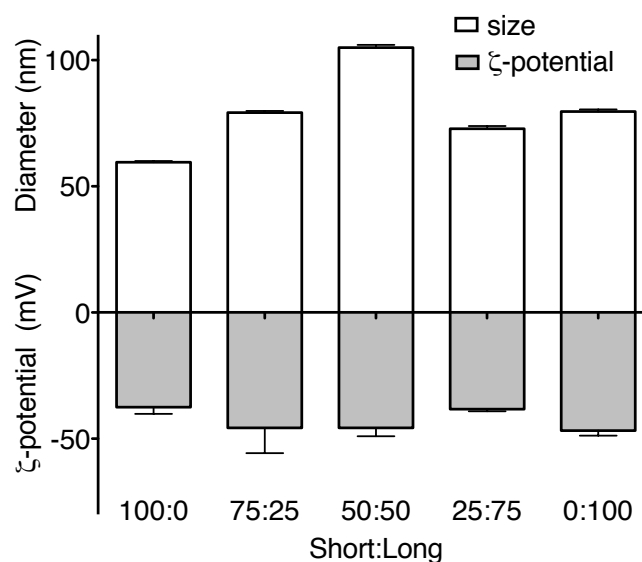


Figure 2.1: Chemical reaction to conjugate PLGA to PEG, with ^1H -NMR of the PLGA (40kDa). The peaks are clearly visible. Similar results were seen for the lower molecular weight PLGA.

The two polymers were combined in different molar percents to a final polymer concentration of 1mg/mL. Five different combinations were used: 100:0, 75:25, 50:50, 25:75, and 0:100, where the first number refers to the percent of moles of the shorter PLGA and the second represents the percent of moles of the longer PLGA. The different combinations of the two polymers were mixed in a final volume of 1mL of acetonitrile and nano-precipitated in water (for further informations see materials and methods). After cleaning the nanoprecipitation size and ζ -potential of the five different combinations were measured (Graph 2.1).

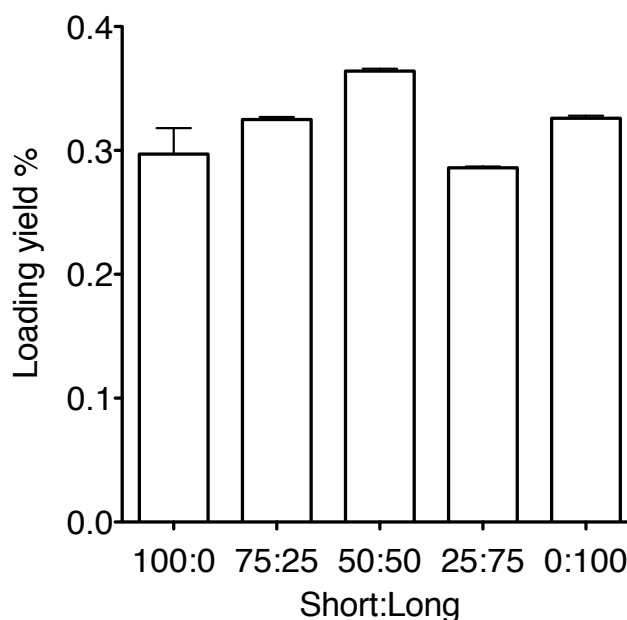
Graph 2.1: Size and ζ -potential distribution of the five different combinations.

The particles made entirely by the shorter polymer show, as expected, a small size (about 60nm), while the particles made with equal molar contribution of the two polymers show the biggest size (about 100nm). The ζ -potential for all five combinations slightly changes with the size showing a slightly higher value for the smaller size particles. The negative charge can be ascribed to the carboxyl group at the end of the PEG, since the PEG distribution is expected to be similar for all the combination, the size difference will be correlate to a ζ -potential difference.

The gradient core structure is expected to have a significant impact on the loading of hydrophobic small molecules. Paclitaxel was chosen as the model hydrophobic drug because it is widely used against many different cancers such as lung, ovarian, breast, head and neck. It is also being considered to treat other illnesses[18]. Paclitaxel is soluble in acetonitrile and when added to the mixture of polymers in acetonitrile it can be encapsulated during nanoprecipitation. In this work, a 5% weight

percent paclitaxel to polymer was loaded into the nanoparticles. After precipitation, the loaded drug was extracted from the washed nanoparticles, and it was quantified by high pressure liquid chromatography (HPLC) (refer to materials and methods for more details). To account for the different sizes, the loading yield was used to compare the different combinations. The loading yield is defined as the weight percent of loaded drug over polymer (Graph 2.2).

Graph 2.2: Loading yield percent of nanoparticles loaded with 5% wt/wt paclitaxel.

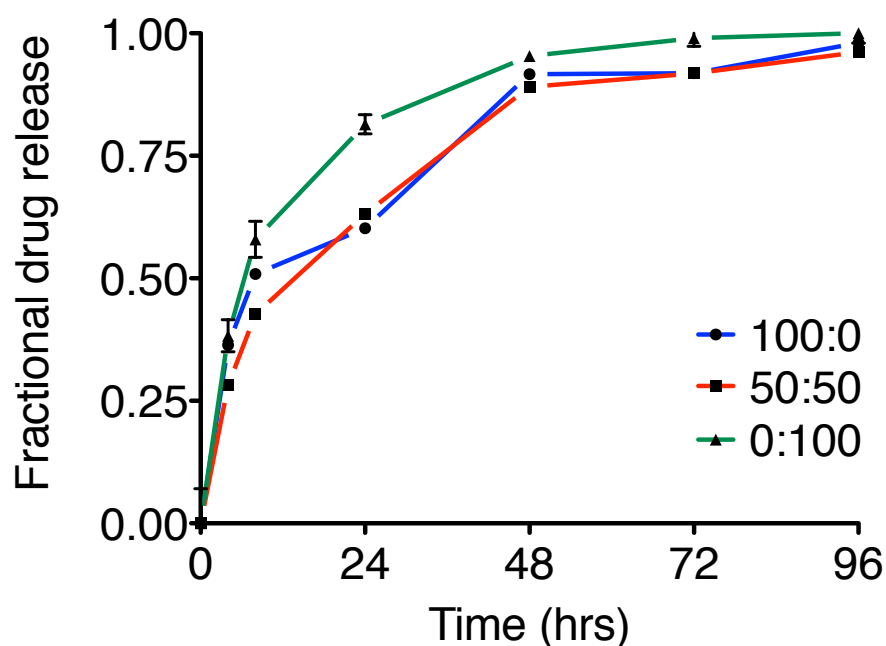


Despite a higher loading yield in the 50:50 polymer formulation, the five different nanoparticles show similar loading yield with insignificant differences.

A drug release study was done to further investigate any possible advantage of the gradient structure. The analysis was limited to the combination which showed the best loading yield. The drug was released from the nanoparticles during dialysis at 37°C

in a 10kDa MWCO floating mini-dialysis cassette (Pierce). The dialyzed samples were collected at different time-points and the drug still encapsulated was extracted and quantified by following the same procedure used for the loading. The fractional drug release from the initial loading was then calculated and plotted over time (Graph 2.3). The release curve was repeated several times displaying similar results, showing little to no difference between the gradient and the smaller polymer non-gradient particles.

Graph 2.3: Drug release of the uniform core nanoparticles for short (100:0) and long (0:100) PLGA chain, compared to the gradient nanoparticles of equal molar percent of the two different length PLGA (50:50).



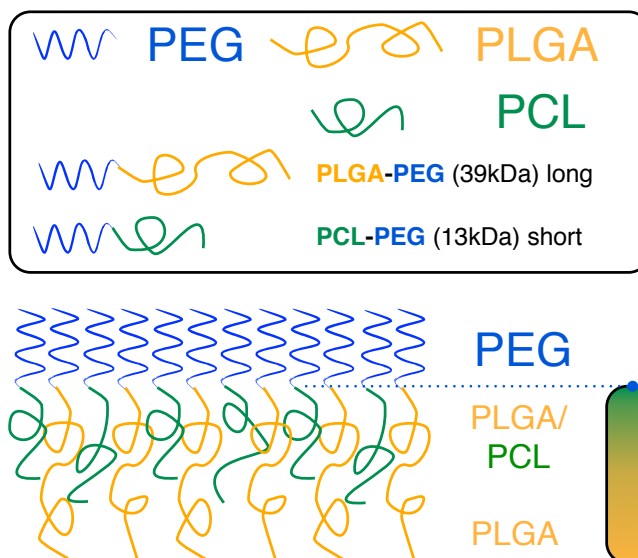
Despite the small improvements achieved with the porosity gradient structure, the results can still be considered promising. The small difference can be mostly ascribed to the fact that the two PLGA used do not dramatically differ in length. The difference between the two polymers could be increased either by making the small

polymer smaller or the long longer. The former solution will lead to significant purification problems during the polymer synthesis (and PEG conjugation), while the latter option will lead to particles of a much bigger size.

2.4 INTRODUCING THE CHEMICAL GRADIENT

The results obtained with the porosity gradient structure show that for nanoparticles of the order of 100nm the gradient effect is not sufficiently large, when limited to porosity. A chemical gradient can add an additional degree of freedom, and therefore, it might be then possible to achieve a more significant difference between uniform and gradient nanoparticles.

Similarly to PLGA, polycaprolactone (PCL) is another widely use biodegradable and biocompatible polymer approved for medical application by the Food and Drug Administration (FDA). PCL is more hydrophobic than PLGA and it degrades at a slower rate. Because of their interesting properties, blends of PCL and PLGA have been studied in film[19], and as shell on PLGA nanoparticles[16] and microspheres[15].



Scheme 2.3: Polymer used in the chemical gradient and expected distribution at the water/polymer interface.

The combination of two PEG-conjugated polymers of both different length and hydrophobicity can offer the ultimate tool for creating one step process, improved gradient nanoparticle. Analogous to the porosity gradient, it is expected that the two polymers will distribute on the surface based on their initial ratio. Polymer tails will interact with each other to a different extent and will create a gradient based on the same theory of the PLGA porosity gradient (Scheme 2.2). Since the chemical gradient nanoparticles involve two different hydrophobic polymer, it is important to test the miscibility of the two composing materials.

2.5 CHEMICAL GRADIENT RESULTS

PCL-PEG was created by ring-opening polymerization, and the resulting polymer was characterized by $^1\text{H-NMR}$ (Figure 2.2). The PCL-PEG chemistry was finely tuned to achieve a similar polymer size as for the porosity gradient nanoparticles. This way any

difference in the results will further highlight the importance of the additional difference in chemical properties.

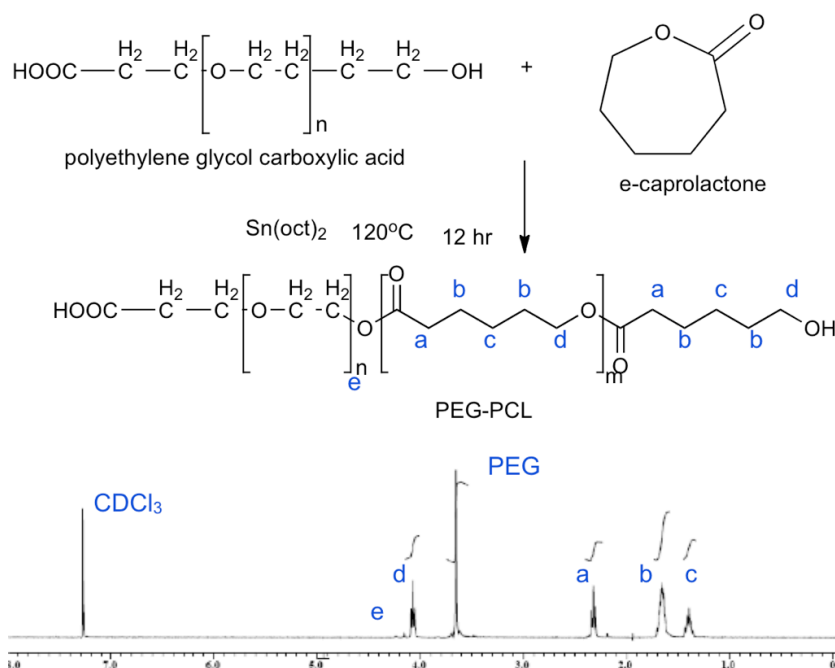


Figure 2.2: Chemistry for the ring opening polymerization of PCL and ^1H -NMR of the product.

As nanoparticle with decreasing hydrophobicity gradient towards the core, it is expected to show a slower release due to increased difficulty of the molecule to diffuse out the more hydrophobic areas [15].

Both PCL-PEG and PLGA-PEG were measured by Gel Permeation Chromatography (GPC) (Table 2.1), to create a more reliable comparison between materials created with two completely different chemistries.

Table 2.1: GPC results for PCL-PEG and PLGA-PEG

MATERIAL	M _n GPC	M _w GPC	M _w /M _n
PCL-(PEG)	6.5k+(3.5k)	9.5k+(3.5k)	1.4
PLGA-(PEG)	26.5k+(3.5k)	35.5k+(3.5k)	1.3

The two polymers were combined in five different sets based on the same moles percents of the porosity gradient: 100:0, 75:25, 50:50, 25:75, and 0:100, and the nanoparticles were created following the same protocol.

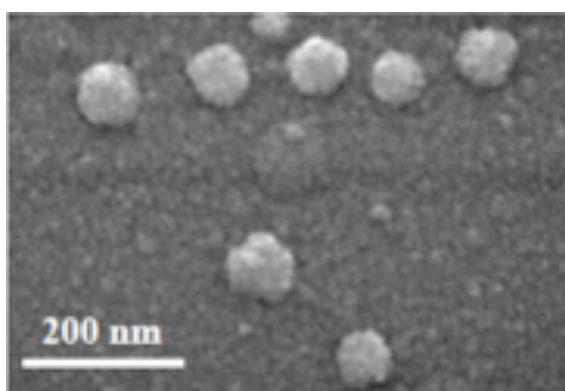
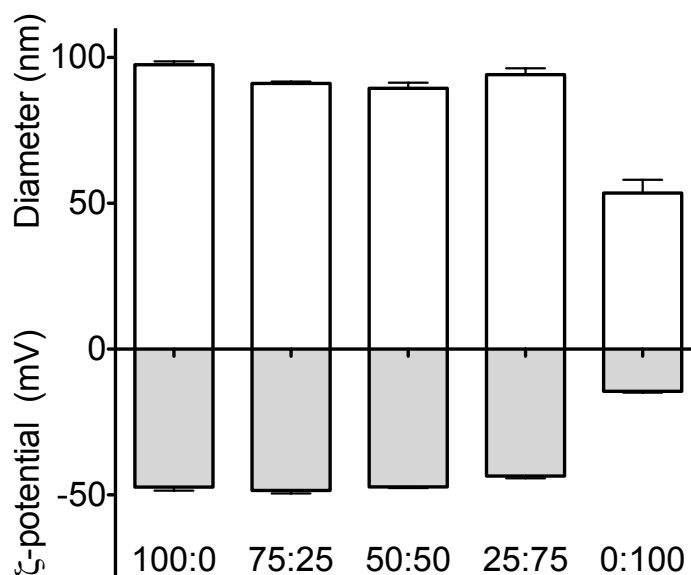


Figure 2.3: SEM image of the 25:75 PLGA:PCL composition representative for the nanoparticles.

SEM (Scanning Electron Microscope) images of the nanoparticles were taken for the different combinations (Figure 2.3). The particles showed a well-defined spherical shape and no clusters were seen in any of the images. This proves a successful nanoprecipitation and suggests the possibility that the two polymers are perfectly miscible.

Graph 2.4: Size and ζ -potential distribution of the five different combinations.



Along with the SEM image, size and ζ -potential were measured by DLS for the five different combinations (Graph 2.4). From the figure, no significant variation of size and ζ -potential can be seen among the samples containing PLGA. It can be deduced that the frame of the particles is highly affected by the presence of the longer PLGA polymer, which seems to provide the particle framework and determine the particles size. The unchanged ζ -potential provides further insight on the polymer distributions within the particles. As previously mentioned for the porosity gradient, the negative surface charge can be associated with the carboxyl terminal group of the PEG; therefore, a similar size and ζ -potential are directly associated to a uniform PEG distribution. Based on this considerations, the smaller pure PCL nanoparticles will be characterized by a less negative ζ -potential. The uniform polymer distribution on the surface, along with the unchanged size, clearly leads to a different porosity in the deepest cores of the different combinations. In addition, the unavoidable interactions

between polymers and the unfavorable presence of a hollow core, further supports the idea of an internal gradient.

For a deeper understanding of the nanoparticles the different combinations were probed with a microenvironment sensitive dye, Laurdan. Laurdan is an uncharged fluorescent dye widely used to study phase transition in biomembranes[20-22]. When lipids membranes phase changes from liquid crystal to gel, the dye shows a characteristic emission shift from 473nm to 416nm. Considering the relative presence of the two emission peaks it is possible to define the net polarization of the surface. Laurdan has the additional quality of being an amphiphilic dye. It is going mainly locate at the interface with water, rendering it a perfect tool to study the surface of the gradient nanoparticles.

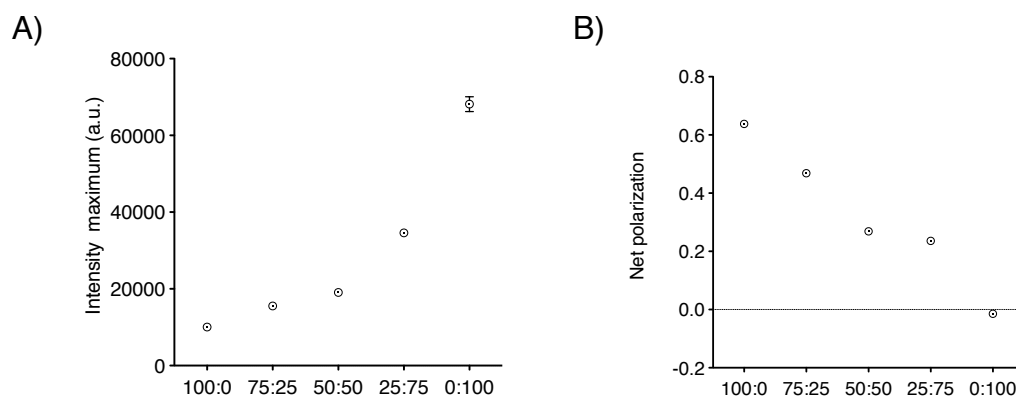
Nanoparticles were loaded with a 0.5 weight percent of Laurdan to polymer. The emission of Laurdan for the different combinations was scanned over the wavelength range 380-550nm, and both wavelength shift and intensity change were seen consistently changing with the presence of one polymer over the other. The intensity increased almost exponentially with the presence of PCL, probably because the dye loading is more efficient in the presence of the more hydrophobic PCL (Graph 2.5A). On the other hand, the net polarization value decreased for the particles with a higher PCL content (Graph 2.5B). The net polarization was calculated according to the formula used for lipid membranes [23]:

$$P \equiv \frac{I_B - I_R}{I_B + I_R}$$

where I_B is the intensity at 416 nm and I_R is the intensity at 473 nm.

The results seem to be contradictory, since the net polarization was expected to be higher for the more hydrophobic surface. This would have been true if PCL and PLGA have the same influence of the apolar lipid tails on the two photon relaxation profiles of Laurdan. Since the use of Laurdan with polymers is fairly new [24] and unexplored, there haven't been any study to relate the effect of the partial negative charge of PLGA or PCL on the dye's emission. Because of this missing information, it is not possible to use the net polarization to quantify the surface's hydrophobicity. However, it is still possible to use it to quantify the change of the chemical's properties on it.

Graph 2.5: A) Intensity maxima and B) calculated Net polarization value for the different nanoparticles combinations.

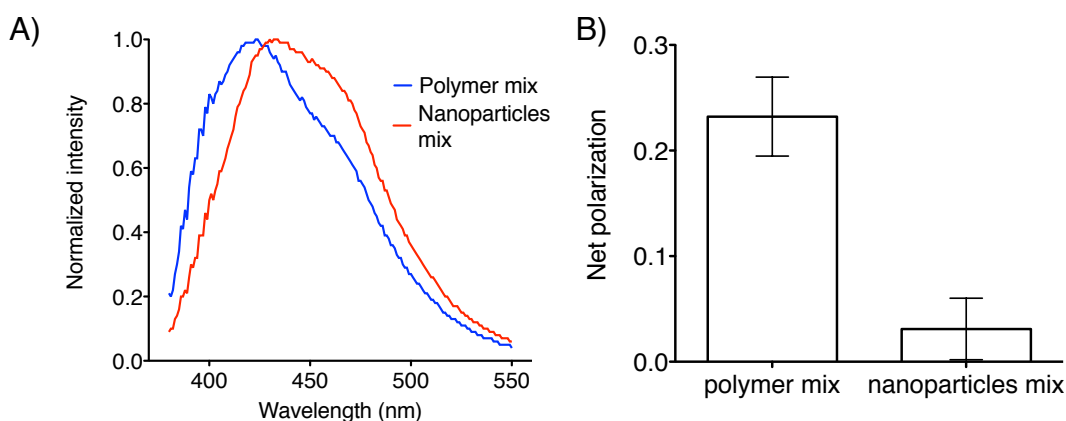


Laurdan can be used to prove the miscibility of the PCL and the PLGA used. It is indeed important to determine that the nanoparticles made in this work are single population composed by both PLGA and PCL and not a combination of two population made by either of the polymers. To prove single population, two different types of nanoparticles' mixes were created, where one was made by co-precipitated PCL and

PLGA polymers (Polymer mix) and the other was made by combining PLGA and PCL nanoparticles after precipitation (Nanoparticles mix).the amount of each polymer was kept the same in both sets. The 25:75 PLGA:PCL combination was used for this investigation.

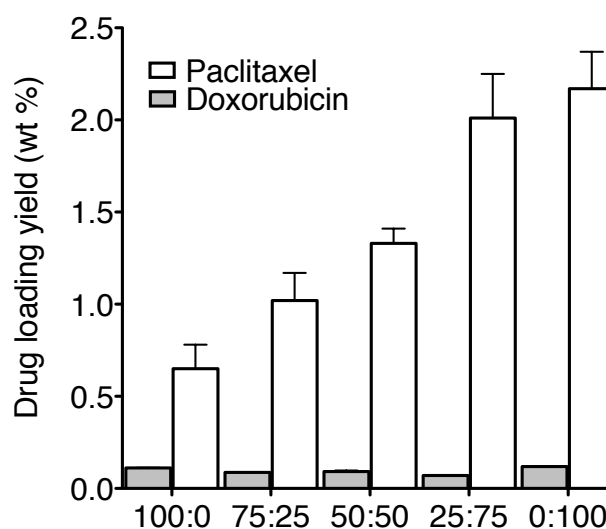
The Laurdan spectra from the polymer mix is completely different from the spectra of nanoparticles mix (Graph 2.6A). This further highlights that the microenvironment seen by Laurdan is not an average micro-environment made by PCL and PLGA independently but a new one created by the co-presence of the two polymers. Consequently, the calculated net polarization of the two is completely different (Graph 2.6B).

Graph 2.6: A) Intensity maxima and B) calculated Net polarization value for polymer mix and nanoparticle mix of 75:25 PLGA:PCL combination.



To further characterize the hydrophobic gradient of the nanoparticles created, the loading yield of paclitaxel, a hydrophobic drug, was compared to the loading yield of doxorubicin (Graph 2.7).

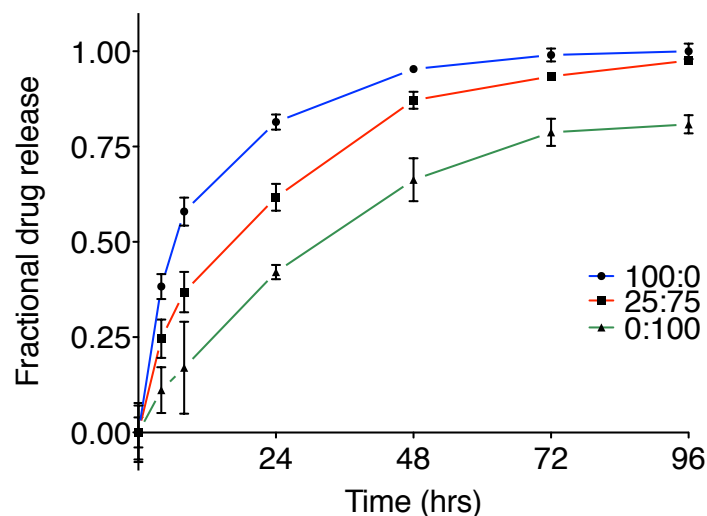
Graph 2.7: Drug loading yield of paclitaxel (hydrophobic) and doxorubicin (hydrophilic) for the five different PLGA:PCL combinations.



The hydrophobic drug (paclitaxel) was loaded in high amounts compared to the hydrophilic one (doxorubicin). In addition, the loading of paclitaxel is proportional to the amount of PCL, while the loading of doxorubicin it is the same for all the combinations (~0.09% loading yield wt%). From these two facts, one could conclude that the increase in hydrophobic environment is the main leading cause for the loading profile of paclitaxel. In addition, the loading profile of doxorubicin precludes the possibility of a hollow core structure, which would result in different doxorubicin loading through physical entrapment.

The chemical difference between the two polymers are not just limited to hydrophobicity but also extends to the degradation rate. PLGA degrades at a faster rate than PCL, and the variation differs larger for polymers with higher molecular weights. A particle that combines both PCL and PLGA at the outer layer is expected to break down slower and therefore release drug at a slower rate than a particle made by PLGA alone.

The 25:75 PLGA:PCL combination was chosen as the composition for the study, because it has the highest loading among the gradient combinations and has the highest molar percent of PCL. The drug release in water at 37°C was observed over a period of 96 hours (Graph 2.8) following the same protocol of the porosity gradient nanoparticles.



Graph 2.8: Drug release for the gradient nanoparticle (25:75 PLGA:PCL) and the individual polymer nanoparticles PLGA (100:0) and PCL (0:100).

The drug release of the gradient nanoparticles is slower than the release of the PLGA particles, but faster than the PCL particles. The release from the PCL particles is somewhat faster than expected, probably due to the particles' small size. A drug release profile is composed of two main aspects: polymer degradation and diffusion through the polymeric matrix. Even though the PCL polymer degrades slower than PLGA, the small size of particle might make the diffusion process the major contribution to the release.

The difference between the single polymer particle and the gradient particle is significant for both loading and release. The loading can be tuned with the amount of

hydrophobic polymer, while the release is intermediate between the one obtained with the individual polymers.

2.6 CONCLUSIONS ON GRADIENT NANOPARTICLES

The characterization studies showed that the gradient nanoparticles have a size dependent mainly on the longer polymer. In addition, the surface charge and chemical properties are directly related to the ratio of the two polymers. This effect is also seen on the loading, where the increase of hydrophobicity of the interface directly relate to the loading of the hydrophobic drug. On the other hand, no big difference between any of the particles, gradient or not, can be seen when a hydrophilic drug such as doxorubicin is used.

Among the several combinations, the one having PCL as 75% of the total polymer content has a high loading and an intermediate release. The advantage of a high loading and intermediate release particle can be seen on cell toxicity studies. In such a scenario, a particle that can steadily deliver a sufficient amount of drug has a higher toxicity than a particle that either doesn't carry enough drug or releases too slowly.

In addition, the gradient nanoparticles loaded either with drugs having different hydrophobicity or affinity toward either of the polymers would offer interesting profiles and qualities to exploit. For example, a drug that tends to clump will tend to show a higher loading in a porosity gradient structure because it will tend to fill the empty spaces of the pores. In a similar way, a drug that has higher affinity with one of the two polymers will show a faster or slower release profile compared to a uniform core nanoparticle. A

drug with affinity with PLGA, will present an extremely slow release followed by a faster release once the PCL shell has degraded.

As a final consideration, the hydrophobicity gradient nanoparticles can be related to the lipid-hybrid nanoparticles made by Chan et. al. [25]. If the PCL in the gradient nanoparticle is made very small to be comparable to a lipid monolayer, then it can be seen as the lipid layer on the hybrid nanoparticles described by Chan. In this scenario, the thin outer shell is much more hydrophobic and helps slowing the release of the hydrophobic drug, while also protecting the PLGA from hydrolysis. Both aspects strongly contribute to a slower controlled release. The main difference between the gradient nanoparticle and the hybrid nanoparticle is the presence of a gradient that will affect the loading. Changing the lipid layer in a hybrid nanoparticle will merely affect stability, while changing the PCL on the gradient nanoparticle will affect both loading and release. This is mostly because in the hybrid nanoparticle the lipid layer protects the PLGA core, but it has minimal interaction with it. On the other hand the PCL of the gradient nanoparticle has a well-established interaction on the shell with the PLGA and this interaction will create a favorable environment for loading of a hydrophobic small molecule.

2.7 ACKNOWLEDGEMENTS

The material for the chemical gradient section (chapters 2.4 to 2.6) was entirely based on the material as it may appear in ACS Nano, 2010, Sartor, Marta; Xu, Zelin; Aryal, Santosh; Hu, Che-Ming J., Pornpattananangkul, Dissaya, Clawson, Corbin; Esener, Sadik; and Zhang Liangfang. The dissertation author was the primary investigator and author of this paper.

In this chapter the help of Dr. Aryal for the chemistry was highly appreciated. Dr. Aryal provided the PCL-PEG polymer and assisted with the ^1H -NMR and GPC measurements.

The porosity gradient initial work was done in a collaboration with Dr. Carson and a special thanks goes to Michael Chan for his assistance on the HPLC measurements. His experience was highly valuable on getting some initial data with their HPLC machine and on setting the proper values for the future work with our new one (used for the chemical gradient project).

This work was possible also thanks to the active comments I've received from all the team members of Prof. Zhang. In particular I would like to thank Zeling Xu for being such a valid student and providing me with a highly valuable assistance on my experiments.

2.8 MATERIALS AND METHODS

PCL SYNTHESIS

Polycaprolactone (PCL) conjugated to the monomethyl ether polyethylene glycol (PEG) was synthesized through ring-opening polymerization of ϵ -caprolactone using PEG as a macroinitiator in presence of catalytic amount of stannousoctoate[26]. In a typical experiment, 200 mg of PEG, 0.8mg of ϵ -caprolactone monomer, and 4.5 mg of stannousoctoate were mixed in a three necked flask under nitrogen. The ring-opening polymerization was carried out under nitrogen at 120°C for 12 hr. The resulting PEG-PCL polymer were dissolved in methylene chloride and precipitated in ether. This

process was repeated for three times to purify the product. The resulting PCL-PEG was subsequently dried under vacuum for 24 h and kept at -20°C for further use.

¹H-NMR MEASUREMENTS

¹H-NMR was taken with a Varian Mercury-400 spectrometer operated at 400 MHz. The ¹H NMR spectra (CDCl₃, δ ppm) of PCL-PEG showed the characteristic peaks of PCL: 4.12-4.02 [t, J=7.0 Hz, -(CO)-CH₂CH₂CH₂CH₂CH₂O, the fifth methylene group connected to carbonyl of repeating unit of PCL], 3.70-3.62 [t, J=5.0 Hz, -(CO)-CH₂CH₂CH₂CH₂CH₂OH, end group of PCL], 2.35-2.24 [t, J=7.5 Hz, -(CO)-CH₂CH₂CH₂CH₂CH₂O, the first methylene group connected to carbonyl of repeating unit of PCL], 1.72-1.56 [m, -(CO)-CH₂CH₂CH₂CH₂CH₂O, the second and fourth methylene groups connected to carbonyl of repeating unit of PCL], 1.42-1.33 ppm [m, -(CO)-CH₂CH₂CH₂CH₂CH₂O, the third methylene group connected to carbonyl of repeating unit of PCL]. The characteristics peaks of PEG segments were observed at 3.4-3.6 ppm (br, -O-CH₂-CH₂-, the repeating unit of MPEG).

GPC MEASUREMENTS

GPC measurements were carried out with OmniSEC (Viscotek, USA) tetrahydrofuran (THF) as the mobile phase with a flow rate of 1 mL/min. Molecular weight averages as well as polydispersity indices were calculated from a calibration curve using a series of polystyrene standards.

SCANNING ELECTRON MICROSCOPE IMAGES

The nanoparticles were diluted in water to 1 µg/mL. Several drops of the nanoparticle solution were deposited on a silicon chip and dried at room temperature for 12 hours. The chip with the dried samples was sputter coated with chromium for 30 seconds at 130 mA before imaging. Emitech K575X Sputter Coater was used. The images were taken with Phillips XL30 ESEM.

MOLAR PERCENT CALCULATIONS

The following equations were used to calculate the amounts of each polymer needed to make the desired molar percent:

$$m_S/(m_S + m_L) = x_S$$

$$M_S + M_L = 1 \text{ mg}$$

$$M_S = m_S * MW_S, M_L = m_L * MW_L$$

where m is the moles, x is the percent of moles, M is the mass, MW is the molecular weight, each of those refers to the shorter (S) or the longer (L) PLGA according to the subscript.

NANOPARTICLES SYNTHESIS

The polymers were combined in a final volume of 1 mL and precipitated drop-by-drop on 3 mL of constantly stirred water. The solutions with the formed particles was left stirring in the hood for two hours before being washed with an 10kDa molecular weight cut off (MWCO) centrifugal Amicon-filter (Millipore). The washes were done by three alternating cycles of concentration and dilution. The concentration was done by spinning

the filter at 3,500 r.p.m. (rotation per minute) for 10 minutes. After the last concentration the solution was resuspended to the desired nanoparticles concentration.

SIZE AND ζ - POTENTIAL MEASUREMENTS

Size and ζ -potential were measured by dynamic light scattering (DLS) with Malvern NanoZeta. The measurement was done using about 1 mL of nanoparticles in water.

LOADING YIELD CALCULATION

Loading yield percent was calculated as the weigh percent of loaded drug over polymer used (it was assumed no loss of polymer during nanoprecipitation).

PACLITAXEL LOADING BY HPLC (POROSITY GRADIENT ONLY)

5% wt/wt paclitaxel (Sigma) was encapsulated during nanoprecipitation (as described). To measure the loaded drug the nanoparticles were broken in a 50% acetonitrile aqueous solution and were gently shaken overnight at 4°C with a vortex at the lowest setting (500rpm). The drug in the solution was then measured by HPLC with Agilent Technology, 1100 Series LC/MSD Trap (USA), using a C18 column. The column used was Discovery HS C-18 (Supelco) 2.1x150mm, flowing 0.2 mL/min with UV detection at 230 nm. The column was left at room temperature. The mobile phase water to acetonitrile ratio started at 90:10 and increased to 90% acetonitrile over 8 mins. 90% acetonitrile was maintained for 4min and then returned to 90:10 water to acetonitrile for the next 7 mins. Each set of data was measured with a calibration curve, both samples and standards were in an aqueous solution of 50% acetonitrile.

PACLITAXEL LOADING BY HPLC (CHEMICAL GRADIENT ONLY)

5% wt/wt paclitaxel (Sigma) was encapsulated during nanoprecipitation (as described). To measure the loaded drug the nanoparticles were broken in a 70% acetonitrile aqueous solution and were gently shaken for two days at room temperature with a vortex at the lowest setting (500rpm). Paclitaxel was quantified by HPLC VARIAN HPLC SYSTEM with starwork integration software. The column used was uBONDAPAK 4.6x150mm flowing 1 mL/min with UV detection at 230 nm. The column was kept at 30°C. The mobile phase water to acetonitrile ratio started at 50:50 and increased to 100% acetonitrile over 10 mins. 100% acetonitrile was maintained for 4min and then returned to 50:50 water to acetonitrile for the next 10 mins. Each set of data was measured with a calibration curve, both samples and standards were in an aqueous solution of 70% acetonitrile.

DOXORUBICIN LOADING AND QUANTIFICATION

Doxorubicin Hydrochloride was bought from Sigma. 1mg/mL particles were loaded with 5% wt/wt drug. A calibration curve was prepared in aqueous solution. The UV measurements were done on the nanoparticle solution with a spectrophotometer (Tecan Infinite M200), the excitation wavelength was 470nm, and the recorded emission wavelength was 584nm.

PACLITAXEL QUANTIFICATION BY HPLC (DRUG RELEASE)

Paclitaxel was measured by HPLC AGILENT HPLC SYSTEM. The column used was Econosphere C-18 5u (Alltech) 250x3.2mm flowing 1 mL/min with UV detection at 230 nm. The column was kept at 30°C. The mobile phase water to acetonitrile ratio

50:70 water to acetonitrile was kept constant during the measurements. Each set of data was measured with a calibration curve, both samples and standards were in the same acetonitrile percent solution.

LAURDAN MEASUREMENTS

Laurdan dye (Invitrogen, USA) was loaded on a 0.5% wt/wt dye/polymer. The emission spectra of the loaded samples were measured with a spectrophotometer (Tecan Infinite 200): the excitation wavelength was 350nm, the emission was scanned from 400nm to 550nm. To create Figure 2.3B the nanoparticles from PLGA-PEG and PCL-PEG were mixed in the same weight proportion of the polymers used for the molar ratio 25:75.

2.9 BIBLIOGRAPHY

- [1] Y. Malam, M. Loizidou, A. M. Seifalian, *Trends in Pharmacological Sciences* **2009**, 30, 592.
- [2] V. P. Torchilin, *Nature Reviews Drug Discovery* **2005**, 4, 145.
- [3] C. J. Chen, D. D. Han, C. F. Cai, X. Tang, *Journal of Controlled Release* **2010**, 142, 299.
- [4] Z. Drulis-Kawa, A. Dorotkiewicz-Jach, *International Journal of Pharmaceutics* **2010**, 387, 187.
- [5] D. Pornpattananangkul, S. Olson, S. Aryal, M. Sartor, C. M. Huang, K. Vecchio, L. Zhang, *Acs Nano* **2010**, 4, 1935.
- [6] S. Parveen, S. K. Sahoo, *Journal of Drug Targeting* **2008**, 16, 108.
- [7] R. C. Mundargi, V. R. Babu, V. Rangaswamy, P. Patel, T. M. Aminabhavi, *Journal of Controlled Release* **2008**, 125, 193.
- [8] D. E. Owens, N. A. Peppas, *International Journal of Pharmaceutics* **2006**, 307, 93.
- [9] M. J. Joralemon, S. Mcrae, T. Emrick, *Chemical Communications* **2010**, 46, 1377.
- [10] G. Pasut, F. M. Veronese, *Advanced Drug Delivery Reviews* **2009**, 61, 1177.
- [11] C. Wischke, S. P. Schwendeman, *International Journal of Pharmaceutics* **2008**, 364, 298.
- [12] F. Gu, L. Zhang, B. A. Teply, N. Mann, A. Wang, A. F. Radovic-Moreno, R. Langer, O. C. Farokhzad, *Proceedings of the National Academy of Sciences of the United States of America* **2008**, 105, 2586.
- [13] J. Cheng, B. A. Teply, I. Sherifi, J. Sung, G. Luther, F. X. Gu, E. Levy-Nissenbaum, A. F. Radovic-Moreno, R. Langer, O. C. Farokhzad, *Biomaterials* **2007**, 28, 869.
- [14] J. Ren, H. Y. Hong, J. X. Song, T. B. Ren, *Journal of Applied Polymer Science* **2005**, 98, 1884.
- [15] J. K. Jackson, T. Hung, K. Letchford, H. M. Burt, *International Journal of Pharmaceutics* **2007**, 342, 6.
- [16] F. Danhier, N. Lecouturier, B. Vroman, C. Jerome, J. Marchand-Brynaert, O. Feron, V. Preat, *Journal of Controlled Release* **2009**, 133, 11.
- [17] Y. Wang, H. Xu, X. Zhang, *Advanced Materials* **2009**, 21, 2849.

- [18] R. T. Liggins, T. Cruz, W. Min, L. Liang, W. L. Hunter, H. M. Burt, *Inflammation Research* **2004**, 53, 363.
- [19] L. L. Lao, S. S. Venkatraman, N. A. Peppas, *Eur J Pharm Biopharm* **2008**, 70, 796.
- [20] T. Parasassi, E. K. Krasnowska, L. Bagatolli, E. Gratton, *Journal of Fluorescence* **1998**, 8, 365.
- [21] T. Parasassi, G. Destasio, G. Ravagnan, R. M. Rusch, E. Gratton, *Biophysical Journal* **1991**, 60, 179.
- [22] T. Parasassi, G. Destasio, A. Dubaldo, E. Gratton, *Biophysical Journal* **1990**, 57, 1179.
- [23] B. Wang, L. F. Zhang, S. C. Bae, S. Granick, *Proceedings of the National Academy of Sciences of the United States of America* **2008**, 105, 18171.
- [24] R. Sachl, M. Stepanek, K. Prochazka, J. Humpolickova, M. Hof, *Langmuir* **2008**, 24, 288.
- [25] L. Z. Juliana M. Chan , Kai P. Yuet , Grace Liao , June-Wha Rhee , Robert Langer , Omid C. Farokhzad, *Biomaterials* **2009**.
- [26] S. Aryal, K. C. R. Bahadur, N. Bhattarai, B. M. Lee, H. Y. Kim, *Materials Chemistry and Physics* **2006**, 98, 463.

CHAPTER 3 COMBINING INCOMPATIBLE MATERIALS: AMPHIPHILIC HYBRID NANOPARTICLES

3.1 INTRODUCTION ON AMPHIPHILIC DELIVERY VEHICLES

Amphiphilic drug delivery vehicles can load materials with two incompatible characteristics: hydrophobicity and hydrophilicity. Liposomes, for example, are amphiphilic delivery vehicles, because they can load both hydrophilic and hydrophobic drugs. While the loading of hydrophilic material is very efficient, the loading of hydrophobic drug is small and limited to the lipid bilayer. Liposomes' loading can be described as compartmental: hydrophilic material inside, hydrophobic material on the shell. Moreover, liposomes are characterized by a burst release, therefore the drug can be kept at effective concentration only for a short period of time.

On the other hand, the loading of hydrophobic drug is highly successfully in poly (lactic-co-glycolic acid) (PLGA) nanoparticles. PLGA-based nanoparticles are stable and with a sustained release, but are not amphiphilic because they cannot easily load hydrophilic drugs.

Several strategies have been used to load a hydrophilic material, such as a small molecule drug, into a PLGA nanoparticle. These methods involve modifications of the PLGA, the drug, or the fabrication process. For example, PLGA can be combined with some hydrophilic polymer to create hydrophilic areas[1]. Similarly, the drug can be either conjugated to the PLGA[2] itself or conjugated to a short hydrophobic segment. In both cases, chemical modifications of the drug itself will have the advantage of creating a pro-drug but at the same time could affect the efficiency of the therapy. Combining PLGA

with another polymer is not ideal either; the chosen polymer must offer the possibility of a successful chemical conjugation, should be compatible with the fabrication process and be approved by the food and drug administration (FDA). This last requisite significantly limits the possible choices. In other words, the modified PLGA should still behave mostly as PLGA chemically during fabrication to create uniformly shaped, non-toxic, stable nanoparticles.

To mediate aforementioned difficulties, several researchers concentrated their effort on finely tuning the fabrication process to trap the highest amount of hydrophilic drugs[3, 4], while keeping the created particles stable and uniform. Often the best loading is achieved with a double emulsion method, but this creates micron size particles [4]. Meanwhile, the most desirable size for a blood circulating drug delivery vehicle is below 200nm[5, 6].

Among the different hydrophilic therapeutic materials, oligonucleotides are attracting increasing attention. In the recent years, with the advances in gene therapy, several oligonucleotides have been identified as possible treatments in different number of illnesses[7]. Oligonucleotides act by inducing or silencing the synthesis of an amino acids's sequence. For instance, they can either induce the production of a foreign protein or halt the fabrication of an essential one. The latter effect can only be transient, while the former has longer effects and can be permanent, often using a viral vector to integrate the sequence into the genomic DNA. Several studies have attempted to create efficient non-viral nanoparticles for gene therapy[7]. Scientists have worked with different types of therapeutical oligonucleotides from the bigger plasmid DNA (pDNA)[8-11], to the lower size antisense DNA[10, 12, 13] and small interferon RNA (siRNA)[12, 14, 15].

pDNA is often used to produce a protein, ranging from 1 to 1,000 kilo base pairs pDNA can be easily produced at a pharmaceutical grade in sufficiently high quantities. Despite its powerful properties, pDNA is poorly up-taken by the cells and it is rapidly degraded in serum by nucleases activity. Intravenous injections of naked pDNA do not induce gene expression[16], therefore to dramatically improve the gene transfer in vivo naked pDNA has been delivered with the aid of physical approaches such as hydrodynamics method, gene gun, electroporation, sonoporation, and laser irradiation [10].

Antisense DNA and siRNA both silence the expression of a specific gene. The short oligonucleotides sequences act by binding to the messenger RNA (mRNA) that encodes the gene and they induce its degradation. The degraded mRNA can no longer be used to translate the genetic code into the amino acids sequence. Antisense DNA are single stranded DNA sequences and range from 13 to 30 bases. siRNA are double stranded RNA sequences and are usually between 20-25 base pairs (bp).

Despite the size differences most oligonucleotides are difficult to deliver because they can be easily degraded by enzymes in the serum. At the same time, they are very sensitive to the materials used during the synthesis of the polymeric nanoparticles. In some preparation methods, oligonucleotides can be degraded or compromised by the liquids such as acetonitrile used during encapsulation. In particular, the organic solvents commonly employed in preparation of PLGA nanoparticles can be deleterious to the functionality of the oligonucleotides.

In the following we describe how small (about 100 nm) PLGA hybrid nanoparticles[17] are created and loaded with different oligonucleotides sequences. Our novel method allows for assisted loading of hydrophilic material into a hydrophobic core.

PLGA hybrid nanoparticles were chosen for their low toxicity, slow release and easy fabrication. The loading into the PLGA core is made possible with the use of an amphiphilic zwitterionic polymer (PMAL-C8) that acts as the interface between the hydrophilic oligonucleotides and the hydrophobic PLGA core. A detailed study was done to observe the interaction of PMAL-C8 with the oligonucleotides and in the hybrid nanoparticles. The biological functionality (or bio-functionality) of the nanoparticles loaded with this new method was tested and exhibited very promising result. We have developed a theoretical understanding of the dynamic involved in the assisted loading. The offered a basic model can be further optimized and its use can be extended to peptides and small molecules towards a combined hydrophobic/hydrophilic drug delivery.

3.2 HYBRID NANOPARTICLES: OVERVIEW

PLGA hybrid nanoparticles have been studied by Chan et. al. [17]. The PLGA core is created by nanoprecipitation and subsequently coated with a lipid layer which self-assembles to cover the hydrophobic surface.

The hybrid nanoparticles can be created with a high range of sizes based on the solvent used during nanoprecipitation and the PLGA's inherent viscosity. In particular, the drug release profile changes based on the mass percent of lipid to PLGA. The hybrid nanoparticles give a more steady drug release profile along with a higher stability in comparison to nanoparticles made with either bare PLGA or poly(ethylene glycol) (PEG) conjugated with PLGA. Experimental results showed that hybrid nanoparticles were stable in serum for at least five days.

Important advantages offered by the hybrid nanoparticles include their biocompatibility and low toxicity. In the study conducted by Chan et. al., the hybrid nanoparticles have shown no toxicity at all in vitro for a concentration up to 5 mg/mL. Moreover, the hybrid nanoparticles are perfectly suited for large-scale production and long shelf life. The production method can be easily scaled to much larger volume and the produced nanoparticles can be lyophilized for storage and subsequently regenerated in water at the time of use.

PLGA hybrid nanoparticles can be attached to targeting ligands for local delivery to the diseased site. For a targeted version, part or all of PEG present on the surface is chemically conjugated to a targeting moiety such as a half-antibody[18].

PLGA hybrid nanoparticles have been used to load a plasmid DNA[19]. Zhong et. al. studied the release of oligonucleotides based on their loading location: surface, core and both. The surface loading was achieved with the use of cationic lipids such as (1, 2-Dioleoyl-3-Trimethylammonium-Propane) (DOTAP) or {3b-[N-(N', N'-Dimethylaminoethane)-carbonyl] Cholesterol} (DC-Chol), whereas the loading in the core was assisted by protamine sulfate (highly cationic peptide). Despite the excellent performance this platform offers, the cationic lipids can have toxic effect for in-vivo applications[20]. In addition, protamine sulfate is used in the clinic to contrast the effect of heparin[21], therefore even if this platform could highly improve the loading of oligonucleotides in clinics, it can lead to potentially lethal drug interactions in some patients. Moreover the peptide is extracted from fish, and people with allergies to fish, might manifest a strong reaction to a delivery vehicle made with it.

In this work DNA has been loaded in the PLGA core with a zwitterionic amphipol rather than a cationic material (polymer, lipid, or peptide). Unlike cationic materials, an

amphiphilic is not limited to negatively charged material. Furthermore the hydrophilic payload doesn't just adsorb onto the surface, but it is "wrapped" by the amphiphilic and made hydrophobic. This concept can be extended to other chemical properties based on the chaperone polymer used.

3.3 PMAL: A POWERFUL ZWITTERIONIC AMPHIPHILIC

An amphiphilic (or apol) is a low molecular weight amphipathic polymer, that has alternating hydrophobic and hydrophilic side chains, which confers amphiphilic (or amphipathic) properties. Amphiphils have been created to offer an alternative to surfactant[22, 23]. They have been mostly used to make water-soluble, and at the same time biochemically stable, membrane proteins[24], peptides[25], and enzymes[26]. The several hydrophobic segments on the amphiphilic interact in different locations on the hydrophobic part of the membrane protein, whereas the polar hydrophilic segments are going to face towards the aqueous environment[27].

Among the several amphiphils described in the literature two in particular have been studied in details: A8[27-32] and PMAL[26, 33]. PMAL is the amphiphilic chosen for this study.

PMAL is the first zwitterionic amphiphilic and it is commercially available from Anatrace. The chemical structure of PMAL-C8 is shown in Figure 1.

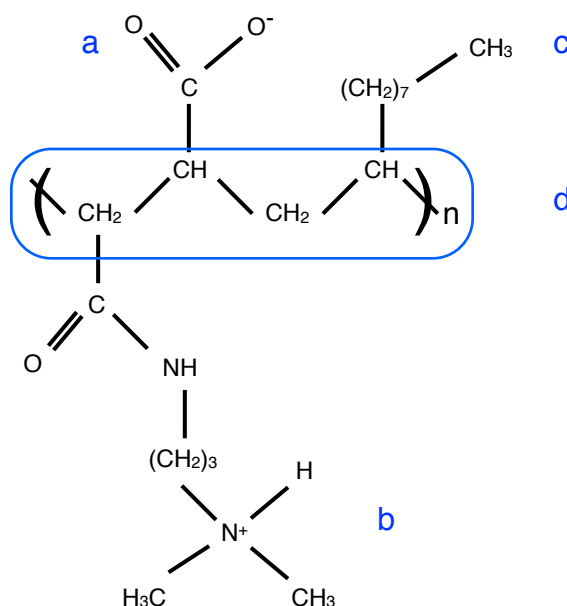


Figure 3.1: Chemical structure of the commercially available PMAL-C8

PMAL is Poly (maleic anhydride-alt-1-decene) substituted with 3-(Dimethylamino) propylamine and C8 is the length of the apolar carbon chain (labeled with c in figure 3.1). As can be seen from Figure 1, PMAL is composed of a hydrophobic backbone (labeled with d in figure 3.1), with alternating polar (labeled with a and b in figure 3.1) and apolar (labeled with c in figure 3.1) units. The polar unit is common to all the different PMALs, while the apolar segment can be as short as eight carbons (PMAL-C8) or as long as sixteen carbons (PMAL-C16). PMAL-C8 has a molecular weight of 18.5 kDa and contains approximately 50 repeating units ($n=50$ in Figure 3.1). PMAL's zwitterionic properties are retained at low and neutral pH[26]. Its use in strongly basic pH is not recommended.

Based on the literature, PMAL efficiently delivers membrane proteins to a lipid bilayer[33] and does not interfere with the functionality of the membrane enzyme it solubilizes[26]. Considering this property, it is expected that oligonucleotides loaded with

PMAL can maintain their functionality and possibly receive an extra help on escaping the endosome. A similar mechanism involved in the protein delivery [33] could be involved in an endosomal escape. PMAL has been used to coat quantum dots with siRNA and the complex have been shown to deliver the oligonucleotides[34]. The study showed not only that siRNA can be delivered with PMAL, but also that the amphipol is not toxic to cells. When using amphipol, particular care should to be taken in the presence of cation such as Ca^{2+} , since both PMAL and A8 had shown to aggregate in presence of Ca^{2+} [35]. Calcium induced aggregation can be a disadvantage in some cases, but advantageous in others. For instance studies made in our lab showed that Ca^{2+} can be used to make PMAL-C16 (PMAL with the longest apolar chain) miscible in acetonitrile. In this study, however, no calcium cations were used since DNA is usually stored in an EDTA (EthyleneDiamineTetraAcetic acid) containing solution. EDTA can bind to cations (such as Ca^{2+}) which are usually necessary for enzymatic activity. That's why oligonucleotides are stored in presence of EDTA to help minimize any DNase induced degradation.

3.4 OLIGONUCLEOTIDES-PMAL COMPLEX

An initial experiment was conducted to verify that PMAL-C8 can indeed interact with oligonucleotides and make them soluble in a solvent such acetonitrile. Acetonitrile was chosen because it is one of the solvent used for the nanoprecipitation of the PLGA hybrid nanoparticles. Owing to its apolar chain, PMAL-C8 can be mixed with acetonitrile, if used at a low concentration ($< 50 \mu\text{M}$). When PMAL-C8 is combined with acetonitrile, it makes a slightly cloudy solution, but it doesn't precipitate out of solution (unlike PMAL-C16). The zwitterionic nature of PMAL makes it soluble either in highly polar liquids such

as water, or non-polar liquids such as chloroform. Acetonitrile is much less polar than water, hence PMAL-C8 has a lower solubility in it.

In the first experiment a 40bp DNA sequence was used to examine whether there is any interaction between PMAL-C8 and DNA. In this work the oligonucleotide sequences used (DNA or RNA) were all double stranded. Different amounts of PMAL-C8 were added to a low volume aqueous solution with 0.5 nano moles of DNA. To quantify the DNA, Sybr Green I was used to label the oligonucleotides. PMAL-C8 was added in amounts varying from 0 to 10 nano moles (Figure 3.2). As a control similar solutions were created without DNA, and Sybr Green I was added to evaluate any “false” positive (Figure 3.3). To the naked eye, Sybr Green I looks orange when not bound to DNA and yellow when bound to DNA. The actual quantification by Sybr was made with a fluorometer (TECAN 2000Infinity) by measuring the green emission (520nm) of the fluorophore excited with 488nm.

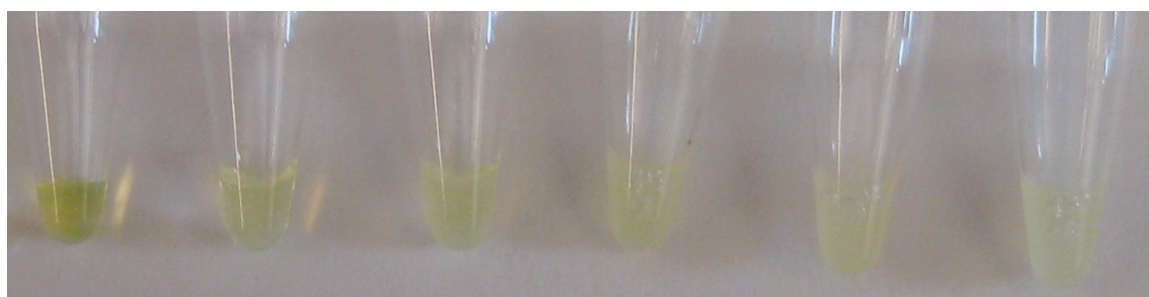


Figure 3.2: Sybr Green I stained DNA mixed with different amounts of PMAL-C8: 0, 2, 4, 6, 8, and 10 nano moles (from the left).

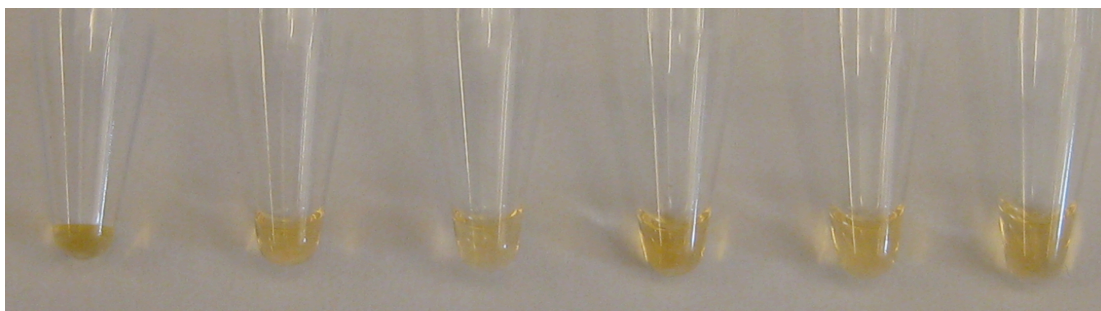


Figure 3.3: Sybr Green I mixed with different amounts of PMAL-C8: 0, 2, 4, 6, 8, and 10 nano moles (from the left).

As shown in Figure 3.2, the solutions containing DNA has increasing opacity with the increase of PMAL-C8 (in absence of DNA PMAL-C8 forms a clear solution in water). On the other hand, the solutions set containing only PMAL-C8 and Sybr Green I did not show the same effect, implying that the cloudiness is most likely from the interaction between PMAL-C8 and DNA. The solutions were left at 4°C for about 40 minutes, during which the Sybr Green I labeled DNA precipitated out of the water solution. The precipitation was observable in the vial containing at least 6 nano moles of PMAL-C8. It was possible indeed to see the clear water on the top and the labeled DNA at the bottom (Figure 3.4).

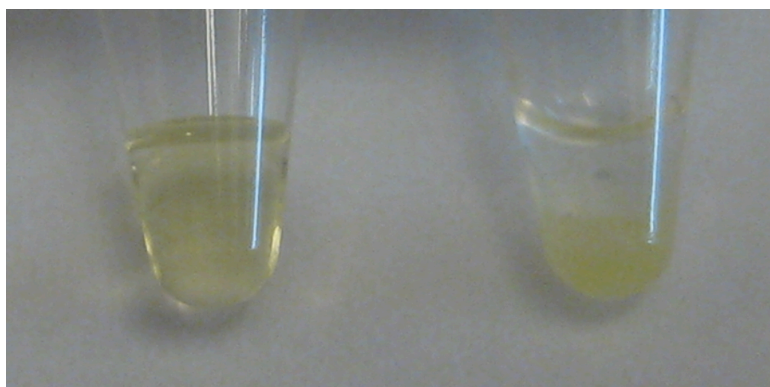


Figure 3.4: DNA-Sybr Green I in water with (right) and without (left) 6 nano moles of PMAL-C8.

For comparison free Sybr Green I was left at 4°C for the same amount of time and didn't show any precipitation out of water (Figure 3.5). Small interaction between the Sybr Green I dye and the polymer PMAL-C8 is expected due to the slightly positive charge of Sybr Green I that could partially affect the signal from the dye. There was no significant difference when the two tubes in figure 5 were closely observed, although in figure 3 slightly varying angles of the tubes produced the apparent color changes.

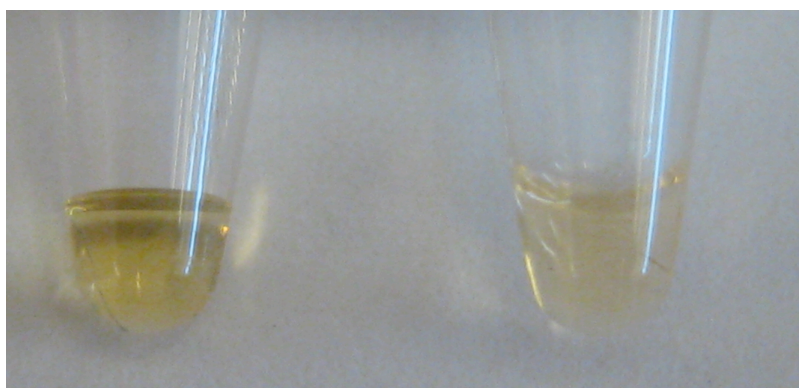


Figure 3.5: Sybr Green I after 40 min incubation at 4°C with (right) and without PMAL-C8.

To eliminate any nonspecific interaction of the DNA staining dye and the polymer, the experiment was repeated with a 20 bp sequence which had a fluorophore (6-FAM) attached on the 5' end. Because the dye is conjugated to the DNA, the signal measured could only come from the DNA.

The same set of solution was prepared with the dye-conjugated 20bp DNA and it mixed with PMAL-C8 (Figure 3.6).

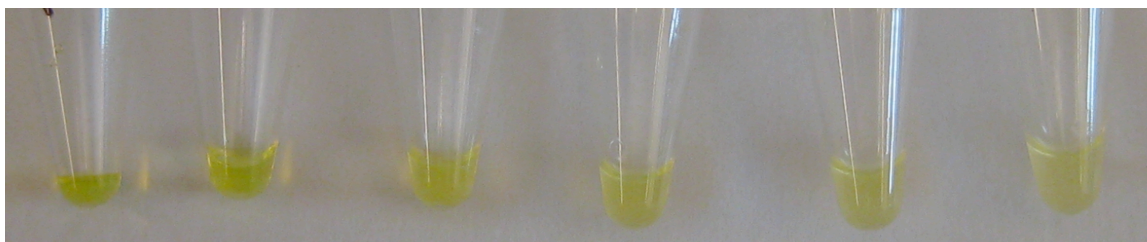


Figure 3.6: 6-FAM labelled DNA mixed with different amounts of PMAL-C8: 0, 2, 4, 6, 8, and 10 nano moles (from the left).

All the samples with the zwitterionic amphipol looked very similar, but the “cloudiness” is more evident with at least 6 nano moles of PMAL-C8. Unlike the longer sequence, no precipitation was seen when the sequence was left at 4°C for 40 minutes, possibly a consequence of the size difference between the two sequences (Figure 3.7).

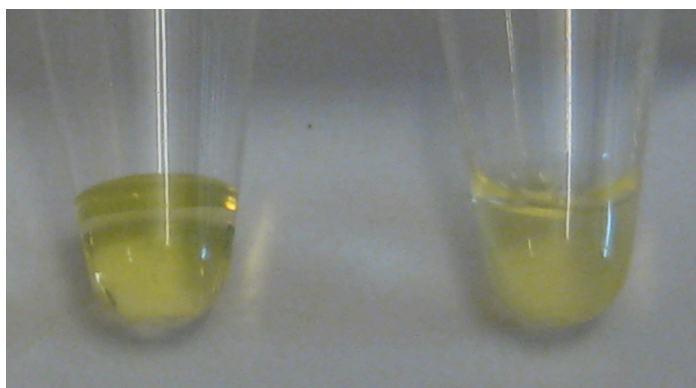


Figure 3.7: 6-FAM labelled DNA in water after 40 minutes at 4°C with (right) and without (left) PMAL-C8.

When mixed with acetonitrile, DNA alone, precipitated out of solution. Small clusters can often be seen on the wall of the test tube. Little to no fluorescence was retained. On the other hand, DNA-PMAL-C8 complex mixed with acetonitrile, and did not form a precipitate (Figure 3.8).

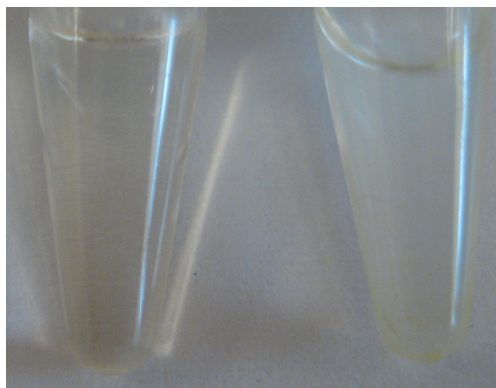
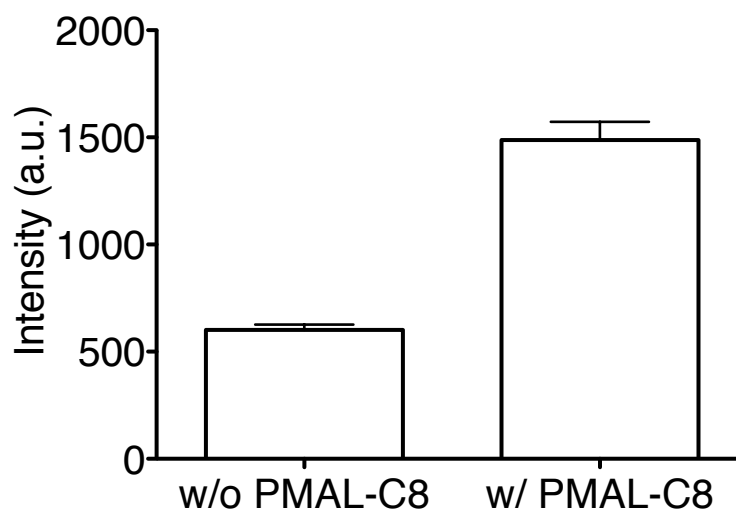


Figure 3.8: 6-FAM labelled DNA in acetonitrile with (right) and without (left) PMAL-C8.

The intensity of the 6-FAM was measured both in the presence and the absence of PMAL-C8, showing clearly a higher number of DNA units with the PMAL-C8 (Graph 3.1). The sample in acetonitrile containing the PMAL-C8 has three times the intensity as the sample without the zwitterionic amphipol.

Graph 3.1: Intensity at 520nm of 6-FAM dye in acetonitrile with and without PMAL.



These first tests show that PMAL-C8 can interact with different DNA sequences. Considering the chemical structure in Figure 3.1, the nucleotides' negative charge interacts with the positive polar arm (labeled as b in figure 3.1). Figure 3.4 shows that following the binding with the DNA, the PMAL-C8/DNA complex became less soluble in water. In acetonitrile the behavior of PMAL with or without DNA is fairly similar (picture not shown), indicating that changes due to the PMAL-C8 complexing with DNA are less evident in a less polar solvent.

3.4 PHYSICAL CHARACTERIZATION OF THE NANOPARTICLES

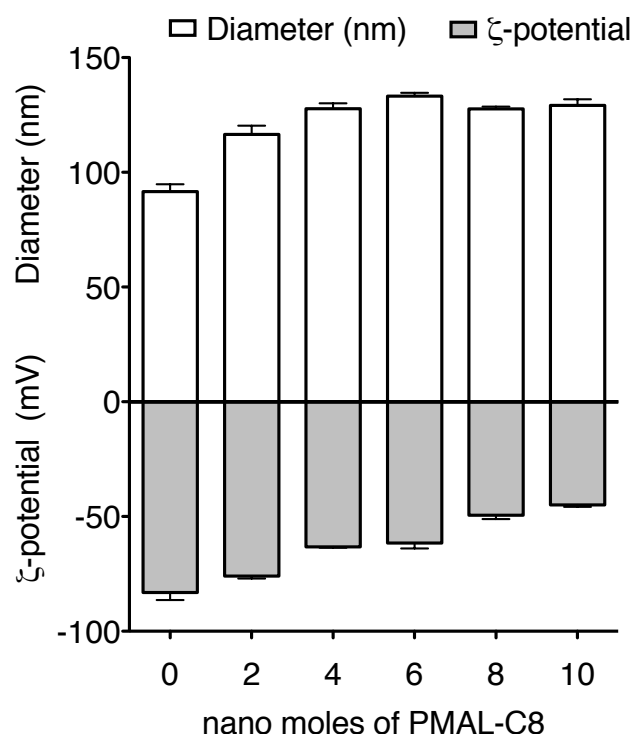
PMAL-C8 and DNA have been proven to interact and to form a fairly stable suspension in acetonitrile. It is now necessary to prove that the new complex can improve the loading of DNA into a PLGA hybrid nanoparticle.

The PLGA hybrid nanoparticles are created based on the published protocol[36] (materials and methods). Different amounts of PMAL-C8 were added to the PLGA solution before nanoprecipitation. The particles were washed with an Amicon filter.

Initially, the nanoparticles were made without DNA, but with varying amounts of PMAL-C8. When more than 10 nano moles of the zwitterionic amphipol were used, the created nanoparticles weren't stable and quickly precipitated out of solution. The instability can be easily explained by considering the interactions between the apolar arms of PMAL-C8 and the tails of the lipids used to coat and stabilize the PLGA core. A high excess of PMAL-C8 can most likely disrupt this stabilizing effect the lipid layer by either removing some of the lipids or by creating some tighter clusters on the surface, which would leave some small areas of exposed PLGA. The size and the ζ -potential of

the different particles were measured in water by Dynamic Light Scattering (DLS) (Graph 3.2).

Graph 3.2: Size and ζ -potential of the hybrid nanoparticles with different amounts of PMAL-C8.

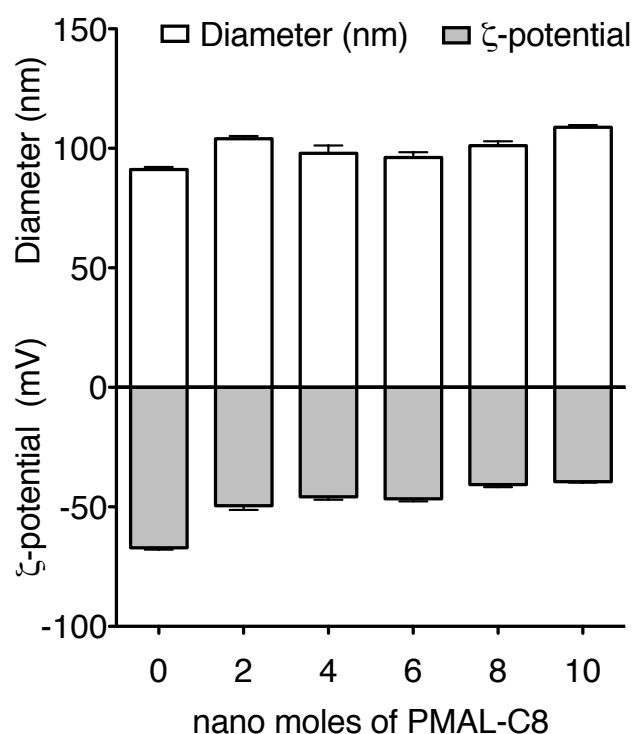


The size of the hybrid nanoparticles is bigger when PMAL-C8 is added. As suspected, the PMAL-C8 can interact with the lipids used to coat the surface, and this would explain the higher ζ -potential. The negative charge is given by the carboxyl terminated lipid-PEG conjugate, therefore a lower degree of lipid-PEG coating would result in a higher ζ -potential. Because PEG is what stabilizes the particles[36], an insufficient amount of PEG coating causes aggregation, which is what happens when less than 10 nano moles of PMAL-C8 are used.

The physical characterization was repeated with a set of nanoparticles loaded with DNA (Graph 3.3). In this test 40bp DNA was labeled with Sybr Green I and was

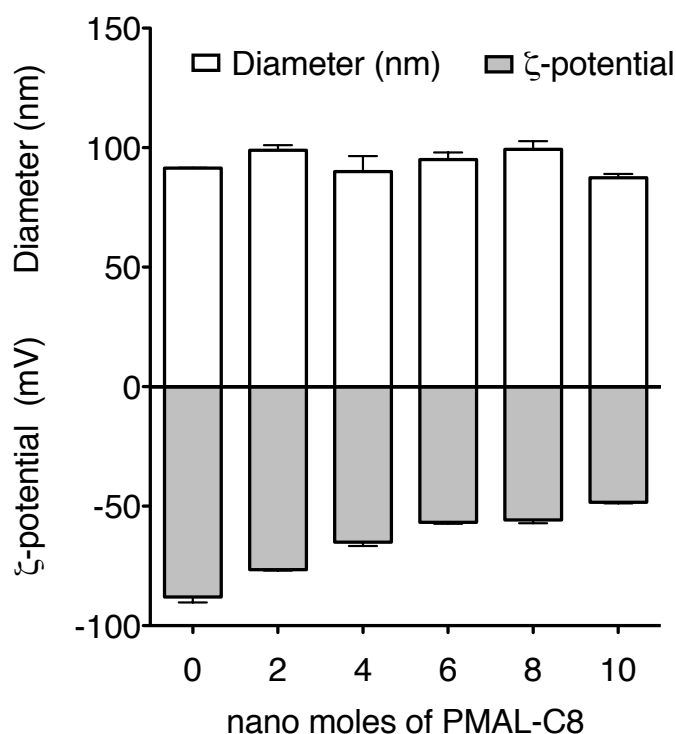
mixed with the PLGA before precipitation. The particles were cleaned with a 100,000 MWCO filter, which eliminates any unloaded DNA. Particles size and ζ -potential were measured in water. No significant interference was expected from the Sybr Green I dye, and a similar experiment without the dye gave comparable results.

Graph 3.3: Size and ζ -potential of the hybrid nanoparticles loaded with DNA (40bp) combined with different amounts of PMAL-C8.



Both size and ζ -potential are similar for all the PMAL-C8 containing nanoparticles. It is possible that some of the PMAL-C8 are interacting with the lipid layer, but it is not dependent from the amount of PMAL-C8 used. The ζ -potential difference suggests a minimum interaction between the PMAL-C8 and the lipids.

Graph 3.4: Size and ζ -potential of the hybrid nanoparticles loaded with DNA (20bp) combined with different amounts of PMAL-C8.



A similar experiment was done with the shorter sequence (6-FAM labeled 20bp) and gave a size profile similar to the nanoparticles loaded with the longer DNA sequence, while the ζ -potential profile was similar to the one of the empty particles (Graph 3.4). Considering these results and on the precipitation observed for the longer sequence, it can be suggested that the shorter sequence interacts with a lower number of PMAL-C8 than the longer one. It is therefore possible that the 20bp length is small enough to limit the DNA interactions to an area within a single PMAL-C8 polymer, while a longer sequence can combine more than one. The PMAL-C8 effect on the ζ -potential is therefore partially masked when a long DNA sequence is used.

To further clarify the effect of the PMAL-C8, two sets of particles were made. One set was loaded with a PMAL-C8/siRNA complex and the other without. The siRNA sequence used in this image is a short one (~20bp) and can be considered comparable to the 6-FAM labeled DNA. The sample was made with 10 nano moles of PMAL-C8, because it was the highest amount that could be loaded without disrupting the stability. Images of the particles were taken with a scanning electron microscope (SEM) and were compared to one another (Figure 3.9). The figures show a uniform particle size distribution for both compositions. The nanoparticles have a well defined smooth shape in either of the set imaged. On the image no small clusters are visible, offering a good sign of a non disruptive behavior. It can be inferred that up to 10 nano moles, the PMAL-C8 added is not affecting the surface stability. It is possible that the PMAL-C8/oligonucleotides complexes are below the lipid layer. From the images the location of the PMAL-C8/oligonucleotides complex does not seem to affect the stability or the uniformity of the structure compared to the unloaded hybrid nanoparticles.

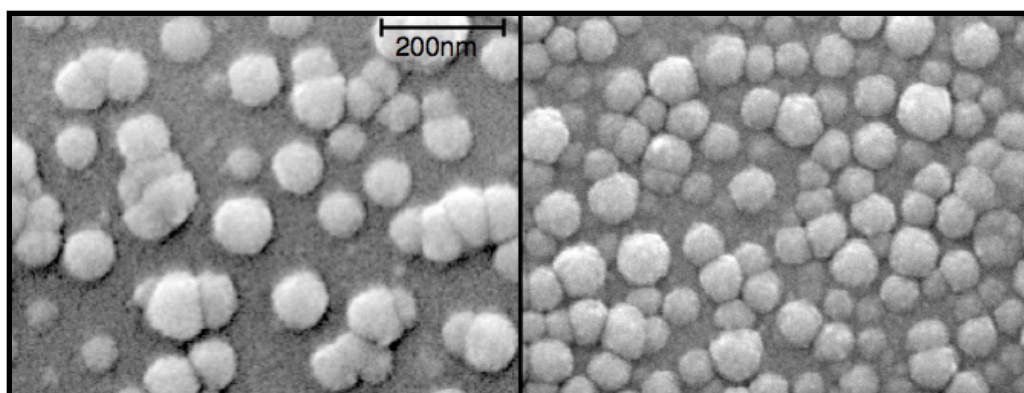
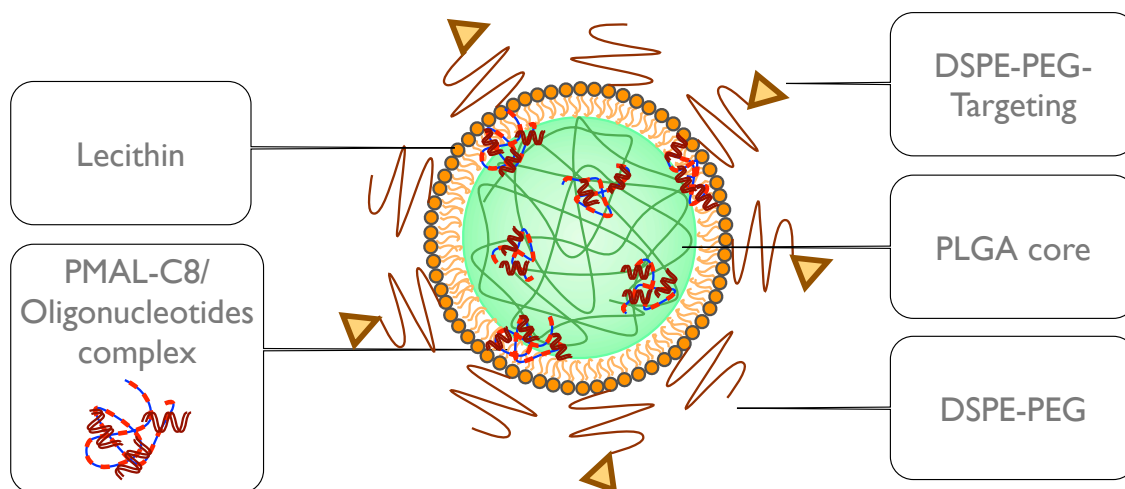


Figure 3.9: SEM images of the nanoparticles loaded with (left) and without (right) the PMAL-C8/DNA complex.

From the data described so far it is possible to draw a detailed schematic that represents the newly created nanoparticles (Scheme 3.1). The representation includes

the option of adding targeting moiety to the PEG conjugated 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE).



Scheme 3.1: Structure of the hybrid nanoparticles with PMAL-C8/Oligonucleotides complex loaded.

The nanoparticle's main structure is expected to be very similar to the hybrid nanoparticles, as described by Chan et. al.[17]. The PMAL-C8/oligonucleotide complexes are most likely going to distribute close to the surface between the PLGA and the lipid tails. The apolar component of the PMAL-C8 is expected to have a good affinity with the lipid tails, but it is not excluded that the complexes could locate inside the nanoparticle. Unreacted PMAL-C8 will form particles smaller than 10 nm in water (7.10 ± 0.07 nm by DLS) and is removed during the washes. No nanoparticles are expected to be made by PMAL-C8/oligonucleotide clusters because it was shown that the complex has low solubility in water. It is possible to create a PMAL-C8/oligonucleotide core coated with lipids, but this structure can be produced only with precise control over the lipid tails. The apolar arms of PMAL-C8 have to be distributed towards the outside of the PMAL-C8 core to interact with the lipid tails. An apolar surface PMAL-C8 core can be

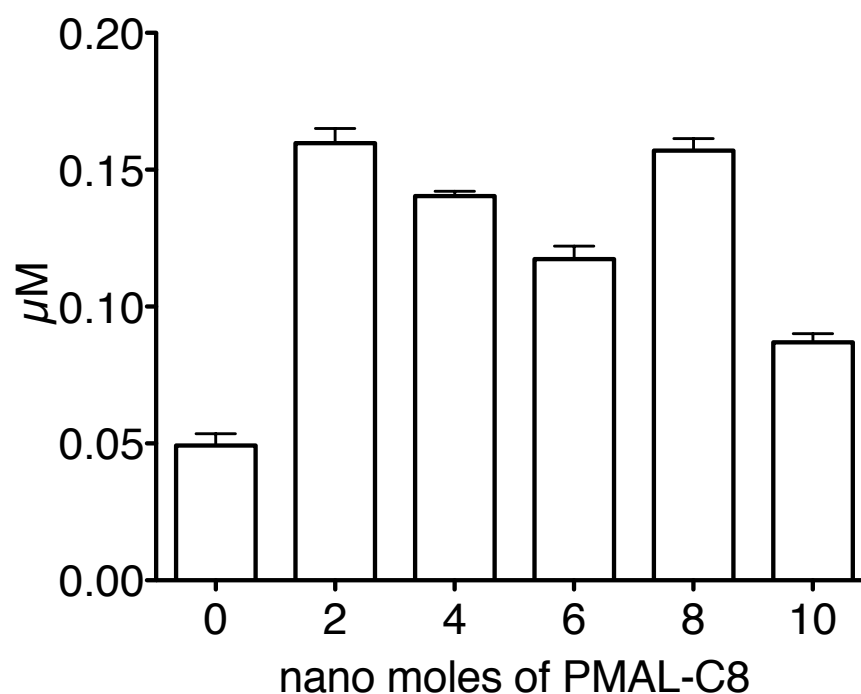
created only if PMAL-C8 is in an apolar environment, whereas the solvents used in this work were polar. The hypothesized structures described above are not expected to possess the smooth surface, spherical shape as observed from the SEM images.

3.6 OLIGONUCLEOTIDES LOADING IN THE NANOPARTICLES

Along with the physical structure of the new nanoparticles it is important to quantify the loading. Initially, it was determined if and how the different amounts of PMAL-C8 could affect the loading of the 40bp DNA sequence. The DNA was stained with Sybr Green I and it was loaded into the nanoparticles with different amounts of PMAL-C8. After nanoprecipitation the nanoparticles were washed with a 100,000 MWCO filter and were diluted to a 2mg/mL solution. The signal was measured from 100 μ L of each set. Because the Sybr Green I can create a background signal, a second set of nanoparticles was created in parallel with the same exact conditions only without oligonucleotides. The signal from the nanoparticles with the DNA was subtracted from the respective Sybr Green I background. From the Sybr Green I intensity profile an increase of the background could be observed corresponding to the amount of PMAL-C8 used, proving an affinity of the Sybr Green I to the PMAL-C8. Such observation is expected since Sybr Green I, like DNA, interacts with the polar fragments of PMAL-C8, even though the interaction is expected to be weaker than that between the dye and the oligonucleotides.

The molarity of the DNA in the nanoparticles solution was calculated based on the intensities from a calibration curve (Graph 3.5).

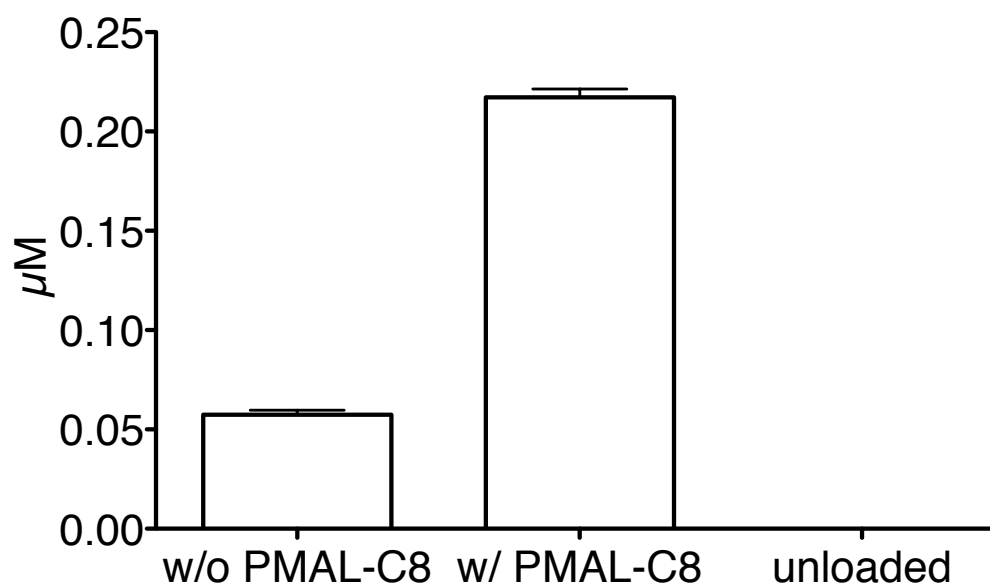
Graph 3.5 : Molarity of the DNA (40bp) nanoparticles with different amounts of PMAL-C8.



The loading is similar for all formulations containing less than 10 nano moles of PMAL-C8. Because it is not possible to define a clear relationship between the amount of PMAL-C8 and the loading, 6 nano moles of PMAL-C8 was chosen as the standard value for future experiments. The chosen amount is the minimum amount required to see a clear interaction with the oligonucleotides, and it is equally distant from the lower and upper limit of PMAL-C8 moles used.

The loading of a nano mole of a shorter sequence (6-FAM labeled DNA) with 6 nano moles of PMAL-C8 was measured and gave similar results of the longer sequence (Graph 3.6). The loaded DNA is at least three times higher when PMAL-C8 is used.

Graph 3.6: Molarity of the DNA (20bp) nanoparticles with different amounts of PMAL-C8.



It can be concluded that PMAL-C8 interacts with DNA and can assist its loading inside the hybrid nanoparticles. The maximum amount of PMAL that can be loaded before instability occurs was determined to be of 10 nano moles. The lower limit wasn't determined, but it is expected to depend on the sequence used: lower for shorter sequences. In addition, to maximize the chances of interaction it is always preferable to work with an excess of PMAL-C8 with respect to the oligonucleotides. During the different tests it was observed that 6 nano moles can work for different amounts and lengths of DNA.

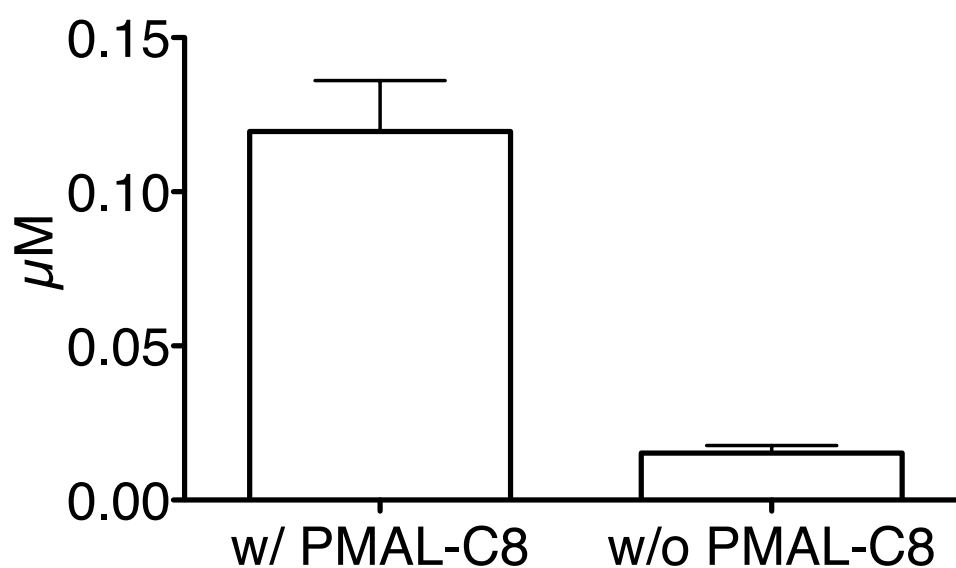
3.7 BIOFUNCTIONALITY: OLIGONUCLEOTIDES DELIVERY

PMAL-C8 offers a good method to load hydrophilic oligonucleotides into a hydrophobic core. Although it improves the loading, the use of PMAL-C8 can potentially

affect the oligonucleotides biofunctionality. A possible application to test the oligonucleotides biofunctionality is siRNA delivery (gene silencing). It is possible to use the siRNA loaded nanoparticles to silence a gene expressed in a cell line. For this application Prof. Dowdy kindly provided a lung cancer cell line (H1299) transfected to constitutively express an enhanced green fluorescent protein (eGFP). The siRNA solution was bought from Invitrogen.

The loading of the siRNA was quantified with the use of Sybr Green I and gave results in agreement with the previous measurements. In the graph the results are compared for nanoparticles loaded with and without the use of PMAL-C8 (Graph 3.7).

Graph 3.7: Quantification of siRNA in the loaded nanoparticles with and without PMAL-C8.



To further strengthen and improve the vehicle, folate was added to the surface as the targeting moiety. Folate is a highly essential vitamin used by cells in various process, such as methylation, DNA repair and DNA synthesis. Cells cannot produce folate,

therefore they depend entirely on how efficiently they can uptake it from the surrounding media. Some cancers have a high expression of the folate receptor, H1299 is one of them[37]. Various papers have exploited the folate as a targeting molecule for delivery of small drugs either directly conjugated to the drug[38] or as targeting ligands on the surface of the loaded nanoparticles[39, 40].

When about 12% of the PEG were conjugated to a folate, the targeted nanoparticles showed to adhered to the cells at a higher degree than the non-targeted ones (Figure 3.10).

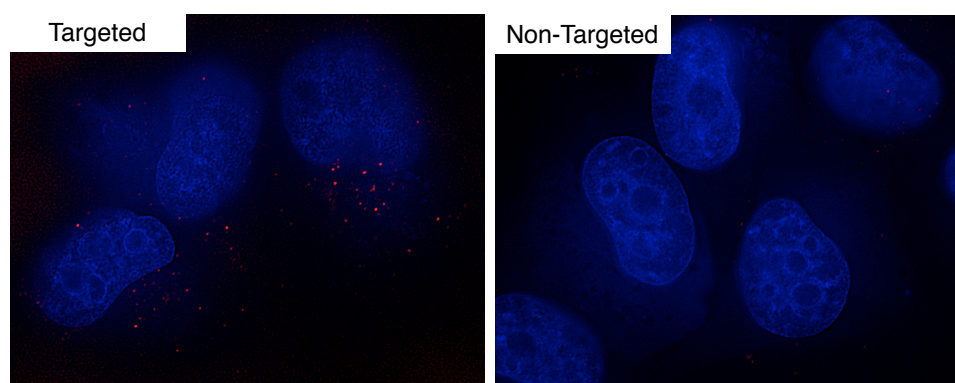


Figure 3.10: Microscope images of cells with targeted (left) and non-targeted (right) nanoparticles. The nuclei are stained with DAPI (blue), while the nanoparticles are labeled with DiD (red)

The targeted nanoparticles loaded with siRNA were incubated with the cells constitutively expressing eGFP for one hour and then washed away. The silencing effect was observed by fluorescence microscope imaging after 48 hours (Figure 3.11) and it was similar to the lipofectamine positive control. The experiment was repeated several times with similar results. The silencing induced by the nanoparticles is sometimes uneven, but definitely different from the negative control (untreated sample).

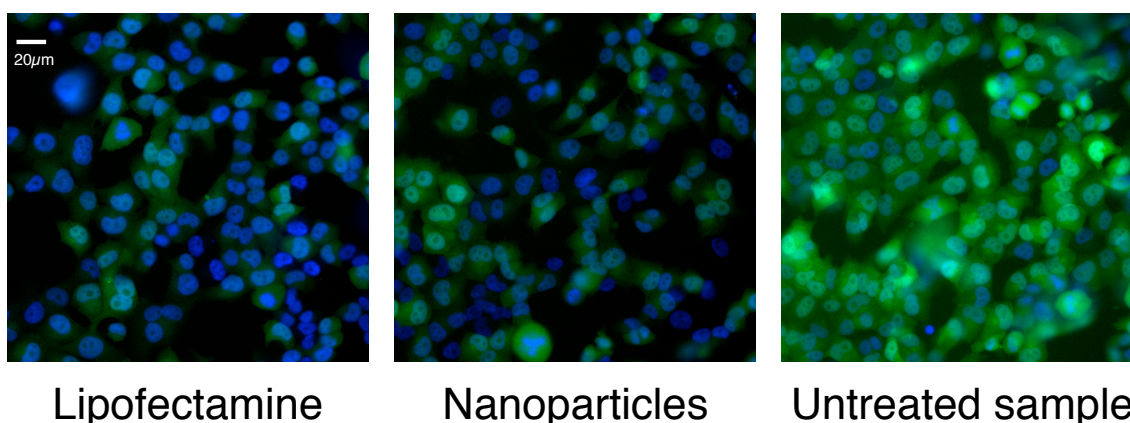


Figure 3.11: Microscope images of the cells after silencing with lipofectamine (left) and nanoparticles (center) compared to the untreated cells (right). The cell nuclei are DAPI stained (blue), while the eGFP protein is green.

The cell line used constitutively expresses eGFP, and it offers the advantage of avoiding any complications due to a co-transfection, in which both plasmid and siRNA are delivered. During a co-transfection there is always the risk of a false positive due to possible interaction between any free siRNA and the vector delivering the GFP-expressing plasmid. In addition, careful and detailed studies are necessary to identify the best time interval to coordinate plasmid and siRNA delivery for the nanoparticles. The working conditions for the hybrid nanoparticle might not be suitable for lipofectamine-assisted delivery. On the other hand, because the fluorescent proteins are constantly produced, best silencing results are usually seen with fast releasing delivery vehicles (like lipofectamine). This silencing model might not offer a platform to highlight the strength of a slow releasing nanoparticle, as the one we propose here, but it is sufficient to show consistent results on a microscope analysis.

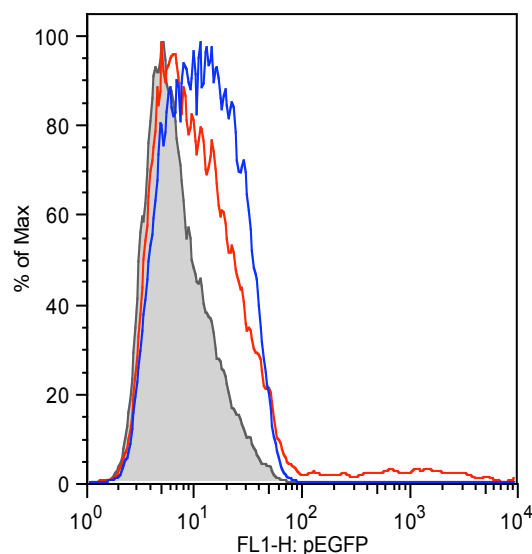
The hybrid nanoparticles created in this study can be loaded also with a bigger oligonucleotide sequence, such as a pDNA. The transfection of a eGFP expressing

plasmid can be accurately measured by FACS, and it is expected to show stable and significant results even for a slow releasing nanoparticle.

A quick experiment was performed to test the pDNA delivery of lipofectamine to the hybrid nanoparticles loaded with the PMAL-C8 polymer. The amount of pDNA used for loading was of 3.2 μg of pDNA per milligram of PLGA. After precipitation the particles were washed and their size was measured by DLS (78.88 ± 0.16 nm). To use similar amounts of total initial pDNA for both nanoparticles and lipofectamine, only 150 μg of nanoparticles were incubated with the cells. HeLa cells were used in this experiment because they are more tolerant in 10% Fetal Bovine Serum (FBS) folate free-RPMI, with antibiotics. The folate-free media is necessary to deprive the cells of folate before administering the folate-conjugated nanoparticles. The cells were plated 24hrs before transfection in a folate-free complete media to improve the uptake of the folate targeted nanoparticles. The folate free media was removed only after the one hour incubation of the nanoparticles with pDNA. Since the incubation was done in serum containing media and for a very short time, no significant effect is expected from free pDNA. In addition, the total amount of pDNA used for both 150 μg of nanoparticles and lipofectamine is about the same, as such any nonspecific effect should be the same for both.

Cells were harvested after 48hrs, washed with PBS and analyzed by FACS (Graph 3.8). The lipofectamine sample shows a small population of cells with increased signals and the majority with a small signal. On the other hand, the cells treated with the nanoparticles showed a broad intensity peak shifted to higher intensities compared to the untreated population.

Graph 3.8: FACS results for eGFP plasmid DNA transfection after 48hrs. The cells transfected with the nanoparticles (blue) are compared to the cells transfected with lipofectamine (red) and the non transfected cells (gray).



Lipofectamine transfection's results peak at 24hrs, after then the effect slowly diminishes, which explains the low number of high intensity cells and the broad low peak. On the other hand, PLGA hybrid nanoparticles follow a different uptake path and show the release effect after 48hrs. The slow release can explain the lower intensity difference, whereas the lower efficiency on oligonucleotides loading relative to lipofectamine explains the absence of a higher intensity population.

The microscope images clearly show that the method described herein to load oligonucleotides doesn't have negative repercussions on the bioavailability of the oligonucleotides. The advantages of a slow release can be highlighted by further improving the loading of the PMAL-C8/oligonucleotides complexes.

3.8 CONCLUSIONS ON AMPHIPHILIC HYBRID NANOPARTICLES

The polar arms of PMAL-C8 can interact with the oligonucleotides and consequently decrease their solubility in water. The complex created by the PMAL-C8 and the oligonucleotides, forms a fairly stable suspension in acetonitrile and can be used to protect the oligonucleotides during the nanoparticle fabrication process. The size of the nanoparticles loaded with the PMAL-C8/oligonucleotide complex is similar to the one of the particles without the complex. On the other hand the ζ -potential varies with the amount of PMAL-C8 used. One possible explanation is that the apolar arms of the PMAL-C8 interacts with the lipid tails affecting the amount of PEG on the surface. The carboxyl terminated PEG on the lipid not only determines the charge seen with the DLS, but also affects the stability of the hybrid nanoparticle.

A possible improvement to the loading can be achieved by using a modified PLGA. A short apolar carbon tail can be added to the end of the PLGA or used to combine two short PLGA segments to create non-polar domains inside the nanoparticle for the PMAL-C8's apolar arms to grab on. Also, an apolar hydrophobic polymer can be used in place of the PLGA, but this might not be FDA approved or compatible with the fabrication process.

The PMAL-C8 interaction with oligonucleotides and their interaction with the lipid tails can definitely be the key of the mechanism for which the oligonucleotides are delivered through the endosomal wall. PMAL-C8 can be the vehicle to escape the endosome base on the same principle it delivers membrane proteins to a lipid bilayer [33]. Further studies are necessary to highlight and understand the mechanism of

escape, but so far PMAL-C8 has shown to be an excellent interface between hydrophobic and hydrophilic materials.

We were therefore able to load and keep oligonucleotides biologically available after delivery. Our method can be extended to upload hydrophilic small molecules or peptides. For practical applications peptides can offer the widest variety of possibilities for a systematic study on the effect of the zwitterionic properties of PMAL-C8. In addition, based on the findings of this research, it is possible to engineer a new amphipol, tailored to load the negatively charged oligonucleotides on a partially negative PLGA polymer.

3.9 ACKNOWLEDGMENTS

The suggestions and experience of Dr. Burkart and Dr. Dangoor were extremely helpful for the success of the siRNA experiments done in this chapter.

Part of the material in this chapter is currently being prepared for publication, Sartor, Marta; Burkart, Christoph; Dangoor, David; Zhang Dong-Er; Zhang, Liangfang. The dissertation author was the primary investigator and author of this paper.

3.10 MATERIALS AND METHODS

OLIGONUCLEOTIDES USED

The double stranded 40bp sequence (actually 39pb) was:

5'-TCC AGC AAA TGT ACC GCG CCA ACA TCA AAT CCC CAA CCT-3'

The double stranded 20bp sequence was:

5'-6 FAM-CTT AGA TTC GTT TCG CAG GC-3'

The EGFP silencing siRNA was bought from Invitrogen (AM4626), and the sequence is not known.

pDNA was from Clontech: eGFP-C1, 500 ng/ μ L, its size was 4.7 kilo bases.

HYBRID NANOPARTICLES

PLGA-ester was dissolved in 500 μ L of acetonitrile at a concentration of 2 mg/mL. A total volume of 2mL of a 4% ethanol solution was made with 15% wt percent lipid to PLGA, 18% of the moles of the lipids were composed by a PEG-conjugated lipid. The folate targeting version had a folate conjugated to 12% of the PEG.

The PLGA in acetonitrile was mixed with the PMAL-DNA complex and precipitated in the heated (68°C) lipid solution while it was stirring at 700rpm. The solution was immediately removed from the heat and vortexed 3 minutes at the maximum speed. The resulting mix was mixed in the chemical hood for about one hour. The particles were washed three times with an Amicon filter (10,000 or 100,000 molecular weight cut off) and resuspended to a final concentration of 2mg/mL.

SIZE AND ζ -POTENTIAL

After washing the nanoparticles were diluted to a 0.5 mg/mL solution and the size and ζ -potential were measured with NanoSize (Malvern).

SCANNING ELECTRON MICROSCOPE IMAGE

Hybrid nanoparticles were made with 15% wt lipid/polymer and 18% moles of PEGylated lipids. The loaded particles were precipitated with 2 nano moles of siRNA

complexed with 10 nano moles of PMAL-C8. After precipitation the nanoparticles were washed and one drop was dried on a silicon surface.

The nanoparticles were diluted in water to 1 $\mu\text{g/mL}$. Several drops of the nanoparticle solution were deposited on a silicon chip and dried at room temperature for 12 hours. The chip with the dried samples was sputter coated with chromium for 30 seconds at 130 mA before imaging. Emitech K575X Sputter Coater was used. The images were taken with Phillips XL30 ESEM.

LOADING

The oligonucleotides were mixed with the PMAL-C8 and stored for a few minutes at 4°C before mixing them with the PLGA for nanoprecipitation. For the several combinations 0.5 nano moles of oligonucleotides were used. When Sybr Green I labeling was required, the oligonucleotides were first mixed with 5 μL of a 100X Sybr Green I solution.

The loading was quantified from a calibration curve based on a serial dilution of: 0.5, 0.4, 0.2, 0.1, 0.05, 0.01 and 0.005 μM oligonucleotides.

INTENSITY MEASUREMENTS

Both 6-FAM and Sybr Green I are green fluorophores. They were detected with TECAN Infinity 2000, the excitation was at 488nm and the emission was measured at 520nm.

CELL LINE

The eGFP transfected H1299 cells were kindly donated by Prof. Dowdy[41]. The cells were cultured in 10% FBS-DMEM media with antibiotics.

The HeLa cells were cultured in 10% FBS in DMEM media with antibiotics, but the day before the experiment the cells were cultured in 10% FBS in Folate Free RPMI with antibiotics.

TARGETING

The nanoparticles were loaded with 0.01% wt of DiD dye (emission 670nm) to polymer, washed with a 100,000 MWCO filter and incubated for one hour with the cells in a folate free media solution. The cells was changed with fresh folate free RPMI during the hour prior the incubation and was removed after the incubation with the nanoparticles. The experiments were done in Lab-Tek II microscope chamber slide, nuclei were DAPI stained for easy identification.

Images were taken with the Deltavision deconvolution microscope and the integration profile was normalized among the different pictures.

TRANSFECTION (siRNA)

Cells were washed and incubated with folate free RPMI at least one hour prior incubation with the nanoparticles. Nanoparticles were loaded with siRNA (0.5 nano moles), washed and incubated with the cells for one hour. Lipofectamine 2000 was used as positive control and was incubated with the cells for one hour as well. Lipofectamine 2000 loading conditions were according to the manufacturer's instruction.

Transfections experiments were done in Lab-Tek II microscope chamber slide, cells were washed and the nuclei were stained with DAPI for easier identification. Images were taken with Nikon upright microscope and normalized with Image J software.

TRANSFECTION (pDNA)

Cells were washed and incubated with folate free RPMI supplemented with 10% FBS, with antibiotics the day before the transfection. Nanoparticles were loaded with pDNA (3.2 μ g), washed and incubated with the cells for one hour. Lipofectamine 2000 was used as positive control and was incubated with the cells for one hour as well. Lipofectamine 2000 loading conditions were according to the manufacturer's instruction.

Transfections experiments were done in a 24 well plate. The cells were collected and washed at 24 and 48 hours post transfection. FACS images were taken of the cells by flow cytometry with FACS Calibur. The results were analyzed with FlowJo software.

3.11 BIBLIOGRAPHY

- [1] T. W. Wang, M. Jiang, Y. Wu, *Journal of Polymer Research* **2010**, 17, 335.
- [2] Y. S. Nam, T. G. Park, *Journal of Microencapsulation* **1999**, 16, 625.
- [3] M. Li, O. Rouaud, D. Poncelet, *International Journal of Pharmaceutics* **2008**, 363, 26.
- [4] F. Ito, H. Fujimori, H. Honnami, H. Kawakami, K. Kanamura, K. Makino, *Journal of Materials Science-Materials in Medicine* **2010**, 21, 1563.
- [5] R. Singh, J. W. Lillard, *Experimental and Molecular Pathology* **2009**, 86, 215.
- [6] S. M. Nie, Y. Xing, G. J. Kim, J. W. Simons, *Annual Review of Biomedical Engineering* **2007**, 9, 257.
- [7] J. F. Guo, K. A. Fisher, R. Darcy, J. F. Cryan, C. O'Driscoll, *Molecular Biosystems* **2010**, 6, 1143.
- [8] P. H. Oliveira, K. J. Prather, D. M. F. Prazeres, G. A. Monteiro, *Trends in Biotechnology* **2009**, 27, 503.
- [9] A. O. Abbas, M. D. Donovan, A. K. Salem, *Journal of Pharmaceutical Sciences* **2008**, 97, 2448.
- [10] S. Kawakami, Y. Higuchi, M. Hashida, *Journal of Pharmaceutical Sciences* **2008**, 97, 726.
- [11] B. Weide, C. Garbe, H. G. Rammensee, S. Pascolo, *Immunology Letters* **2008**, 115, 33.
- [12] E. Fattal, A. Bochota, *International Journal of Pharmaceutics* **2008**, 364, 237.
- [13] J. H. Jeong, T. G. Park, *Bioconjugate Chemistry* **2001**, 12, 917.
- [14] D. M. Cun, C. Foged, M. S. Yang, S. Frokjaer, H. M. Nielsen, *International Journal of Pharmaceutics* **2010**, 390, 70.
- [15] K. A. Woodrow, Y. Cu, C. J. Booth, J. K. Saucier-Sawyer, M. J. Wood, W. M. Saltzman, *Nature Materials* **2009**, 8, 526.
- [16] K. Kawabata, Y. Takakura, M. Hashida, *Pharmaceutical Research* **1995**, 12, 825.
- [17] J. M. Chan, L. F. Zhang, K. P. Yuet, G. Liao, J. W. Rhee, R. Langer, O. C. Farokhzad, *Biomaterials* **2009**, 30, 1627.
- [18] C. M. J. Hu, S. Kaushal, H. S. T. Cao, S. Aryal, M. Sartor, S. Esener, M. Bouvet, L. F. Zhang, *Molecular Pharmaceutics* **2010**, 7, 914.

- [19] C. Qiu Zhong, D.M.D., Pamujula, S., Haifan Wang, Xin Yao, Mandal, T.K., Luftig, R.B., *Journal of Nanobiotechnology* **2010**, 8, 10.
- [20] H. T. Lv, S. B. Zhang, B. Wang, S. H. Cui, J. Yan, *Journal of Controlled Release* **2006**, 114, 100.
- [21] M. M. Fiore, I. M. Mackie, *Thrombosis Research* **2009**, 124, 149.
- [22] Y. Gohon, J. L. Popot, *Current Opinion in Colloid & Interface Science* **2003**, 8, 15.
- [23] J. L. Popot, E. A. Berry, D. Charvolin, C. Creuzenet, C. Ebel, D. M. Engelman, M. Flotenmeyer, F. Giusti, Y. Gohon, P. Herve, Q. Hong, J. H. Lakey, K. Leonard, H. A. Shuman, P. Timmins, D. E. Warschawski, F. Zito, M. Zoonens, B. Pucci, C. Tribet, *Cellular and Molecular Life Sciences* **2003**, 60, 1559.
- [24] C. Tribet, R. Audebert, J. L. Popot, *Langmuir* **1997**, 13, 5570.
- [25] A. M. S. Duarte, C. J. A. M. Wolfs, R. B. M. Koehorst, J. L. Popot, M. A. Hemminga, *Journal of Peptide Science* **2008**, 14, 389.
- [26] B. M. Gorzelle, A. K. Hoffman, M. H. Keyes, D. N. Gray, D. G. Ray, C. R. Sanders, *Journal of the American Chemical Society* **2002**, 124, 11594.
- [27] C. Tribet, R. Audebert, J. L. Popot, *Proceedings of the National Academy of Sciences of the United States of America* **1996**, 93, 15047.
- [28] Y. Gohon, T. Dahmane, R. W. H. Ruigrok, P. Schuck, D. Charvolin, F. Rappaport, P. Timmins, D. M. Engelman, C. Tribet, J. L. Popot, C. Ebel, *Biophysical Journal* **2008**, 94, 3523.
- [29] Y. Gohon, G. Pavlov, P. Timmins, C. Tribet, J. L. Popot, C. Ebel, *Analytical Biochemistry* **2004**, 334, 318.
- [30] T. Dahmane, M. Damian, S. Mary, J. L. Popot, J. L. Baneres, *Biochemistry* **2009**, 48, 6516.
- [31] C. L. Pocanschi, T. Dahmane, Y. Gohon, F. Rappaport, H. J. Apell, J. H. Kleinschmidt, J. L. Popot, *Biochemistry* **2006**, 45, 13954.
- [32] M. Zoonens, F. Giusti, F. Zito, J. L. Popot, *Biochemistry* **2007**, 46, 10392.
- [33] J. K. Nagy, A. K. Hoffmann, M. H. Keyes, D. N. Gray, K. Oxenoid, C. R. Sanders, *Febs Letters* **2001**, 501, 115.
- [34] L. F. Qi, X. H. Gao, *Acs Nano* **2008**, 2, 1403.
- [35] M. Picard, T. Dahmane, M. Garrigos, C. Gauron, F. Giusti, M. le Maire, J. L. Popot, P. Champeil, *Biochemistry* **2006**, 45, 1861.
- [36] L. Z. Juliana M. Chan , Kai P. Yuet , Grace Liao , June-Wha Rhee , Robert Langer , Omid C. Farokhzad , * *Biomaterials* **2009**.

- [37] C. P. Leamon, P. S. Low, *Drug Discovery Today* **2001**, 6, 44.
- [38] G. Pasut, F. Canal, L. D. Via, S. Arpicco, F. A. Veronese, O. Schiavon, *Journal of Controlled Release* **2008**, 127, 239.
- [39] Y. T. Liu, K. Li, J. Pan, B. Liu, S. S. Feng, *Biomaterials* **2010**, 31, 330.
- [40] S. Q. Liu, N. Wiradharma, S. J. Gao, Y. W. Tong, Y. Y. Yang, *Biomaterials* **2007**, 28, 1423.
- [41] A. Eguchi, B. R. Meade, Y. C. Chang, C. T. Fredrickson, K. Willert, N. Puri, S. F. Dowdy, *Nature Biotechnology* **2009**, 27, 567.

CONCLUSIONS AND FUTURE WORK

The specific aims of the work described in this thesis include:

- a) The use a new building material to made a nanoparticle with targeting qualities.
- b) Modification of the internal structure of a polymeric nanoparticle for a higher loading and sustained release.
- c) Combine two incompatible materials such as a hydrophilic payload and a hydrophobic core.

The results of our studies led to the creation of three new platforms based on completely new design approach including:

- a) DNA nanoparticles created by concatameric repeat of a template sequence.
- b) Polymeric nanoparticles made with a physical and chemical internal gradient.
- c) Amphiphile assisted loading of hydrophilic material into a hydrophobic core.

However, our accomplishment is preliminary and all three technology proposed have the potential to address many future applicaitons.

DNA NANOPARTICLES

DNA was proposed and used as building material to create DNA nanoparticles from repeating concatameric unit. The DNA nanoparticles were bio-panned to select for

a sequence that specifically binds to dendritic cells. Dendritic cells were chosen as a target because they would have allowed possible future applications in a vaccine.

After bio-panning, the different sequences from the enriched pool were extracted and individually tested for binding by flow cytometry. Among the 14 different sequences tested, one in particular was shown to bind best to the dendritic cells harvested from the blood of different donors. The sequence was tested for binding against other cell lines showing no affinity for other than dendritic cells.

The selection of the DNA nanoparticle does not only offer a specific particle, but it also offers a new method. The selection protocol produced with this work was used in collaborations with other scientists and led to identify nanoparticles specific for pancreatic and breast cancer.

The selection for dendritic cells was done with a vaccine application in mind. Future work with the selected sequence can involve their use as an adjuvant by either presenting an antigen or by incorporating immunostimulatory CpG sequences. Current experiments are evaluating the identified sequence for possible in-vivo applications.

In addition, the sequence obtained in this work is very specific to dendritic cells and future work is necessary to investigate such a specificity by identifying which receptor or receptors it binds to. The results can open the door to additional applications based on the receptors involved.

Additional work can be done to combine the targeting ability of the DNA nanoparticle to their drug delivery potentials. A DNA binding drug such as doxorubicin, or cisplatin can be loaded to the DNA nanoparticle and delivered only to the cancer it selects for. The drug will be selectively delivered to the cancer leaving the healthy cells unaffected. In a similar application, the DNA nanoparticles can be used for tumor

imaging by integrating to make them fluorescent. The high selectivity of the DNA nanoparticles can result in a brighter and sharper image compared to the standard antibodies conjugated to a fluorophore.

GRADIENT NANOPARTICLES

A gradient structure was created in the polymeric core of nanoparticle with one-step method. Two hydrophobic poly(lactic-co-glycolic) acid (PLGA) of different length were each conjugated to hydrophilic polyethylene glycol (PEG). The two amphiphilic block-polymers were combined at different ratio and nanoprecipitated to self-assemble into nanoparticles with a porosity gradient. The gradient porosity particles created showed slightly higher loading and slower release when the two polymers are used on a 1:1 ratio. Due to the small size ($\sim 100\text{nm}$) of the nanoparticle considered, the effects of the porosity gradient were very small and were not deemed significant. In particular, the release profile was not strongly affected from the porosity gradient structure. Slow release was expected based on the fact that the drug in the core had to diffuse towards increasingly dense layers. This effect is greater when both the nanoparticle's size and the molecular weight difference between the two polymers are sufficiently big. As alternative to a work with a bigger particle, the gradient structure difference was improved by creating a chemical gradient structure. Poly(caprolactone) (PCL) was used in place of the shorter PLGA. The hydrophobicity difference between the two polymers significantly affected the loading of the hydrophobic drug paclitaxel. In addition to the porosity difference, PCL on the densely packed outer layers degraded slower than the less dense inner PLGA layers further contributing to the slower release.

A chemical gradient structure gave the desired high loading and slow release profile.

The gradient nanoparticles are suitable for a broad array of different applications: from testing different polymer combinations to essential studies on drug release profiles.

In fact, future work could involve the use of materials other than PLGA and PCL. Two polymer with a polarity difference can be combined into the gradient structure. The new array of different gradients will provide a high variety of loading and release profiles based on the payload and its interaction with the material composing the core.

On the other hand, the material-drug interaction can be further explored in a detailed study that relates the interaction drug-drug and drug-polymer to the loading and release profiles. Future work in this direction would provide deeper knowledge of the drug release and can offer a reliable platform for mathematical models to represent the drug release based on porosity and materials.

In addition, the release profile of two drugs with opposite affinity for the polymers used, can provide a new vehicle for combined drug therapy. The release profiles will need to be related to polymer compositions and length. It is also possible to use of more than just two different polymers' length.

The work presented was done with systemic delivery in mind of only particles smaller than 200nm considered good candidates. On the other hand, particles used for topical applications on the skin or mucosae, do not have the same size restriction limit. Future work can be done to create a micron size porosity gradient nanoparticle, in which the gradient structure can be used for its high loading and sustained release. In addition, the bigger size might be more flexible for direct proofs of a gradient internal structure and the use of more than two different length of polymers.

During this work we found that more work is necessary to understand the interaction of Laurdan dye with different materials. The dye has the potential to become an universal tool to investigate the chemical properties of several surfaces besides lipids' bilayer.

AMPHIPHILE ASSISTED LOADING

PMAL-C8 was successfully used as interface to load oligonucleotides inside a PLGA core. Oligonucleotides and PLGA are incompatible materials, the former are hydrophilic, and the latter hydrophobic. PMAL-C8 is an amphiphilic polymer (apol), being both hydrophobic and hydrophilic, that can act as transition material between the two environments.

The complex formed by the oligonucleotides and the apol is not soluble in water and, when long DNA sequences (40 base pairs) are used, the complex tends to slowly precipitate out of water. Different length of oligonucleotides were evaluated, all of them showed a higher encapsulation inside the PLGA core with the assisted loading provided by the oligonucleotides/PMAL-C8 complex.

Hybrid PLGA nanoparticles are characterized by a slow steady release and for this reason were used as platforms in this work. Based on the physical and chemical characterization, it is possible to deduce that most of the PMAL-C8 is located between the PLGA and the lipid tail. In fact, PMAL-C8 is not only amphiphilic, but also zwitterionic, having alternating polar and apolar arms. It is the apolar arm that can interact with the lipid tails, destabilizing the particle. As a consequence, not more than 10 nano moles of PMAL-C8 can be used without affecting the stability.

The bioavailability of the oligonucleotides post-loading was tested by loading and delivering to cells a silencing RNA short sequence (siRNA). The siRNA was loaded with the amphiphile assisted technique. The experiment showed a successful silencing effect, proving that the sequence can be released from the nanoparticle. A quick test done with a longer sequence (plasmid DNA) indicated similar results.

The use of amphiphilic polymers to load oligonucleotides is a fairly new idea that very few other groups have explored (L. F. Qi, X. H. Gao, *Acs Nano* 2008, 2, 1403). Cationic lipids or peptides are often the choice for inducing high loading of oligonucleotides even in a nanoparticle with a hydrophobic core. Our approach is different from the current techniques, because it uses an interface polymer rather than exploiting a charge driven clustering of oligonucleotides. Even if this research uses oligonucleotides, the concept can be easily extended to any payload and loading core made of incompatible materials. In particular, future work will involve the use of peptides with low or no polarity, to take full advantage of the zwitterionic properties of PMAL-C8.

PMAL-C8 wasn't created for this application, therefore further work will be focusing on adapting it or providing similar short polymers to improve the loading, while still acting as a "buffer" between core and payload. Alternatively, PLGA can be combined with some apolar segments, to increase the interactions with PMAL-C8's apolar chains. Both modifications aim to increase the loading of the amphiphile/DNA complex into the polymeric core. The improved structure will offer a sustained release and will present even higher differences on silencing or inducing the expression of a gene, compared to the burst release of the commercial lipofectamine.

After minor modifications, the platform described is ready to be used in vivo studies. In the field of oligonucleotides delivery, a very limited number of particles had

achieved such a maturation level. Future studies in this direction can combine the use of gene therapy and efficient delivery. For instance an enzyme activated hydrophobic pro-drug can be co-delivered with the gene encoding for the enzyme.

Some of the results or technique used in this work were shared with other scientists in different collaborations. Based on these collaborations several papers were either published or are in currently in preparation.