Photothermal Liposomal Drug Delivery Systems

Physicochemical Aspects in Particle Characterization and Drug Release from Light-Sensitive Drug Carriers

Lauri Viitala

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and Drug Release from Light-Sensitive Drug Carriers

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Abstract

Liposome is a phospholipid structure that surrounds its aqueous cavity with a lipid bilayer. Hydrophilic drug molecules can be encapsulated in liposomes and delivered to a given target. Ideally, liposomes are internalized by the target cell via endocytosis and the hydrophilic drug is released therein. In many cases, liposomes reach their target cell but the rate of passive release remains insufficient. Luckily, this challenge can be tackled with a suitable triggering mechanism. One option is to use light.

Light triggered drug release can be obtained with several strategies. One of the most interesting methods is to employ materials that convert light into heat. In this case, a photothermal agent absorbs light and releases the absorbed energy as heat. Such materials include a wide selection of gold nanoparticles that can be tuned to any relevant wavelengths by adjusting their size and shape. Another example is the fluorescent dye indocyanine green (ICG). It absorbs in the near infrared region that is safe for tissue irradiation (i.e. at the physiological window).

A photothermal liposomal drug delivery system is composed of drug-encapsulated liposomes with photothermal agents. In essence, a lipid bilayer prevents drug from releasing below its phase transition temperature. However, when the photothermal agents heat up locally, the drug molecules are released as the bilayer undergoes a phase transition.

This thesis addresses photothermal liposomal drug delivery systems from the physicochemical point of view and provides some new methods to characterize such systems. This work can be divided in three sections. In the first section, liposomes were coupled with photothermal agents and light-inflicted changes in the lipid bilayer were monitored with the quartz crystal microbalance and fluorescence spectroscopy. The main effect was the thermal phase transition in the lipid bilayer followed by the contents release. In the second section, methods of liposome detection were examined. Surface plasmon resonance imaging microscopy (SPRIM) was used to determine the number of encapsulated gold nanoparticles residing inside the liposomes with a new analysis method. In the third section, the prospects of surface engineering of liposomes was investigated with the addition of lipid-bound poly(ethylene glycol) (PEG). A new method, based on laurdanC, was developed to monitor the shape of the particulates. In addition, PEGylation caused changes in the phase transition behavior and shape of the lipid particulates. These features can provide new opportunities for drug delivery. These could be e.g. drug release systems with multiple release sequences and shape-shifting drug carriers with trigger polymers.

Keywords light triggering, photothermal effect, phase transition, drug release, detection methods, surface plasmons, PEGylation, shape transformation

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Tekijä

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Fototermit liposomi-lääkkeenkuljetusjärjestelmät: partikkelien karakterisoinnin ja lääkkeenvapautuksen fysikaalinen kemia

Julkaisija Kemian tekniikan korkeakoulu**Yksikkö** Kemian ja materiaalitieteen laitos**Sarja** Aalto University publication series DOCTORAL DISSERTATIONS 36/2019**Tutkimusala** Fysikaalinen kemia**Käsikirjoituksen pvm** 23.01.2019**Väitöspäivä** 15.03.2019**Julkaisuluvan myöntämispäivä** 13.02.2019**Kieli** Englanti☐ **Monografia**☒ **Artikkeliväitöskirja**☐ **Esseeväitöskirja****Tiivistelmä**

Liposomi on fosfolipideistä koostuva rakenne, jonka sisällä on sen lipidikaksoiskerroksen suojaama vesifaasi. Hydrofiilista lääkeainetta voidaan ladata liposomiin, jonka sisällä ne kulkeutuvat kohdesoluunsa esimerkiksi endosytoosin avulla. Liposomit saavuttavat usein kohdesolunsa, mutta niiden passiivinen lääkkeenvapautus voi olla tehotonta. Lääkkeenkuljetussysteemit voidaan kuitenkin varustaa aktivointimekanismeilla. Eräs vaihtoehto on lääkkeenvapautus valoa käyttäen.

Valoherkistetty lääkkeenvapautus voidaan saavuttaa useilla strategioilla. Eräs kiinnostavimmista on valoa lämmöksi muuttavien materiaalien käyttö. Tässä tapauksessa fototerminen materiaali muuntaa absorboimansa valon lämmöksi. Fototermitä materiaaleja ovat esimerkiksi monet kultananopartikkelit, joiden absorptio-maksimi voidaan säätää lähes mille tahansa relevantille valon aallonpituusalueelle nanopartikkelien kokoa ja muotoa muuttamalla. Toinen esimerkki on fluoresoiva merkkiaine indosyaanivihreä. Se absorboi lähi-infrapuna-alueella, joka on yleisesti turvallinen aallonpituusalue kudoksille (so. fysiologinen ikkuna).

Fototerminen liposomi-lääkkeenkuljetussysteemi koostuu lääkkeellä ladatusta liposomista ja fototerimisestä materiaalista. Käytännössä lääkeaine ei vapaudu lämpötiloissa, jotka ovat lipidin faasimuutoslämpötilan alapuolella. Kun fototerminen materiaalia lämpenee lokaalisti, lääkeaine vapautuu kalvon faasimuutoksen seurauksena.

Tämä väitöskirja tarkastelee fototermitä liposomi-lääkekuljettimia fysiko-kemiallisesta näkökulmasta ja tarjoaa joitakin uusia menetelmiä näiden systeemien karakterisointiin. Työ voidaan jakaa kolmeen osa-alueeseen. Ensimmäisessä osassa fototermitä liposomi-formulaatioihin kohdittavia valo-lämpö-ilmiöitä tutkittiin kvartsikidevaan ja fluoresenssispektroskopian avulla. Pääasiallinen havainto tutkimuksessa oli lipidikaksoiskerroksen faasimuutos ja sitä seurannut lääkkeenvapautus. Toisessa osassa tutkittiin menetelmiä liposomien havainnoimiseksi. Vakiokulman pintaplasmoniresonanssimikroskopian avulla voitiin määrittää kultananopartikkeleita sisältävien liposomien lukumäärä käyttämällä tässä työssä kehitettyä uutta analyysimenetelmää. Kolmannessa osassa liposomien pintarakennetta muokattiin lipidi-polyetyleeniglykolin (lipidi-PEG) hybridin lisäyksiä. Uusi laurdanC-merkkiaineen käyttöön perustuva tekniikka kehitettiin partikkelien muodon määrittämiseksi. Lisäksi havaittiin muutoksia lipidi-kalvon faasimuutoskäytöksessä PEG-pitoisuuden kasvaessa. Muodonmuutos ja faasimuutoskäyttäytyminen voivat tarjota joitakin hyötynäkökohtia lääkkeenvapautusta ajatellen. Yksi tällainen voisi olla lääkkeenvapautus useammassa jaksossa. Toisaalta myös polymeeri voisi toimia vapautuksen aktivoijana.

Avainsanat valoaktivointi, fototerminen efekti, faasimuutos, lääkkeenvapautus, havaitsemismenetelmät, pintaplasmonit, PEGylointi, muodonmuutos

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Our international collaborators are also gratefully acknowledged. I wish to thank Prof. Rob Corn for giving me the opportunity to visit his laboratory in

Irvine. He and his research group are gratefully acknowledged for introducing me to the subject of surface plasmon resonance, in connection with our Publication III, and for showing me some of the bits at the Golden State of California.

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Helsinki, 2/2019

Lauri Viitala

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List of Abbreviations

CBF	Critical Bicellation Fraction
Cryo-TEM	Cryogenic Transmission Electron Microscope
DDS	Drug Delivery System
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DSC	Differential Scanning Calorimeter
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
DSPE-PEG(2000)	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]
ECDF	Empirical Cumulative Distribution Function
EE	Encapsulation Efficiency
EMA	European Medicines Agency
FDA	U.S. Food and Drug Administration
FFT	Fast Fourier Transformation
GNP	Gold Nanoparticle
GNR	Gold Nanorod
GUV	Giant Unilamellar Vesicle
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICG	Indocyanine Green
IR _x	A photothermal agent absorbing at 780 nm (IR780), 820 nm (IR820) or 825 nm (IR825)
LUV	Large Unilamellar Vesicle
LysoPC	Lysophosphatidylcholine
MD	Molecular Dynamics
MLV	Multilamellar Vesicle
MUAM	11-Mercaptoundecylamine
PEG(x)	Poly(ethylene glycol) with molar mass of x (g mol ⁻¹)
PEG _x	DPPC:DSPE-PEG(2000) 100- <i>x</i> : <i>x</i>
PL	Photoluminescence
QCM	Quartz Crystal Microbalance
QD	Quantum Dot
QY	Quantum Yield
RC	A photothermal agent
SAXS	Small Angle X-ray Scattering
SPR(I)	Surface Plasmon Resonance (Imaging)
SPRIM	Surface Plasmon Resonance Imaging Microscope
SUV	Small Unilamellar Vesicle
TRIS	Tris(hydroxymethyl)aminomethane
WAXS	Wide Angle X-ray Scattering

List of Symbols

a	Mean area of lipids or a geometrical factor for Γ
a_0	Optimal area of lipid head groups
$\tilde{a}$	Acceleration
$\tilde{a}$	Complex wave number
A	Surface area
c	Speed of light ($2.998 \cdot 10^8$ m s ⁻¹) or concentration
C	Capacitance
C_f	Constant in Sauerbrey equation (17.7 ng cm ⁻² s)
C_p	Heat capacity at constant pressure
d	Thickness or diameter
D	Dissipation (QCM) or diffusion coefficient
E	Energy or magnitude of an electric field $ \vec{E} $
$\vec{E}$	Electric field
f	Frequency
$\vec{F}$	Force
G	Complex shear modulus
GP	(Laurdan) generalized polarization
h	Thickness (QCM)
I	Intensity
k	Thermal conductivity, spring constant (Hooke's law), or absorption coefficient
K	Total curvature
K_0	Spontaneous total curvature
l_0	Hydrocarbon tail length
L	Inductance or depolarization factor
m	Mass
n	Refractive index or number density
N	Number of particles or the frequency overtone number
p	Parameter in ECDF fitting: intensity (p_1), mean value (p_2), and variance (p_3)
$\vec{p}$	Local or time invariant polarization
P	Pressure or packing parameter
$\vec{P}$	Polarization
q	Charge
Q	Heat of transition or source term (in SPRI microscopy)
r	Radius or reflection
$\vec{r}$	Displacement vector (also $\vec{r} = \vec{r} - \vec{r}_0$ in SPRI microscopy)
R	Radius, resistance, drug release percentage, or reflectivity
t	Time
T	Temperature
u	Temperature increment
v	Hydrocarbon volume
v_f	Fermi velocity ($1.395 \cdot 10^6$ m/s)
$\vec{v}$	Velocity
x	Molar fraction
Z	Impedance

γ	Collision frequency or surface tension
Γ	Size-dependent collision frequency
δ	Skin depth
$\epsilon = \epsilon_1 + i\epsilon_2$	Complex dielectric Constant
ϵ_0	Vacuum permittivity ($8.854 \cdot 10^{-12} \text{ F m}^{-1}$)
ϵ_r	Relative permittivity
η	Viscosity or aspect ratio
θ	Angle
θ_c	Critical angle in SPR
κ	Thermal diffusivity or bending modulus
$\bar{\kappa}$	Gaussian curvature modulus
λ	Wavelength
μ	Elasticity
$\nu = 1 + \bar{\kappa}/\kappa$	Poisson's ratio
π	Surface pressure
ρ	Density
σ_i	Absorption (i = abs), scattering (i = sca) or excitation (i = ext) cross section
τ	Perfusion time, time of localized SPR half cycle, or relaxation time
v	Field enhancement factor
$\chi = \frac{\Delta R}{-\Delta f/f_0}$	QCM parameter related to viscoelasticity
χ_e	Electric susceptibility
$\psi = \frac{\Delta D}{-\Delta f}$	QCM parameter related to viscoelasticity
$\omega = 2\pi f$	Angular frequency
ω_p	Plasma frequency

List of Publications

This doctoral dissertation consists of a summary and of the following publications which are referred to in the text by their Roman numerals:

- I.** L. Viitala; T. Lajunen; A. Urtti; T. Viitala; L. Murtomäki. Detection of Phase Transition in Photosensitive Liposomes by Advanced QCM. *The Journal of Physical Chemistry C*, **2015**, 119, 37, 21395. DOI:10.1021/acs.jpcc.5b04042.
- II.** L. Viitala; S. Pajari; T. Lajunen; L.-S. Kontturi; T. Laaksonen; P. Kuosmanen; T. Viitala; A. Urtti; L. Murtomäki. Photothermally Triggered Lipid Bilayer Phase Transition and Drug Release from Gold Nanorod and Indocyanine Green Encapsulated Liposomes. *Langmuir*, **2016**, 32, 18, 4554. DOI:10.1021/acs.langmuir.6b00716
- III.** L. Viitala, A.M. Maley, H.W.M. Fung, R.M. Corn, T. Viitala, L. Murtomäki. Surface Plasmon Resonance Imaging Microscopy of Liposomes and Liposome-Encapsulated Gold Nanoparticles. *The Journal of Physical Chemistry C*, **2016**, 120, 45, 25958. DOI:10.1021/acs.jpcc.6b09503.
- IV.** L. Viitala, S. Pajari, L. Gentile, J. Määttä, M. Gubitosi, J. Deska, M. Sammal-korpi, U. Olsson, L. Murtomäki. Shape and Phase Transitions in a PEGylated Phospholipid System. Submitted to *Langmuir*, **2019**.

Author's Contribution

Publication I: Detection of Phase Transition in Photosensitive Liposomes by Advanced QCM

LV, TV and LM defined the experimental design. LV and TV built the QCM setup. LV performed the experiments, did the analyses, and wrote the manuscript and refined it to its final form according to comments from the reviewers and other authors.

Publication II: Photothermally Triggered Lipid Bilayer Phase Transition and Drug Release from Gold Nanorod and Indocyanine Green Encapsulated Liposomes

LV, T. Lajunen and T. Laaksonen defined the research plan. LV set up the experiments with PK. LV and SP (under LV's supervision) prepared the samples and performed the laurdan measurements (ca. 25:75). T. Lajunen performed the calcein release experiments and wrote the drug release chapter in the first manuscript. LV performed the other analyses and wrote the rest of the manuscript refining it to its final form according to comments from the reviewers and other authors.

Publication III: Surface Plasmon Resonance Imaging Microscopy of Liposomes and Liposome-Encapsulated Gold Nanoparticles

LV, AM, RC and LM defined the research plan. LV prepared the samples and performed the DLS, SPRI and fluorescence spectroscopy measurements. LV, AM and MF performed the SPRI microscopy imaging. LV designed the analyses tools and performed the analyses. LV wrote the manuscript and refined it to its final form according to comments from the reviewers and other authors.

Publication IV: Shape and Phase Transitions in a PEGylated Phospholipid System

LV, LG, MG, UO and LM defined the research plan. JD synthesized laurdanC. JM performed the MD simulations and wrote a portion of the initial manuscript with MS. LV and SP prepared the samples (70:30). SP performed the laurdanC measurements and took part in the cryo-TEM image analyses. LV performed measurements with the DSC and cryo-TEM. LV, LG and MG performed the SAXS and WAXS measurements. LV did the analyses for the experimental work and wrote the manuscript refining it to its final form according to comments from the pre-examiners, reviewers and other authors.

1. Introduction

1.1 Overview

Liposomes offer a biocompatible option for targeting contents into various target sites. The content release can occur either passively or with some internal or external trigger. One of the most interesting triggers is the light in the wavelength region that is not significantly absorbed or scattered by the tissue.

Photothermal liposomal drug delivery systems (DDSs) are relatively simple in contextual sense. They contain two key components. The first is the lipid carrier containing the drug. The second is the photothermal agent that converts light into heat. As the heat transports to the surrounding lipid bilayer, lipids undergo a phase transition that increases the permeability of the membrane. The contents of the liposome are thus released.

The broad scope of this thesis are the physicochemical effects and novel detection methods of photothermal liposomal DDSs with gold nanoparticles (GNPs) or indocyanine green (ICG). The work is carried out in four research papers. The main scope in Publications I and II is the photothermal heating, the resulting phase transition, and its significance to drug release. Publication III focuses on a specific surface sensitive microscopy technique, SPRI microscopy that was used to determine the amount of photothermal material (*i.e.* GNPs) inside the liposomes. Publication IV focuses on PEGylation, a standard practice in lipid applications, and discusses about some of the new possibilities in drug release via extended phase transition region and shape transition. Together, these publications provide a pervasive viewpoint of the effects taking place in photothermal lipid systems and encompass some new research tools that can be used to study such soft nanomaterials in the future.

1.2 Outline of this Compendium

The outline of this compendium is as follows. Chapter 2 provides a brief literature review of the thesis topic. Chapter 2.1 compiles some of the literature regarding liposomal DDSs. Photothermal agents are reviewed in chapter 2.2. The main work of this thesis, Publications I – IV, are summarized in Chapters 3 – 6. These papers share no common theoretical basis. Therefore, they are detailed in their own chapters. Since Publications I – IV are part of this written work (provided in the back), the aim in this compendium is not to repeat their narration, but to give a brief overview and to extend some of their viewpoints in order to compliment the original works.

2. Review of Literature

2.1 Liposomal Drug Delivery Systems

2.1.1 Liposomes and Other Lipid Aggregates

Phospholipids are amphiphilic molecules with a hydrophilic head group and a hydrophobic tail group. In an aqueous solution, they form lipid structures by self-assembly. The shape of the aggregates can be often predicted with so-called packing parameter P , which is the ratio of the average hydrocarbon area v/l_0 , where v is hydrocarbon volume and l_0 is its length, versus the optimal area of the lipid head groups a_0 .^{1, 2} With the ratio $1/2 < P = v/(l_0 a_0) \leq 1$,¹ a bilayer is formed, that is then instantaneously folded to a spherical liposome. This is due to the unsustainable line tension at the interface of bilayer edges and water. Similarly, a spherical micelle can be formed when $P \leq 1/3$, a cylindrical micelle between $1/3 < P \leq 1/2$, and inverse structures when $P > 1$, with cylinders appearing between $2 \leq P < 3$ and inverse spherical micelles when $P \geq 3$.^{3, 4}

The packing parameter describes the macroscopic particle shape with excellent agreement when the system contains identical lipids or when the lipids are ideally mixed. It also describes the shape of the segregated lipid systems⁴ since the parameter characterizes a singular monomer.² In this case, the packing parameter is often used to explain local curvature values (K). For example, bicelles (*i.e.* disc-like micelles with a bilayer) are formed when the lipids of low packing parameter are transported to the bilayer edges, where they stabilize the highly curved interface, and the formation of liposomes is prevented. Hence, the packing parameter predicts the curvature in some cases. However, reverse thinking may be advised in many occasions,^{5, 6} as the curvature values are actually needed to determine the packing parameter.⁴⁰ Curvature is the main contributor in the bending energy:

$$H = \frac{1}{2} \kappa [K_0, K_1, K_2] \begin{bmatrix} 1 & -1 & -1 \\ -1 & 1 & \nu \\ -1 & \nu & 1 \end{bmatrix} \begin{bmatrix} K_0 \\ K_1 \\ K_2 \end{bmatrix} \quad (1)$$
$$= \frac{1}{2} \kappa (K_1 + K_2 - K_0)^2 + \bar{\kappa} K_1 K_2$$

(generalized from Ref. 7), where $\nu = 1 + \bar{\kappa}/\kappa$ is the Poisson's ratio, κ is the bending modulus, $\bar{\kappa}$ is the Gaussian curvature modulus, K_1 and K_2 are the two principal total curvatures of the bilayer, and K_0 is the spontaneous total curvature. Together with surface tension, minimizing the surface area, the bending contribution aims to minimize the difference $K_1 + K_2 - K_0$ throughout the bilayer. Hence, it determines the particle shape, as demonstrated by Helfrich⁸ and Deuling with Helfrich⁹.

The most common bilayered lipid structures in drug delivery applications are liposomes and bicelles. In addition to these, monolayered structures are also

¹ Kumar (Ref. 3) has set the lower bound to 0.74 for the phosphatidylcholine.

used. These include small globular micelles and cylindrical micelles. All of the lipid systems can be used in the delivery of lipophilic drug molecules by embedding them into the lipid core.¹⁰⁻¹³ Liposomes have one clear benefit over the others: they contain an aqueous phase that can encapsulate hydrophilic molecules. In this case, the bilayer offers protection against the fast clearance or the hostile conditions in the blood stream that may degrade the precious cargo. On the other hand, uncontrolled drug activity is also inhibited by the bilayer prior to the target.¹⁴

Liposomes can be categorized according to their size and lamellarity (*i.e.* unilamellar vesicle, ULV, and multilamellar vesicle, MLV). The small unilamellar vesicles (SUVs) are typically less than 200 nm in diameter. Large unilamellar vesicles (LUVs) are found in the size region of 200 nm to 800 nm, and giant unilamellar vesicles (GUVs) are in the micrometer scale. MLVs are typically either “large” or “giant”, reaching from 500 nm up to 5 μm .¹⁵ In all cases, the lipid bilayer thickness is close to 5 nm and varies only when the bilayer goes through a phase transition.

2.1.2 Drug Delivery Pathways

Liposomes have achieved a firm position among new drug formulations due to their excellent biocompatibility and superb track record in receiving FDA approvals. Amongst others, FDA approval has been given to formulations such as DaunoXome[®] that is used to treat Kaposi’s sarcoma, Doxil[®] that is used to treat refractory Kaposi’s sarcoma, ovarian cancer, and recurrent breast cancer, and Myocet[®] that is used in the combinational therapy of recurrent breast cancer.¹⁶⁻¹⁸ Injection is the most common and effective technique to administer liposomal drugs¹⁹ but also other administration routes are available. The digestive system tract is uncommon but not unheard of.²⁰ Transdermal administration is a viable and pleasant non-invasive option, although permeation through stratum corneum (*i.e.* the outer layer of the skin) may be difficult in some cases. This can be bypassed, however, by using natural water ducts, *i.e.* hair follicles and sweat glands, with or without the enhancement of an iontophoretic device.²¹ In addition, nasal cavities or the lung offers another bypass. In fact, one of the first commercial liposome product includes the synthetic lung surfactant Alveofact[®] that was used for pulmonary instillation for the treatment of respiratory distress syndrome.^{22, 23} Liposomes can also enter the body with inhaled aerosol droplets, typically at the size range of 1-5 μm .²³

In the most typical scheme, a drug carrier is delivered into the target cell via endocytosis whereby a hydrophilic drug is released into the cytoplasm or delivered further into the lysosome, as depicted in Fig. 1. Other possible interactions with the cell surface may take place but not inhibit the drug permeation. In the case of endocytosis, the size of the liposome is important, although much depends on the target cell type as well. SUVs and LUVs are both uptaken into the phagocytosing cells with ease²⁴ as they can internalize bigger entities than themselves.²⁵ In the case of a non-phagocytic target, endocytosis is restricted to four mechanisms: 1) clathrin-mediated endocytosis, 2) caveolae-mediated endocytosis, 3) micropinocytosis and 4) other (clathrin or caveolae-independent) type

of endocytosis,²⁶ such as the one mediated by the enzyme dynamin.²⁷ In Caco2 cells (*i.e.* human colorectal adenocarcinoma cells), for example, it has been shown that SUVs of the diameter of 100 nm to 160 nm were mainly uptaken through clathrin pathway, whereas SUV of ca. 40 nm in diameter followed the dynamin-mediated endocytosis, and intermediately-sized liposomes were adapt to follow both pathways.²⁷

Figure 1. Drug-bearing liposome interactions and pathways into the target cell: 1) Specific and 2) non-specific adsorption on the cell surface; 3) drug release near the target cell; 4) micropinocytosis; 5) protein-mediated exchange of lipid components with the cell membrane; 6) specific and nonspecific endocytosis, with eventual endosomal delivery into the lysosome (6a) or release into cytoplasm (6b) via provoked endosomal destabilization; 7) provoked endocytosis due to certain viral components on the liposome surface, followed by an endosomal delivery into the lysosome (7a) or release into cytoplasm (7b). Modified from Ref. 15.

2.1.3 PEGylation

Liposomal drug delivery systems are subject to clearance in the blood stream. Serum opsonin targets liposomes and specialized phagocytic cells remove the opsonized liposomes in a matter of few hours.^{28, 29} Hence, the dose that reaches the target may remain below the therapeutic limit, and the drug delivery system can become unsustainable with respect to the costs-to-effectiveness ratio.

One of the most commonly used method to prolong circulation times in blood and in storage is the addition of poly(ethylene glycol) (PEG) moieties on the surface of the liposomal carrier. This procedure is called “PEGylation”, and it is portrayed as “the gold standard” among researchers developing drug delivery systems.^{30, 31} For instance, it was one of the cornerstones in the first FDA approved nanodrug, Doxil®.³²

The PEG decoration of liposomes is obtained by adding PEG-conjugated lipids into the bilayer. Most commonly used PEG-lipid is DSPE-PEG(2000) with generally accepted optimum amount of ca. 5 mol%.³¹ This fraction is in the PEG-brush conformation. Lower PEGylation would result a mushroom conformation and less tight packing order,³³ which can influence the durability of the liposome in blood.³⁴

PEGs provide a stealth sheath for the drug carrier due to the repulsion between the polymeric units and added steric barrier against opsonin and other bioactive molecules.³⁴ In addition, PEG moieties can protect the cargo by other

means. For instance, PEG can influence the lipid packing in the bilayer by controlling the extent of hydration in the lipid head groups.³⁵ A PEG layer blocks divalent ion (*e.g.* Ca^{2+}) interactions with the lipid headgroups, and thus inhibits membrane fusion, improving membrane stability.^{36, 37} PEG can also couple with some bioactive molecules, such as albumin, to increase the biocompatibility and circulation times.³⁸

PEG is soluble in both hydrophilic and hydrophobic solvents.³⁹ Therefore, it can bind hydrophilic molecules, like albumin³⁸, but also more hydrophobic molecules, for example indocyanine green (ICG).⁴⁰ The latter is a fluorescent dye, but it acts as a photothermal agent as well.⁴¹ Due to some hydrophobicity in the PEG chain, it has been hypothesized that PEG moieties (with low level of PEGylation) may also penetrate into the bilayer in some cases.³⁷

2.1.4 Internal and External Triggers for Drug Release

Small compounds permeate the lipid bilayer faster than large molecules. Hydrophilic and charged molecules have a slower passive permeation rate through the bilayer than lipophilic components. Macromolecules need a complete phase transition in the bilayer to pass through it.⁴² Typically, at temperatures below the phase transition temperature of the lipid mixture, passively targeted liposomes have drug release rates of less than 1 % (*in vitro*) of their total drug capacity per hour.¹⁵ This is sufficient in some special cases. For example, Doxil® can obtain a therapeutic effect via passive release in tumorous tissues.³² In some other cases, passive release is not sufficient. Liposomes may reach the target cell, but the released dosage remains below the therapeutic window and does not have desired effect without a trigger that would destabilize the bilayer.⁴³ Hence, there is a keen interest to liposomal DDSs with triggering mechanisms of endogenous or exogenous type.

Endogenous or internal triggers include changes in *e.g.* pH⁴⁴⁻⁴⁸ and enzymatic activity⁴⁹. Most commonly, these triggers are relied on when the selected pathway is endocytosis: the pH 7.4 of the extracellular fluid drops during this process to 5.5 or below in the lysosome,⁴⁴ and many enzymes reside inside the lysosome. In the case of utilizing the decreasing pH as a trigger, 1,3-Diolein and cholesterol hemisuccinate pairing can be used, for example. This formulation destabilizes the bilayer and releases the drug molecules.^{46, 48} However, even with pH sensitive lipid formulations, the drug release into cytoplasm may have a weaker effect than desired if the endosome wall remains intact. In this case, systems with pH trigger may need another triggering mechanism.⁴⁸

External triggers include changes in temperature⁵⁰, and electromagnetic radiation in the spectrum of ultraviolet, visible light or near-infrared. Temperature is a powerful trigger, as the lipid bilayer is a temperature-sensitive structure. A typical phospholipid bilayer undergoes two consecutive phase transitions upon temperature raise. The first one is the pre-phase transition from the gel phase into the rippled gel phase. The second one is the main phase transition from the rippled gel phase into the fluid phase.⁵¹ The phase order determines the perme-

ability of the drug.⁵²⁻⁵⁴ Maximum permeation occurs in the main phase transition temperature region,^{53, 54} and water permeates through a curved bilayer hundred times faster above the phase transition temperature than below it.⁵²

The light triggered drug release can be obtained in the UV region via isomerization of azobenzene^{55, 56}, by a photocleavage mechanism⁵⁷, or through a singlet-oxygen-mediated photochemical decomposition⁴⁵. Polymerization⁵⁸ and photo-oxidation⁵⁹ are used in the visible light region. In addition to these, all light wavelengths can be used to convert light energy into heat (see *e.g.* Refs. 20, 40, 48, and 60-73). In this case, temperature sensitive liposome formulations are used with specific nanoparticulates, or other photothermal agents that are embedded into the bilayer or encapsulated into the aqueous cavity of the liposome. The photothermal agent absorbs light and converts the absorbed energy into heat. The heat is then locally transported, and the nearby bilayer is melted, and the drug thus released. This is addressed in the next chapter.

2.2 Photothermal Agents

2.2.1 Physiological Windows

When light passes into a medium, it is absorbed and scattered on its path. The intensity of the transmitted light is attenuated:

$$I = I_0 \cdot e^{-dn\sigma_{\text{ext}}}; \sigma_{\text{ext}} = \sigma_{\text{abs}} + \sigma_{\text{sca}}, \quad (1)$$

where I_0 is the intensity of the light prior to the medium, d is its path length, n is the number density, and σ_{ext} , σ_{abs} , and σ_{sca} are the excitation, absorption, and scattering cross sections, respectively. The absorbed energy can be released as luminescence. This may occur by emitting photons of a different wavelength than the absorbed one or by emitting phonons; effectively transporting heat from the surface of the absorber to the surrounding medium. The latter is known as the photothermal effect and it occurs on a photothermal material.⁷⁴

All media absorb light. Therefore, a tissue experiences background heating and light attenuation even without having a photothermal agent involved. This is readily understandable by placing a finger on flashlight light and gazing upon the red glow that passes the fingernail with a small sensation of warmth. Unfortunately, the nice and warm sensation in the finger is the power that is lost from utilization and to make matters worse, the loss may become unsafe with high intensities of light, because the tissue may suffer irreversible damage due to excess heating or photochemical reactions. Obviously, the penetration of light into tissues and the sensitivity of epithelia to light varies significantly. In general, biological systems have two optical windows (Fig. 2) in which the light penetrates effectively and causes less damage to the cells and tissues.⁷⁴⁻⁷⁷ The first physiological window situates between the visible absorption band of hemoglobin (and melanin) at 650 – 700 nm and the characteristic absorption band of water at 980 nm.^{74, 77} This is the region that transmits freely through a finger, and makes it glow red on the other side. Similarly, the light in this region can permeate few centimeters through the skin, eye, and other tissue unobstructedly.^{74, 78}

The second physiological window is invisible to the naked eye. It is limited by the water absorption bands and locates between 1000 nm and 1400 nm.⁷⁴ Here, water still absorbs some light and causes some background heating. The benefit of the higher wavelength is the reduction in scattering and autofluorescence, often producing substantial background noise in a biological tissue.^{74, 77}

Figure 2. Optical windows I and II for the photothermal drug release are situated between hemoglobin and water absorption bands. Thick black line represents the effective attenuation coefficient of the whole blood (fully saturated with oxygen) that is composed of absorption (thinner line) and scattering (dashed line) coefficients. Data from Ref. 79.

In some cases, *e.g.* during tumor ablation, tissue damage is the desired effect and biological windows are irrelevant. In most cases, tissue damage is inadmissible and the light trigger can be excited in the biological windows only. This poses a disadvantage to some promising light triggering options, such as azobenzene isomerization in the UV range^{55, 56}, and photo-oxidation alternatives in the visible light range⁵⁹. Fortunately, many photothermal agents operate in the near-infrared region.

2.2.2 Gold Nanoparticles

Introduction

Gold nanoparticles have been an increasingly popular topic in the scientific literature. One of the most interesting properties of gold nanoparticles is their capability to convert light to heat at certain wavelengths. Today, a Web of Science search (obtained 26th of April 2018) “TOPIC:(photothermal) AND TOPIC:(Gold nanoparticles)” results a total number of 3607 publications till the end of the year 2017. Interestingly, ca. 54 % of these publications are published between 2015 and 2017, and more than 20 % are from the year 2017. Only two publications are found before the millennium, both from the year 1999. This is surprisingly recent in contrast to the fact that the first gold nanoparticle solutions were prepared in the 1850s by Michael Faraday⁸⁰, optical properties of spherical particles were defined by Gustaf Mie⁸¹ in 1908, and hyperthermia therapies has

been around for millennia^{ii,82} In fact, the first paper on the subject of photothermal heating is by Hodak *et al.*⁸³ (1998), followed by the first proof-of-concept study by Hüttmann and Birngruber⁸⁴ (1999), who used light triggered gold colloids to denature proteins.⁸⁵

Gold nanoparticles are plasmonic meaning that their surface plasmon starts to resonate in a specific resonance condition when the particle is exposed to an external electric field, *e.g.* under of a certain wavelength of light, and the absorbed energy is released either by luminescence of photons or heat.⁷⁴ The heating is commonly referred as “plasmonic heating”. From a practical point of view, the photoluminescence concerns gold nanoparticles of the diameter smaller than 5 nm.⁸⁶ This size range absorbs at wavelengths lower than the physiological window, making them uninteresting in bio applications. To access the correct absorption range, nanoparticle geometry can be engineered so that the plasmon band reacts to desired wavelengths.⁸⁷⁻⁹⁰ This is demonstrated in the following chapter and Appendix A. The contributions of the surface plasmons are also later examined in Chapter 5, where we introduce the surface plasmon resonance imaging microscopy (SPRIM) as part of Publication III.

Surface Plasmons

Surface plasmons were first discovered by Wood in 1902⁹¹, when he observed a pattern of “anomalous” dark and light bands in a spectrum of diffracted light leaving from a diffraction grating mirror surface. Fano (1941)⁹² associated these anomalies with electromagnetic surface waves before Ritchie (1957)⁹³ gave a more complete description of the phenomenon, and imprinted the term “plasmons” (proposed by Pines in 1956⁹⁴) to the English vocabulary. Finally in 1968, independently to each other, Otto⁹⁵ and Kretschmann and Raether⁹⁶ established means to excite plasmonic waves conveniently on a smooth metal surface. This gave final rise to the field called plasmonics.

A plasmon is an electron charge density wave occurring at the metal-dielectric interface. The plasmonic oscillations occurs at a well-defined frequency, classifying a plasmon as a bosonic excitation, corresponding to a quantum of plasma oscillation and adding the suffix “-on” to the term “plasmon”.⁹⁷⁻⁹⁹

The plasmonic effect occurs on a plasmonic metal (*e.g.* Au and Ag) that possess a cloud of free electrons (d-electrons in Au and Ag) with a large negative real part on its dielectric function at the given wavelength.⁹⁸ Surface plasmons cannot be excited in bulk, *i.e.* the continuum underneath the skin depth of the plasmonic metal

$$\delta = \frac{c}{\omega} \sqrt{\frac{\epsilon - \epsilon_m}{\epsilon^2}} \approx \frac{\lambda}{2\pi k}, \quad (2)$$

where c is the speed of light, k is the absorption coefficient of the metal, λ is the wavelength of light, ϵ_m and $\epsilon = \epsilon_1 + i\epsilon_2$ are the dielectric constants of the medium and metal, and $\omega = 2\pi f$, where f is the frequency of light. The free-electron cloud can be displaced and put into an oscillatory motion by an external electric field, usually generated with a parallel polarized (p-polarized) light in

ⁱⁱIn hyperthermia therapy, a tumorous tissue is exposed to elevated temperatures. According to Hippocrates (ca. 400 BC): if a tumor “cannot be cut, it should be burned. If it cannot be burned, then it is incurable.”⁸²

an angle above the critical angleⁱⁱⁱ θ_c , *i.e.* in the total internal reflection region. The total internal reflection can be easily understood considering a perfectly mirror-like water surface that blocks the visibility through the interface. At the surface plasmon resonance (SPR) angle, the momentum and the energy of the incident photon coincides with the plasmonic wave, and the energy of the incident beam is dissipated into the interfacial propagation. This is shown as a drop in the reflected light intensity (see Fig. 18) near the SPR angle.⁹⁸ The SPR angle depends on the dielectric functions of the interfacial materials where the plasmonic propagation occurs and the evanescent wave penetrates inside the non-metallic material. This observation has put forth a surface scientific technique¹⁰⁰, commonly referred plainly as surface plasmon resonance (SPR) where changes in the reflected light intensity indicate changes in the dielectric material, for example biofilms. The surface scientific SPR is detailed in chapter 5.2. In this chapter, we focus on the special case of SPR waves occurring on the surface of a nanoparticle smaller than the skin depth of its metal. This is referred as localized surface plasmon resonance.

The difference between surface plasmons on a nanoparticle and on a thin film is that nanoparticles experience non-propagating plasmonic oscillations. This means that the oscillation takes place in the entire body of a particle, and the electronic cluster is displaced with respect to the positive ion background. Hence, when the light frequency is equal to the resonance frequency condition, plasmons start to oscillate over the particle, making it a dipole (Fig. 3).⁹⁷ This kind of system can be described as a nanosized antenna.

Figure 3. Non-propagating plasmon excitation on plasmonic metal nanoparticle (of size smaller than the skin depth of the metal) with light wave of frequency $f = 1/2\tau$.

The plasma frequency condition depends on the shape and the size of the nanoparticles. A spherical nanoparticle resonates in a single wavelength, as all principal axes are uniform. The optical properties of this shape can be determined analytically by the Mie theory.^{88, 89} In anisotropic shapes, the principle axes might have their own unique resonance condition and thus three or more eigen-frequencies can be found. Since the shape and the size of the nanoparticles are readily engineered today¹⁰¹, the application-required frequency of light is readily accessible. For drug delivery applications, this frequency is in the physiological window (see Fig. 2), and the optimal particle shape of gold is a nanorod with suitable aspect ratio.^{69, 102} The effect of the aspect ratio $\eta = r_x/r_y$ to the excitation

ⁱⁱⁱ Snell's law states that $n_1 \sin \theta_1 = n_2 \sin \theta_2$, where θ_i is the angle of the incident ($i = 1$) or the refracted beam ($i = 2$), and n_i is the refractive index of metal or dielectric, respectively. Illumination from angle $\theta_1 > \theta_c = \sin^{-1} n_2/n_1$ requires that $\sin \theta_2 > 1$, which is impossible for any real θ_2 . This means that all light will be reflected when $\theta_1 > \theta_c$. This is called "total internal reflection".

spectra of gold nanoparticles is displayed in Fig. 4. These plots follow the equation:

$$\sigma_{\text{ext}} = \frac{2\pi}{3\lambda} \epsilon_m^{3/2} \sum_j^3 \frac{\epsilon_{r2,j}}{L_j^2} \left[\left(\epsilon_{r1,j} + \frac{1-L_j}{L_j} \epsilon_m \right)^2 + \epsilon_{r2,j}^2 \right]^{-1}, \quad (3)$$

that has been derived in Appendix A. A spherical particle (green line) follows the equation

$$\sigma_{\text{ext}} = \frac{18\pi}{\lambda} \frac{\epsilon_2 \epsilon_m^{3/2}}{(2\epsilon_m + \epsilon_1)^2 + \epsilon_2^2}. \quad (4)$$

Figure 4. Simulated excitation spectra of gold nanorods with aspect ratio ($\eta = r_x/r_y$) of 1.0, 1.5, 1.8, 2.0, 2.5, 2.9, 3.5, 3.8, 4.1, 4.5, 5.0, 5.9, 6.7, and 8.1 (from left to right). Aspect ratios between ca. 2.5 (or 2.9) and 5.0 absorb in the physiological window I and $\eta > 5$ in window II.

2.2.3 Photosensitizers

Near-infrared fluorophores can be excited in the physiological window. They are used to visualize various biomedical processes including targeted drug delivery *in vivo* and *in vitro*, and in diagnostics of tumor growth in various parts of the body.^{41, 103} In addition, many of them can be used in photothermal heating and photodynamic therapy.

Fig. 5 presents the photophysical processes in a photosensitizer.¹⁰⁴⁻¹⁰⁶ By default, a fluorophore absorbs light at its excitation wavelength (1) and releases emission at a higher wavelength (2). The photothermal effect, or other mechanisms can take place when the absorbed energy surpasses the energy of the emitted photons. The ratio of these energies is the fluorescence quantum yield (QY). A low fluorescence QY means that the molecules remain in a higher energy state after the fluorescence emission, and the excess energy must be released through some other mechanism. The primary mechanism in the fluorophore photothermal agents is the emission of phonons and dissipation of heat (3). However, other mechanisms are commonly involved. The photosensitizer goes often to the triplet state T_1 via internal conversion (4), and may release the rest of the energy as phosphorescence (5). In another case, triplet state molecule undergoes a photodynamic effect. This means that it forms cytotoxic singlet oxygens (6) or other radicals (7) that may be used in cancer treatments, for example. In the drug delivery applications, these compounds may cause harm in the target tissue and some precautions may be needed.¹⁰⁴⁻¹⁰⁷

Figure 5. Modified Jablonski diagram for a photosensitizer. Photophysical processes include: 1) absorption of light, 2) fluorescence emission, 3) internal conversion and vibrational relaxation (photothermal effect), 4) inter-system crossing to triplet state T_1 , 5) phosphorescence, 6) singlet oxygen production, and 7) formation of free radicals. Based on Refs. 104-106.

Interestingly, the research history of dye molecules in photothermal therapy extends longer than that of the gold nanoparticles that have been dominating the field for decades due to their plasmonic elegance and general appeal. Nonetheless, in 1995, Chen *et al.*¹⁰⁸ published a pioneering work “Chromophore-enhanced in vivo tumor cell destruction using an 808-nm diode laser” that introduced indocyanine green (ICG, Fig. 6) as a novel candidate for cancer treatment via the photothermal effect. Despite some clear benefits over some other photothermal agents, the enthusiasm around ICG and other cyanine-based fluorophores has been moderate with only a couple of papers published on the subject of photothermal heating before 2012. For example, a Web of Science search: “TOPIC:(Indocyanine green) AND TOPIC:(photothermal) AND TOPIC:(photodynamic)” (accessed in 3rd of May 2018)^{iv} shows a rapid growth in the number of research papers in the past recent years. Four papers were published in 2012. In 2017, the number was 13-fold with 52 publications. In total, the number of papers in 2017 counts a whopping 31% of all published work within the search.

Figure 6. Chemical structure of indocyanine green (ICG).

The success of ICG as a photothermal agent is based on its photophysical properties. ICG has a minuscule free molecule fluorescence QY of ca. 3-4 %.^{107, 109} Approximately 11 % of the absorbed energy forms triplet state ICG that generates singlet oxygen radicals from the ground state molecules at its proximity and causes the decomposition of the neighboring ICG molecules.¹¹⁰⁻¹¹² The rest, ca. 85 % of the energy, is released as heat via the photothermal effect.¹⁰⁷

^{iv} “Photodynamic” is included in the search because ICG is a dual photothermal/photodynamic agent, and this topic is usually covered whenever ICG is illuminated as a photothermal agent.

In addition to high photothermal efficacy, ICG is an inexpensive standard fluorescent marker that has been used in medical imaging and *e.g.* cancer diagnostics throughout the body.^{41, 103} ICG is excited in the physiological window with two characteristic excitation peaks (see Fig. 7). As a free molecule, the maximum absorption can be found at 780 nm. The shape of the excitation peaks depends on the concentration. At low concentrations (*i.e.* <60 μM), the higher wavelength peak is more pronounced than the lower one, whereas at higher concentrations, the lower wavelength peak becomes more intense.¹¹³ Yet, the absolute size of the upper wavelength peak also increases with the concentration. Thus, the peak shift has no major effect to the optical heating. However, interaction with other molecules shifts the excitation bands upwards. In plasma, or *e.g.* inside liposomes, ICG excitation occurs at wavelengths closer to 800 nm.

Figure 7. The molar excitation spectra of ICG (black) in water and ICG in plasma (gray) with concentrations of 6.5 μM (solid line), 65 μM (dashed line), 650 μM (dotted line). Data acquired from Ref. 113.

The safety of ICG is superior to most photothermal agents. ICG has been approved for clinical use by the European Medicines Agency (EMA) and FDA.^{114, 115} It is sold as a prescription drug “Verdye” in Austria, Belgium, Germany, Italy, the Netherlands, Portugal, Sweden, Russia, the United Kingdom and the United States. The injection dose of Verdye is 5 mg in 1 ml (*i.e.* 6.5 mM) for adults.¹¹⁶ This is well above the dosage needed for the photothermal therapy or liposomal drug release. For the photothermal/photodynamic therapy of tumors, the threshold levels are above 25 μM but some estimates, taking into account the cumulative effect at the beginning of cell incubation, reach up to 0.5-1.7 mM.^{111, 117} In drug delivery applications, the required ICG concentration is lower because the photothermal effect occurs in the proximity of the lipid bilayer, and therefore, the disruption of the bilayer occurs locally. More important is the amount of photothermal agent versus the amount of drug carrier constituents. For example, in the work by Lajunen *et al.*⁴⁰, the optimum ICG:lipid ratio is near 1:50. If the ICG-liposome dose is 0.3 mM, only 6 μM of ICG is injected with the liposomes. The desired therapeutic effect is obtained, nonetheless.⁷²

Like several other substances, ICG suffers from a few drawbacks in physiological conditions. Free ICG interacts with the proteins in the blood stream and the half-life in plasma remains under 4 min.^{118, 119} The major challenge is that ICG is

rather an unstable molecule in aqueous solutions, especially when the temperature of the system is raised by *e.g.* the photothermal effect. ICG also photo-oxidizes and can break in half by a radical reaction.¹¹²

The photodecomposition of ICG splits the cyanine groups that are coupled via the conjugated carbohydrate bridge (see Fig. 6).¹¹² In order to increase its photostability, ICG structure can be stabilized by *e.g.* substituting chlorocyclohexane moiety between the hydrocarbon bridge. The resulting structure is called IR820 (Fig. 8, left). It acts as a photothermal agent, similarly to ICG, but with longer degradation half-times in aqueous solutions, a higher fluorescence quantum yield, and a slightly lower photothermal effect.⁴¹ Similar structures like IR780¹²⁰, IR825¹²¹, and RC¹²² (Fig. 8, right) have been studied as photothermal agents that have an enhanced stability compared with ICG. However, the stability of ICG can also be successfully increased by encapsulating the molecule into the body of micelles¹²³, liposomes^{40, 48, 72, 124}, or *e.g.* antibodies¹²⁵. For instance, liposomes were able to stabilize ICG for one month at temperatures below the lipid phase transition.⁴⁰ At higher temperatures, ICG was exposed to water and thus decomposed.

Figure 8. Chemical structures of IR820 (left) and RC (right) ions (from Ref. 122).

2.2.4 Photothermal Heating

In addition to absorption spectra, the heat conversion efficiency is the most important quality of a photothermal agent.^{67, 69} For instance, some GNRs produce heat with a rate of 95%,⁶⁹ while ICG releases heat with the efficiency of 85 % (at 800 nm).¹⁰⁷ These values are part of the effective absorption cross-section $\sigma_{\text{abs,eff}}$, which is one of the source terms in the heat equation

$$\nabla^2 u - \frac{1}{\kappa} \frac{\partial u}{\partial t} - \frac{u}{\kappa \tau} = - \frac{nI\sigma_{\text{abs,eff}}}{k}, \quad (5)$$

where $u = T - T_0$ is the temperature increment, k is the heat conductivity (of solvent), κ is the thermal diffusivity, τ is the perfusion time,^ν n is the number density, and I is the intensity of light. A solution to this equation is¹²⁶

$$u = \frac{nI\sigma_{\text{abs,eff}}}{k} \left\{ -\kappa \int_0^t e^{-\frac{t'}{\tau}} \operatorname{erfc} \frac{R}{2\sqrt{\kappa t'}} dt' - R \int_0^t e^{-\frac{t'}{\tau}} \sqrt{\frac{\kappa}{\pi t'}} e^{-\frac{R^2}{4\kappa t'}} dt' + \kappa \tau \left(1 - e^{-\frac{t}{\tau}} \right) \right\}, \quad (6)$$

where R is the radius of a sphere influenced by the photothermal agents (set as a boundary condition¹²⁶). Obviously, the heat equations (5) can be expressed with alternative source and sink terms with some optional boundary conditions (see *e.g.* Ref. 67). Eq. (6) it is later used in Chapter 4.4.

^ν Relevant for biological systems as *e.g.* blood flow can dissipate heat. $\tau \rightarrow \infty$ when no perfusion occurs.

3. Light Triggered Effects on QCM

3.1 Background for Publication I

The work for Publication I was conducted between the years 2013 – 2015. As discussed in Chapter 2.2.2, the light-triggered heating of gold nanoparticles had been known for some 15 years prior to this publication. Nevertheless, only a few papers had utilized this effect to liposomal DDSs. These early works include Paasonen *et al.*^{61, 63} in 2007 and 2010, and Troutman *et al.*⁶² in 2009.

The aim of Publication I was to determine the action taking place in the lipid bilayer during the illumination. Paasonen *et al.*⁶³ had reported a phase transition in the lipid bilayer by using small angle x-ray scattering (SAXS) and hydrophobic gold nanoparticles (GNPs) under UV irradiation. In Publication I, this was repeated using hydrophilic citrate capped GNPs (10 nm in diameter) and the phase order monitoring was done with a quartz crystal microbalance (QCM).

3.2 Methods

3.2.1 Preparation of the Samples

A dry lipid film (5 mg of pure DPPC or 1:9 DSPC:DPPC) was prepared by solute (chloroform) evaporation. To increase the encapsulation efficiency, lipid films were first hydrated with a solution of 0.9 nM GNPs in deionised water. This was followed by the addition of buffer (140 mM NaCl, 20 mM HEPES, pH7.4) to a volume of 0.5 ml. The dispersion (60 °C) was briefly sonicated and then extruded 11 times through a double-stack of polycarbonate membranes (pore size: 200 nm). The dispersion was then cooled down to room temperature and diluted to ca. 0.4 mg ml⁻¹ of lipids. The concentration of GNPs inside the liposomes was ≥90 pM. The cryo-TEM sample contained tenth of the gold contents.

3.2.2 Cryogenic transmission electron microscope

A cryogenic transmission electron microscope (cryo-TEM, FEI Tecnai T12) was used to obtain micrographs of vitrified samples, and to ensure the encapsulation of nanoparticles. Here, the preparation method of Kuntsche *et al.*¹²⁷ and Iancuet *et al.*¹²⁸ was used. In brief, 5 µl of sample was pipetted on a TEM grid inside a humidity chamber (humidity 100 %). The grid was blotted between two filter papers and dropped into a condensed 1:1 ethane–propane mixture. Sample was then moved to Cryo-TEM under liquid nitrogen for imaging.

3.2.3 Quartz Crystal Microbalance

Measurement

A QCM instrument (KSV Instruments QCM-Z500) was used to detect changes in the viscoelastic properties of adsorbed liposomes under UV irradiation. The measurements were performed using a gold-plated QCM sensor coated with

C₁₀PEO_dC₁₀SH. The liposomes were first immobilized onto a surface. The unbound particulates were washed with a buffer flow and the system was left to stabilize. The surface was then illuminated for 5 min (flux ca. 2.8 W cm⁻²)^{vi} and the changes in the frequency and resistance were collected.

Analysis

A gold coated quartz crystal oscillates at the fundamental resonance frequency of ca. 5 MHz. When the crystal surface is coated, the frequency f shifts, and the change is connected with the change of the adsorbed mass¹²⁹

$$\Delta m = -\frac{C_f}{N} \Delta f, \quad (7)$$

where $C_f = 17.7 \text{ ng cm}^{-2} \text{ s}$ and N is the frequency overtone.^{vii} This equation is the well-known Sauerbrey equation and works well for fully elastic films. However, when a viscoelastic film (*e.g.* film of adsorbed liposomes) is formed, the Sauerbrey equation is no longer valid and another model is needed to describe the surface response. Most commonly used is the Voigt model that can be derived from the transmission line.¹³⁰ In this case, the impedance response is

$$Z_s = Z_f \left[\frac{Z_1 + Z_f \tanh \gamma_f h}{Z_f + Z_1 \tanh \gamma_f h} \right]; \quad Z_f = \sqrt{\rho G}, \quad \gamma_f = i\omega \sqrt{\frac{\rho}{G}}, \quad (8)$$

where Z_1 and Z_f are the impedance of the solution and the film, and ρ and h are the density and the thickness of the film. Changes in the physical properties are seen in the complex shear modulus

$$G = \mu + i\omega\eta, \quad (9)$$

that can be used to determine the ratio of the viscosity η and the elasticity μ , known as the relaxation time:

$$\tau = \frac{\eta}{\mu}. \quad (10)$$

It is the time needed to recover from a physical deformation. It goes to zero when the film follows a rigid behavior and increases as the material becomes softer.

Modern QCM systems measure changes in frequency and dissipation

$$\Delta D = \frac{E_{\text{dissipated}}}{2\pi E_{\text{stored}}}, \quad (11)$$

where the dissipated energy $E_{\text{dissipated}}$ is higher in a viscous layer and goes to zero in the case of fully elastic layer. Thus, a qualitative relation to τ can be easily drawn.

Viscoelastic films are often characterized qualitatively by a parameter¹³¹⁻¹³³

$$\psi = \frac{\Delta D}{-\Delta f'}, \quad (12)$$

where Δf represents the mass increase on the quartz surface. In addition to ψ , many earlier works and Publication I uses a similar parameter

$$\chi = \frac{\Delta R}{-\Delta f/f_0}. \quad (13)$$

This can be justified in some cases, since $\Delta R \propto \Delta D$ and $\chi \propto \omega\tau$.¹³⁴ Since $f = (2\pi\sqrt{C_1 L})^{-1}$ and $R = 2\pi DfL$,^{135, 136} we can also derive a simple relation between ψ and χ . Here, the frequency is a function of the inductance L but capacitance

^{vi} Erratum: 35 W light was irradiated to a circular radius of $r = 2 \text{ cm}$ (instead of 4 cm).

^{vii} Fundamental resonance frequency ($N = 1$) is accompanied by its integer multiples, overtones. Overtones penetrate to the surface film on quartz crystal. 1st, 3rd, 5th, 7th, 9th, and 11th overtones have penetration depths of 238, 138, 107, 90, 79, and 72 nm, respectively.

C_1 is a constant during the mass or liquid loading.¹³⁵ Therefore, the change in frequency is essentially^{viii} $\Delta f = -(4\pi L\sqrt{C_1 L})^{-1} \Delta L$, and

$$\frac{\Delta f}{f} = -\frac{\Delta L}{2L} \approx \frac{\Delta f}{f_0}. \quad (14)$$

Similarly,

$$\Delta D = \sqrt{\frac{C_1}{L}} \left(\Delta R - R \frac{\Delta L}{2L} \right) \Rightarrow \chi \approx \frac{\psi}{2\pi C_1} + R. \quad (15)$$

This shows that parameters ψ and χ are closely connected. The benefit of ΔR is that it can be constantly measured from the system via electrical impedance. ΔD is measured indirectly by exciting the quartz crystal with a radiofrequency pulse and then determining the decay exponent. Many authors prefer ψ over χ , because ΔR is more sensitive to calibration errors.¹³² This can be justified, because ΔR is an electrical parameter, whereas the decay exponent connects ΔD more closely to the actual oscillation taking place on a quartz crystal (ΔD is essentially the imaginary part of the complex frequency¹³²). Nonetheless, both ψ and χ can be used to represent the qualitative viscoelastic changes in a film.

3.3 Results and Discussion

The main results in Publication I include light triggered changes in the film thickness h and the relaxation time τ in samples that contained GNPs (showed to reside within the liposomes with cryo-TEM). These factors were determined by Voigt analyses, *i.e.* by fitting measured data to Eq. (8). In addition, a qualitative parameter χ was determined and tested against the relative change of the relaxation time:

$$\frac{\tau}{\tau_{\text{ref}}} \propto \frac{\chi}{\chi_{\text{ref}}} \quad (16)$$

Here, χ_{ref} and τ_{ref} are the values of the reference sample. χ was shown to offer a commendable method to monitor viscoelastic changes in real time in the films of adsorbed liposomes. However, the best indication of the photothermal effect was obtained from the Voigt analyses after the measurements. The effects of photothermal heating to the film thickness h , relaxation time τ , and parameter χ are shown in Fig. 9. These changes are relative to their initial values prior to the illumination.

^{viii} Change in a function $z(x, y)$ can be estimated with a total derivative $\Delta z(x, y) = \partial_x z(x, y) \Delta x + \partial_y z(x, y) \Delta y$.

Figure 9. Relative change in film thickness h , relaxation time τ , and parameter χ for liposome samples (DPPC: black; DSPC:DPPC 1:9: gray) without (hollow circles) and with encapsulated gold nanoparticles (filled circles) with the diameter of 10 nm after the illumination on QCM sensor.

The most prominent feature in Fig. 9 is the shift of the relaxation time (with GNPs). A similar trend was also obtained for the parameter χ and layer thickness. This clearly demonstrated that irradiation caused an effect in the adsorbed liposomes. The temperature rise was observed based on the size of the ΔR - Δf hysteresis, that were larger in the case of encapsulated liposomes. However, the slight problem of the results was the lack of detailed information of the mechanisms that took place there. As clear as the effect was, it did not explain whether the liposomes were fused together, GNPs released from the liposomes, or nanobubbles formed due to the photothermal heating. The temperature increment remained also undetermined in the publication.

3.4 Supplementary Results

The photothermal heating was also measured with a common thermocouple (Fig. 10). This measurement shows that the temperature increase was ca. 8 °C from the initial 37 °C when the background heating was subtracted. This consolidates the effect observed in QCM and proves that a phase transition ($T_m = 41$ °C for DPPC) must have taken place during the measurement.

Figure 10. Temperature of a gold nanoparticle solution (circles) and pure water (triangles) during the illumination with UV light.

4. Photothermal Effect in Liposomal DDSs

4.1 Background for Publication II

The work for Publication II was conducted in years 2015 and 2016. The aim was to acquire detailed information of the photothermal effect, and the resulting phase transition and drug release (of calcein). In addition to gold nanorods (GNRs) with aspect ratio 4.1, this work introduced ICG as a prospective photothermal agent for liposomal DDSs. Both photothermal agents absorb in the physiological window (compare Fig. 11 and Fig. 2).

The thermometry was conducted with two fluorophores: CdSe quantum dots (QDs) and laurdan. Laurdan was also used to determine the lipid phase order. The inspiration to use these fluorophores were given by Maestro *et al.*^{66, 69, 102} (QDs) and Parasassi *et al.*¹³⁷⁻¹³⁹ (laurdan).

Figure 11. The attenuation coefficient of gold nanorods (black) and indocyanine green (gray).

4.2 Methods

4.2.1 Preparation of the Samples

A lipid film (1.5 μmol DPPC and 5.7 nmol laurdan) was dried out of its organic solute on a round flask under a stream of nitrogen. The system was heated to 60 $^{\circ}\text{C}$ and the film was hydrated with 750 μl of buffer solution (100 mM NaCl, 10 mM TRIS-HCl, pH 7.4) with or without one of the photothermal agents. The dispersion was then extruded 11 times through double-stacked polycarbonate membranes with the average pore size of 400 nm. The solution was eluted through a Sephadex G-50 gel column, and the third fluorescent aliquot was collected with the help of the fluorescence emissions of laurdan (and a UV lamp).

4.2.2 Fluorescence Thermometry and Lipid-Order Detection

Quantum Dots

QDs are nanocrystals possessing a bright and narrow emission band that can be excited in a wide range of wavelengths. The maximum wavelength of the emission band depends on the size and the geometry of the QDs, but some QDs can be used as *nanothermometers* because the peak position is also redshifted as a function of temperature.¹⁴⁰ In Publication II, CdSe QDs with a peak maximum near 560 nm was used. These QDs show a peak shift of ca. 0.1 nm K⁻¹.⁶⁶

Laurdan

Laurdan molecule exhibits two energy states that are called a *locally excited state* and a *charge transfer state*. These two energy states are expressed as a function of the electric polarity in the proximity of the fluorophore.^{137, 139, 141} The polarity of the lipid-water interface depends on the phase order of the bilayer.^{137, 139, 141-143} Thus, a phase order can be detected in the *generalized polarization*¹³⁷

$$GP = \frac{I_0 - I_1}{I_0 + I_1}, \quad (17)$$

where I_0 and I_1 represent the emitted intensities of the locally excited lauridan molecule and the charge transfer state molecule, respectively.¹³⁷⁻¹³⁹ As the $GP(T)$ decreases monotonically, lauridan can be also used as a temperature probe.

Measurement and Analysis

A fluorescence spectrometer PerkinElmer LS5 was used to detect changes in the phase order (lauridan) and temperature (lauridan and QDs). The emission spectra of the samples were measured as a function of temperature (example in Fig. 12A; excitation at 365 nm). After the measurement, the peak at ca. 560 nm was baseline corrected (Fig. 12A inset), and the emission maximum $\lambda_{\max}$ of the QDs was determined (example in Fig. 12B). I_0 and I_1 were determined at 430 nm and 510 nm, respectively, and the GP value of the given temperature was calculated with Eq. (17) (example in Fig. 12C).

Figure 12. A) Emission spectra of liposomal DPPC-lauridan and CdSe QDs versus temperature. Inset is the baseline corrected QD response. The arrow points to the direction of the temperature raise from 25 °C to 70 °C. B) Peak maxima of the QD emissions as a function of temperature. C) Laurdan GP as a function of temperature.

In the light triggering experiments, calibration curves (equal to Fig. 12B and Fig. 12C) were used to determine the temperature versus time.

4.2.3 Drug Release with Calcein

Calcein emissions can be excited at 495 nm and measured at 515 nm. The drug release studies were thus performed separate to thermometry.

Calcein is a self-quenching fluorophore. Its emission is quenched inside the liposomes due to high concentration. Once released, however, its intensity I increases due to dilution. Therefore, the calcein release percentage is

$$R = \frac{I - I_0}{I_{100} - I_0} 100\%, \quad (18)$$

where I_0 and I_{100} are the intensities of the sample with 0% and 100% of calcein released. I_{100} was measured after the addition of 10 μl of 10% Triton X-100.⁴⁸

4.3 Results and Discussion

Fluorescence Thermometry

The sample volume influenced the magnitude of the measured photothermal temperature rises (Fig. 3 in Publication II). Thermometers thus rendered bulk effects rather than the local temperature rises. As an estimate, the GNR surface reached the temperature of 65 °C instantaneously under the irradiation of 3W, and the temperature was increased ca. 0.5 °C min⁻¹ until the laser was switched off. In a water circulation (set to 37 °C), the mean temperature of the solution increased by 13 °C irrespective to the GNR concentration (0.5, 1, or 2 nM).

With GNRs inside liposomes, a part of the energy goes to the phase transition of the lipid bilayer. Fig. 13A shows the *GP* with illumination of 1 W and 3 W. With 3 W, the light power was enough to cause a phase transition, showing potential towards drug release. The temperature rise (Fig. 13B) was around 12-13 °C, reaching the final temperature of 50 °C. The size of the liposomes and the presence of QDs had no effect to the temperature rise.

Figure 13. A) Laurdan GP as a function of temperature. The dots represent the *GP* values during the illumination. B) The temperature during a 3 W irradiation of liposomes with GNRs. The solid line and the hollow dots represent the temperature of a system containing 400 nm liposomes, measured with QDs and laurdan, respectively. The filled circles are the temperatures of 100 nm liposome systems with QDs (gray) and without QDs (black), measured with laurdan.

The major obstacle of ICG is its poor photostability and water sensitivity. During the illumination, ICG decomposed and heating decreased rapidly. As discussed in Chapter 2.2.3, ICG goes to the triplet state and promotes radical reactions, decomposing the nearby ICG molecules. To increase the time of the photothermal effect, 100 mM of ascorbate was added to the buffer solution. This influenced the calibration curves of laurdan and QDs (Fig. 14A), as the photodynamic effects impacted both, the ascorbate and ICG molecules. The calibration curves followed the same shape before and after the illumination, but a shift from the original GP and $\lambda_{\max}$ was observed. In any case, the temperature increase was of the same magnitude as detected with the GNRs (Fig. 14B).

Figure 14. A) Laurdan calibration curves of ICG-embedded (40 μM) liposomes in buffer containing 100 mM ascorbate (black line) and without ascorbate (gray). The GP values during the illumination are shown as red and purple dots. B) The temperature of the system during the photothermal heating, measured with laurdan (black dots with ascorbate and gray dots without ascorbate) and QDs (black line with ascorbate and gray line without ascorbate).

From Fig. 13 and Fig. 14, it is clear that phase transitions took place in the lipid bilayers during the (3W) illumination with both photothermal agents. This effect occurred throughout the bulk, as measured with fluorescent thermometers. In an application, photothermal triggering causes the leakage of the contents locally, and the effect occurs almost instantaneously. The temperature increments measured in this publication were virtually same as the measurements with a thermocouple. Hence, these measurements do not count as nanothermometry, which might be possible with advanced microscopy techniques. Nonetheless, the magnitude of the local temperature rises should be prominent in the bulk when the time scales reach the time scales of this publication. Thus, it is likely that both photothermal agents heat up to ca. 65-70 $^{\circ}\text{C}$ instantaneously (as we have estimated for GNRs), and the contents can be released with much smaller doses. This is actually demonstrated in next chapters.

Calcein Release

Calcein was released by local heating. Therefore, only tenth of the photothermal material was used (*i.e.* 0.3 nM of GNRs or 3 mM of ICG). Fig. 15 shows the release percentages of calcein after the 1 W and 3 W irradiation.

Figure 15. Calcein release percentage of liposome samples containing 0.3 nM GNRs, and 3 mM ICG after the 5 min irradiation of 1 W or 3 W light.

Fig. 15 shows that the 3 W irradiation, *i.e.* the light power sufficient to cause a phase transition in the lipid bilayer, was also able to release calcein from the liposomal cavity. 1 W did not cause this effect, although, ICG was decomposed (see Publication II) and somewhat enhanced release was observed. This may stem from the fact that ICG is embedded in the bilayer and a more imminent photothermal heating may take place. The release may also occur via other photodynamic effects (see Fig. 5).

4.4 Supplementary Results

With GNRs, the temperature rise of the bulk against the water circulation was ca. 13 °C. Based on the theoretical work by Norton *et al.*¹²⁶, the temperature can be predicted using the formulae of Appendix A and Eq. (6), which result to a rise of 13 °C at 808 nm (Fig. 16). This result uses parameters: $\tau = 100$ s, $R = 5$ cm, and $I = 18$ W cm⁻² (3 W). The heat conversion efficiency is 95 %.

Figure 16. The temperature rise vs. time and incident wavelength according to Eq. (6) for a system of 0.5 nM GNRs with aspect ratio 4.1 in a water circulating bath set to 37 °C.

The liposomal structure and the ICG concentration were not optimized in Publication II. It is likely that higher concentrations cause higher photodynamic perturbation and decomposition of ICG. Luckily, Fig. 17 shows that a very small amount of ICG suffices to release drug molecules. Calcein release in this case

was executed in DPPC:DSPC:LysoPC:DSPE-PEG(2000) 75:15:10:4 liposomes in phosphate buffered saline containing only 1 μM of ICG in the hydration step. Here, 1W had no effect whatsoever, whereas 2 W and 3 W illuminations were able to release ca. 40 % and 80 % of the contents, respectively. This result is in line with Lajunen *et al.*⁴⁰, who showed that the optimum ICG:lipid fraction is close to 1:50, meaning 6 μM of ICG in the hydration solution.⁷²

Figure 17. Calcein release from DPPC:DSPC:LysoPC:DSPE-PEG(2000) 75:15:10:4 liposomes containing 1 μM of ICG with irradiation powers of 1, 2 and 3 W.

5. SPRIM: Encapsulation Efficiency of GNPs

5.1 Background for Publication III

The work for Publication III was conducted in years 2015 and 2016. The aim was to determine the number of liposomes ($d \leq 200$ nm) with encapsulated gold nanoparticles using a new microscopy technique called the surface plasmon resonance imaging microscopy (SPRIM)^{ix}. Here, the idea was to determine the encapsulation efficiency (EE) of the photothermal liposomes containing GNPs ($d \sim 10$ nm or 40 nm) that was still undetermined from Publications I and II.

In addition to EE, the aim of Publication III was to provide a new analysis method for the SPRIM images. This work was based on Halpern *et al.*¹⁴⁴, whose image filtering method was adopted to this paper.

The initial overview to plasmons was given in Chapter 2.2.2. In Publication III, the main concept is the microscopic plasmonic propagation. Before that, it may be justified to start with the “normal” SPR and SPRI first.

5.2 General View on Surface Plasmon Resonance

The implementation of this theory can be found in Appendix B (for Matlab). The Kretschmann configuration (Fig. 18A) is often used to excite plasmons in the metal-dielectricum interface. The Kretschmann configuration is essentially a four-phase system, containing a prism on a plasmonic metal on a dielectric material lying on the phase of exterior. The transmission and reflection balance of this system is as depicted in Fig 18B. Here the boundary incidents are the $\{E_{p1}, E_{p2}, E_{p3}, E_{p4}\}$ and the reflections are $\{E_{n1}, E_{n2}, E_{n3}, 0\}$.^x Naturally, the transmission through the layers is $t = E_{p4}/E_{p1}$, giving the fraction of the original incident field that is passed to the exterior. Similarly, the reflection is $r = E_{n1}/E_{p1}$, signifying the fraction that has returned. Reflections and transmissions can be calculated for each boundary alike. This is the simple beauty of the SPR system. It can be extended to multiple layers with a linear algebraic form¹⁴⁵

$$\begin{bmatrix} E_{p1} \\ E_{n1} \end{bmatrix} = S_1 \begin{bmatrix} E_{p2} \\ E_{n2} \end{bmatrix} = S_1 S_2 \begin{bmatrix} E_{p3} \\ E_{n3} \end{bmatrix} = S_1 S_2 S_3 \begin{bmatrix} E_{p4} \\ 0 \end{bmatrix}, \quad (19)$$

where

$$S_j = \frac{1}{t_j} \begin{bmatrix} e^{ik_0 n_j d_j \cos \theta_j} & r_j e^{ik_0 n_j d_j \cos \theta_j} \\ r_j e^{-ik_0 n_j d_j \cos \theta_j} & e^{-ik_0 n_j d_j \cos \theta_j} \end{bmatrix}, \quad (20)$$

with $d_1 = 0$, $k_0 = \omega/c$ and

$$\cos \theta_j = \sqrt{1 - \frac{n_1^2}{n_j^2} \sin^2 \theta_1} \quad (21)$$

that we obtain from the Snell's law (see footnote iii). The reflections can be also calculated by

^{ix}“The surface plasmon resonance imaging microscopy” refers to a surface-scientific SPR where the incident and the detection angles are fixed (“SPR imaging”) but with a microscope objective that puts system into the total internal reflection condition.

^xSubscripts p and n denote the “positive” and “negative” directions respect to incident beam

$$r_j = \frac{n_j \cos \theta_{j+1} - n_{j+1} \cos \theta_j}{n_j \cos \theta_{j+1} + n_{j+1} \cos \theta_j} \text{ (p-polarized)}$$

$$r_j = \frac{n_j \cos \theta_j - n_{j+1} \cos \theta_{j+1}}{n_j \cos \theta_j + n_{j+1} \cos \theta_{j+1}} \text{ (s-polarized).}$$
(22)

Now only the first incident angle θ_1 , layer thicknesses d_2 and d_3 , and refractive indices n_1 , n_2 , n_3 and n_4 are needed to describe the whole SPR phenomenon. This is, when the layer properties change, the reflectivity ($R = |r|^2$) changes, and the SPR peak shifts. When *e.g.* liposomes with a refractive index $n_3 \approx 1.45$ are adsorbed to the surface (gray line in Fig. 18C), the SPR peak shifts. A special measurement condition of SPR is the SPR imaging (SPRI). In this case, the angle of incidence is fixed and the shifts in the SPR curve are only shown as an increase or decrease in the reflectivity.

Figure 18. A) Kretschmann configuration for plasmonic generation. B) Schema of the transmission (t_{ij}) and reflection (r_{ij}) balance in four phase system. C) Simulated SPR curve with $d_3 = 10$ nm and $n_3 = 1.45$ on $d_2 = 45$ nm gold on glass (gray line). Black line is the initial SPR curve.

5.3 Methods

5.3.1 Preparation of the Samples

A lipid film containing 90:3:7:0.5 DPPC:DSPE-PEG:DPPE-Biotin:DHPE-Fluorescein was prepared as in Publication II. The film hydration was performed in a bath sonicator (60 °C) with addition of 750 μ l phosphate buffered saline (pH7.4) containing GNPs (if any). The dispersion was then extruded 11 times through two sets of double-stacked polycarbonate membranes; first through membranes with 400 nm pores and then through 200 nm pore membranes. The stock solution was eluted through a Sephadex G-50 gel column to remove the excess GNPs. The concentration of the aliquot was determined based on the emission intensities of fluorescein. Final samples were diluted to ca. 20 pM.

5.3.2 Surface Plasmon Resonance Imaging

A prism-coupled SPRI with a microflow channel was used to compare different surface chemistries on gold (layer thickness ca. 50 nm). The surface was thiolated with SH-PEG, SH-MUAM, and SH-MUAM-PGlu-NHS-PEG₄-Biotin (RBiotin). From these, the PEG surface did not bind liposomes while the MUAM and

RBiotin-streptavidin surface immobilized them. Hence, these chemistries were chosen to SPRIM.

5.3.3 Surface Plasmon Resonance Imaging Microscopy

Measurement

The operation principle of the SPRI microscope has been detailed by Halpern *et al.*¹⁴⁴. The general idea is that the condition of the total internal reflection is obtained through a microscope objective. Typically, the intensity of the reflected beam is set to an angle matching the displacement of 30 % of the reflectivity, where the intensity changes during the adsorption events are readily detectable due to the sharpness of the SPR peak.

The SF10 glass slides were first coated with layers of ca. 1 nm chromium, ca. 45 nm gold, and thiolated functional group (MUAM or RBiotin). An adhesive microscope well grid was attached on the glass surface, and the RBiotin glass slides were treated with streptavidin solution for 30 s. The glass slides were then moved to the SPRIM with the grid holes filled with buffer. The microscope (Olympus IX51) was focused, and the reflected images, constructed in Andor Neo sCMOS camera, were used to set the mean reflectivity to ca. 30% between the SPR angle (the angle of minimum reflectivity; see Fig. 18C) and the critical angle (*i.e.* the kink at ca. 41° in Fig. 18C). The adjusting was done with a gold-coated knife-edge mirror located under the microscope objective. The lights were switched off and the buffer was replaced with the sample dispersion. 30 11-bit images were accumulated to acquire one raw image every 3 s.

Image Filtering and Particle Detection

Pixel intensities $I_t^{\{x,y\}}$ of the raw image at time t were used to calculate the raw difference image in position $\{x, y\}$:

$$I^{\{x,y\}}(t_2) = 100 \frac{I_{t_2}^{\{x,y\}} - I_{t_1}^{\{x,y\}}}{I_{t_1}^{\{x,y\}}}. \quad (23)$$

Typical adsorption event appears as a clear intensity maximum accompanied with a few micrometers long fan-like propagation pattern with the orientation in the same general direction as the inherent plasmonic propagation obtained with the p-polarized light (Fig. 19A). A similar, but opposite pattern is obtained in the case of particle desorption or due to an adsorption event of a particle with lower refraction index than the host medium.^{144, 146} In many cases, the raw difference images are noisy. Some of this is unspecific noise but some part is caused by the inherent plasmonic propagation that is readily detectable in the images and can hide some of the adsorption events. One method to reduce this problem is to apply 2D Fast Fourier Transformation (FFT) (Fig. 19B). In this case, the FFT image has a characteristic shape of a double sphere that emerges due to the critical resonance condition in the Fourier space (ω_x, ω_y):

$$\tilde{a}^2 = \omega_x^2 + \omega_y^2, \quad (24)$$

where $\tilde{a}$ is the complex wave number. The image noise is then reduced by zeroing the area inside and outside of the double spheres (Fig. 19D) prior to an inverse Fourier transformation that returns the final FFT filtered image (Fig. 19C).

Figure 19. Fast Fourier filtering of an SPRi microscope image. Raw image showing liposomal adsorption events (A) is Fourier transformed (B). A mask that covers everything except the double sphere shape is applied (D), and the inverse FFT is calculated, showing the original adsorption events with reduced noise levels (C). The circle in the middle of the FFT images is a mask that makes the circles more detectable but has no influence in the filtration.

Data Analyses

To analyze the adsorption events of photothermal liposomes, we measured the average of the nine pixels surrounding each intensity maxima. These were used to build an empirical cumulative distribution function (ECDF) that was fitted with the well-known CDF equation

$$P(x) = \frac{p_1}{2} \left(1 + \operatorname{erf} \left(\frac{x - p_2}{\sqrt{2}p_3} \right) \right) \quad (25)$$

or its log-normal alternative assuming that one or two particle types were present in the system. In addition to intensity maxima, a phenomenological equation was derived:

$$I_{\text{particle}}^{\text{fit}} = I_b + \frac{v^2 |H_0^{(2)}(\tilde{a}\sqrt{x^2+y^2})|^2 + 2v\Re\{-iH_0^{(2)}(\tilde{a}\sqrt{x^2+y^2})(-i\tilde{a}^{-1}e^{-i\tilde{a}x})^* \cos\theta\}}{|-i\tilde{a}^{-1}e^{-i\tilde{a}x}|^2}, \quad (26)$$

where v is the field enhancement parameter and I_b is the intensity of the baseline. This equation was obtained by solving the differential equation

$$(\nabla^2 + \tilde{a}^2)\vec{E} = -Q\vec{E}_{\text{ext}}\delta(\vec{r}) \quad (27)$$

leading to 1D and 2D solutions

$$E_{1D} = -iQ'_1 \sqrt{\frac{\pi}{2}} \frac{1}{\tilde{a}} e^{-i\tilde{a}x}, x = |x - x_0|, \text{ and} \quad (28)$$

$$E_{2D} = -\frac{iQ'_2}{4} H_0^{(2)}(\tilde{a}r), r = |r - r_0|,$$

where Q'_1 and Q'_2 are the source terms and $H_0^{(2)}$ is the zeroth order Hankel function of second kind. The field enhancement v , presented earlier, is the ratio of the effective source terms. Fitting Eq. (26) was applied to adsorption events and the fitting parameters v and $\tilde{a}$ were used to build an ECDF that was further analyzed.

5.4 Results and Discussion

Publication III showed that LUVs ($d \leq 200$ nm) were easily observed in the SPRIM images on MUAM and streptavidin modified gold surfaces. The intensity maxima at the binding site were used to detect differences in a set of particles. The most visible separation of liposomes and liposomes with gold nanoparticles of diameter 10 nm or 40 nm was detected in the intensity maxima ECDF^{xi} as shown in Fig. 20. Here, one particle type fit was sufficient to explain the distribution of liposomes without gold nanoparticles, but two particle type fit was needed in the case of photothermal liposomes. In the case of 10 nm GNPs, the EE of liposomes were 23 %. With 40 nm GNPs, the fraction was 24 %. This is in the limits of normal EE (*i.e.* 10-30 %) ¹⁴⁷.

Figure 20. ECDF (black dots) of the measured intensity maxima on streptavidin (A) and MUAM (B) with the best CDF fit without GNPs (blue lines), with 10 nm (red lines) and 40 nm (yellow lines) GNPs, assuming one particle type (dashed lines) or two particle types (solid lines) in the liposome samples.

In addition to EE detection, the DLS size distribution was showed to have a $d_{DLS} \propto \sigma(I^3)$ relation with the intensity. This is in line with Halpern *et al.* ¹⁴⁴.

Similar to Fig. 20, the fitting parameter v lead to the EE of ca. 26 % and 23 % for 10 nm and 40 nm GNPs, respectively (Fig. 21). The complex wave number also gave a similar result on MUAM (Fig. 5 in Publication III).

^{xi} Intensity maximum value is the average of nine pixels surrounding the peak intensity of the adsorption pattern.

Figure 21. ECDF (black dots) of the fitted intensity parameters ν of the liposomes on streptavidin (A) and MUAM (B) with the best CDF fit without GNPs (blue lines), with 10 nm (red lines) and with 40 nm (yellow lines) GNPs assuming one particle type (dashed lines) or two particle types (solid lines) in the liposome samples.

6. The Effects of PEGylation

6.1 Background for Publication IV

The work for Publication IV was conducted in years 2017 and 2018. The aim was to determine the impact of PEGylation to the relevant lipid drug carriers^{xii} by using fluorescence spectroscopy, differential scanning calorimetry (DSC), cryo-TEM, small/wide-angle x-ray scattering (SAXS/WAXS) and molecular dynamics simulations (MD simulations).

One of the initial motivators of this work was the computational work by Lajunen *et al.*⁴⁰. They had reported that, in addition to binding ICG, PEG may also enter the lipid bilayer due to its hydrophobic moieties. This claim was not directly corroborated in the results of Publication IV, but other effects were observed. For instance, it was demonstrated that PEGylation causes an increase in the phase transition temperatures (also observed in Ref. 148). Due to the preparation method and uneven PEGylation, this effect is manifested as a liposomal drug release temperature window, potent to *e.g.* photothermal drug release.

PEGylation can also alter the shape of the particulates.¹⁴⁹⁻¹⁵² Hence, this effect was examined as a potential drug release mechanism. As predicted by Ref. 4, the effect of the shape transformation from liposomes to bicelles was showed to result from segregation of lipids that were driven to match their spontaneous curvature.^{xiii} As the curvature depends on the size of the lipid head, this effect could possibly be triggered with a suitable trigger-responsive polymer. However, this work counted just the fundamental effects: a separate study would be needed in order to demonstrate the drug release from a trigger-polymer system.

Finally, Publication IV provided a rather pervasive viewpoint of the effects of PEGylation. Unfortunately, many of these were already known. New insight was nonetheless given by the MD simulations, for example, and a new method to measure the average shape of the lipid aggregates was put forward. The shape-recognition was based on the emissions of laurdanC, a fluorophore similar to that used in Publication II (laurdan). With this fluorophore and cryo-TEM, the critical bicellation fraction was determined at ca. 9.6 mol%.

6.2 Methods

6.2.1 Preparation of the Samples

LaurdanC samples were prepared according to Publication II with a change in buffer (here 150 mM NaCl, 20 mM HEPES in pH 7.4) and lipid composition. Otherwise, 20 μ mol lipid films containing DPPC and 0, 3, 6, 9, 12, 15 or 24 mol% of DSPE-PEG(2000) were dried on a round flask. The dispersion was hydrated in a path sonicator (60 °C) with 2 ml of HEPES buffer. The dispersion was then

^{xii} The importance of PEGylation has been established in Chapter 2.1.3.

^{xiii} The curvatures were briefly introduced in Chapter 2.1.1.

extruded 11 times through double-stacked polycarbonate membranes (pore sizes: 200 nm). Samples were not diluted or purified further in order to keep the integrity of the particulate sizes and shapes.

6.2.2 Cryogenic Transmission Electron microscope

A Cryo-TEM (FEI Tecnai T12) was used to determine the shape and the size of the PEGylated lipid systems (*i.e.* DPPC:DSPE-PEG(2000)). The samples were prepared as in Publication I, following Kuntsche *et al.*¹²⁷ and Iancuet *et al.*¹²⁸.

The monomeric bicelle fraction was determined as

$$x_{\text{bicelle}}^{\text{TEM}} = \frac{N_{\text{bicelle}}}{N_{\text{bicelle}} + \tau N_{\text{liposome}}}, \quad (29)$$

where τ is the area-scaling factor

$$\tau = \frac{\langle A_{\text{liposome}} \rangle}{\langle A_{\text{bicelle}} \rangle}, \quad (30)$$

a fraction of the mean liposome surface area versus the average area of bicelles.

6.2.3 Phase Transition Temperatures with DSC and LaurdanC

LaurdanC

Carboxylated laurdan (laurdanC) was synthesized using the pathway described by Cheniour *et al.*¹⁵³. Just like laurdan in Publication II, LaurdanC exhibits *locally excited* and *charge transfer* energy states. Hence, it provides the *GP* value exactly like laurdan (see Chapter 4.2.2).

Measurements

A MicroCal VP-DSC calorimeter and a Perkin-Elmer LS5 fluorescence spectroscope with laurdanC were used to obtain the phase transition temperatures of the samples. The DSC measurement was conducted between 10 °C and 60 °C. The heating and cooling rates were 1 °C min⁻¹ and 0.5 °C min⁻¹, respectively. The laurdanC spectra were measured after 3 min in each temperature from 25 °C to 70 °C, with temperature intervals of 0.5 °C between 34 °C and 45 °C (and 1-5 °C elsewhere). The lipid order was determined from *GP* (Eq. (17)), and its derivative $\partial GP / \partial T$ was determined to portray the data like the DSC results.

Thermographic Analysis

The DSC and $\partial GP / \partial T$ peaks were deconvoluted by fitting the data with multiple Voigt profiles (*i.e.* the convolution of Gaussian distribution and Cauchy-Lorentz distribution) concurrently. Usually, the DSC data contained 3 Voigt profiles, but *e.g.* sample PEG9^{xiv} contained 5 distributions in the upward scan and 4 distributions in the downward scan. The peaks were labelled in the order of their position and compared with the like measurement (*e.g.* downward scan of PEG9 with the other downward scans). $\partial GP / \partial T$ peaks were counted as “liposomal” or “bicellar”, and the fraction of bicelles $x_{\text{bicelle}}^{\text{LC}}$ was calculated.

6.2.4 Shape Recognition with LaurdanC

The polarity, measured by LaurdanC,¹³⁹ is related to the surface area of the lipid-water boundary.¹⁵⁴ If LaurdanC is mixed evenly, it should render the cryo-TEM

^{xiv} From here on, the number after “PEG” indicates its mol% in the formulation.

results. Yet, $x_{\text{bicelle}}^{\text{LC}} \rightarrow 0.684$ as $x_{\text{bicelle}}^{\text{TEM}} \rightarrow 1$ and a discontinuity was observed at 9.6 mol%. Thus, it was assumed that laurdanC is mixed evenly into the bilayers of liposomes, but only the face region was resided in bicelles:

$$x_{\text{bicelle}}^{\text{LC}} = \frac{GP_{\text{bicelle}}}{GP_{\text{tot}}} \approx \frac{A_{\text{flat}}}{A_{\text{flat}} + A_{\text{curved}}} = \frac{2(\eta-1)^2}{2(\eta-1)^2 + \pi(\eta-1) + 1}, \quad (31)$$

where A_{flat} and A_{curved} are the surface areas of flat and curved surfaces, and η is the aspect ratio.

6.2.5 Small/Wide-Angle X-ray Scattering

Measurement

A SAXSLab Ganesha 300XL instrument was used to determine the particulate shapes and sizes, as well as, the structural properties of the bilayer in 25 °C, 41 °C, and 50 °C. The scattering intensity $I(q)$ was calculated by radial averaging for the scattering vector region $q \in \{0.05, 20.0\} \text{ nm}^{-1}$.

Analysis

The implementation of this theory can be found in Appendix C (for Matlab). The total scattering cross-sections is a sum of the liposome and bicelle:

$$I_{\text{fit}} = x_{\text{bicelles}} I_{\text{bicelle}} + (1 - x_{\text{bicelles}}) I_{\text{liposome}}, \quad (32)$$

where the fraction of bicelles $x_{\text{bicelles}} = x_{\text{bicelles}}^{\text{TEM}}$.

The PEG-covered particle has the total scattering cross-section^{155, 156}

$$I_{\text{tot}} = n(P_s + N_c[P_c + 2P_{\text{sc}} + (N_c - 1)P_{\text{cc}}]), \quad (33)$$

where n is the number density; P_s is the self-correlation term of the lipid core;^{xv}

$$P_c = 2(\Delta\rho_{\text{PEG}}^w v_{\text{PEG}})^2 (e^{-(qR_G)^2} + (qR_G)^2 - 1)(qR_G)^{-4} \quad (34)$$

is the self-correlation term of the PEG chain;^{157, 158} N_c is the number of polymer chains on a single particle; and P_{sc} and P_{cc} are the cross-correlation terms of the core-chain and the chain-chain interactions.

The core-shell structures (*i.e.* P_s) of liposomes and bicelles are constructed from their component layers.^{xvi} This is presented in Scheme 1.

Scheme 1. A: The core-shell structure of a liposome. On the left, the full representation; on the right, its divisions into component layers. B and C are two cylindrical core-shell structures with three layers (1, core; 2, face; and 3, rim). D represent a bicelle.

^{xv} $\Delta\rho_i^w = \rho_i - \rho_0$ is the scattering length density contrast, v_{PEG} is the volume of PEG molecule, and R_G is the radius of gyration

^{xvi} Each component layer contains $\pm V_i \Delta\rho_i^w F_i$, where V_i is the volume of the component layer and F_i is the form factor amplitude.

The factor $f_{k,i} = V_i \Delta \rho_i F_i$ in Scheme 1 depends on particle geometry k . As the polydispersity is relatively high in lipid dispersions, equations in Scheme 1 were replaced with

$$P_{s,k} \mapsto P_{s,k} = \sum_j \Gamma_j \sum_i f_{k,i,j}^2, \quad (35)$$

where $\Gamma_j(\xi_j) = (\sqrt{2\pi} \text{PDI} \cdot r_1)^{-1} \exp(-\xi_j^2 (\sqrt{2\pi} \text{PDI} \cdot r_1)^{-2})$ is the Gaussian weight coefficient with the polydispersity index PDI, the radius of the first component layer r_1 , and the deflection length $\xi_j \in \{x | x \in \mathbb{R}, |x| \leq 3\text{PDI} \cdot r_1\}$. Similarly, the cross-correlation terms in Eq. (33) are

$$\begin{cases} P_{sc,k} = f_c \int_0^{\frac{\pi}{2}} \sum_j \Gamma_j f_{k,j} \Xi_{k,j} \sin \alpha \, d\alpha \\ P_{cc,k} = f_c^2 \int_0^{\frac{\pi}{2}} \sum_j \Gamma_j^2 \Xi_{k,j}^2 \sin \alpha \, d\alpha \end{cases}, \quad (36)$$

where $\Xi_{k,j}$ is the formfactor amplitude of an infinitely thin shell.¹⁵⁶ For a liposome with a PEG fraction x_{out} on its outer leaflet,

$$\begin{cases} f_{\text{liposome},j} = \sum_i^4 \Delta \rho_i V_{i,j} 3 \frac{\sin q r_{i,j} - q r_{i,j} \cos q r_{i,j}}{(q r_{i,j})^3} \\ \Xi_{\text{liposome},j} = x_{\text{in}} \frac{\sin q(r_{1,j} - R_G)}{q(r_{1,j} - R_G)} + x_{\text{out}} \frac{\sin q(r_{4,j} + R_G)}{q(r_{4,j} + R_G)} \end{cases} \quad (37)$$

The equivalent formulations for a bicelle are^{159, 160}

$$\begin{cases} f_{\text{bicelle},j} = \sum_i^4 \Delta \rho_i V_{i,j} \frac{J_1(q r_{i,j} \sin \alpha) \sin(q l_i \cos \alpha)}{q^2 r_{i,j} l_i \sin \alpha \cos \alpha} \\ \Xi_{\text{bicelle},j} = x_{\text{face}} \frac{2J_1(q r_{4,j} \sin \alpha)}{q r_{4,j} \sin \alpha} \cos(q(l_4 + R_G) \cos \alpha) \\ \quad + (1 - x_{\text{face}}) J_0(q(r_{4,j} + R_G) \sin \alpha) \frac{\sin(q l_4 \cos \alpha)}{q l_4 \cos \alpha} \end{cases} \quad (38)$$

6.2.6 Computational Methods

MD simulations of DPPC:DSPE-PEG(2000) systems were performed to support the experimental observations. The MD runs were performed in the NVT ensemble with the total duration of 0.5 μs . A DRY-MARTINI model¹⁶¹ with the 3-to-1 CG mapping for PEG (following Refs. 162 and 163) and the 4-to-1 CG mapping for lipids was used. The DSPE-PEG model was constructed based of Refs. 164 and 165.

Four different molecular compositions were studied with the total number of 10500 lipids and their Na^+ counter-ions. These were 1) PEG0, 2) PEG2.2, 3) PEG10.5, and 4) PEG50 (see footnote xiv). PEG50 was simulated in a $100 \times 100 \times 100 \text{ nm}^3$ simulation box. A simulation box of $50 \times 50 \times 50 \text{ nm}^3$ was used otherwise. To assess the lipid segregation and bicelle thickness, 1050 lipids were also self-assembled in a $30 \times 30 \times 30 \text{ nm}^3$ simulation box to produce single bicelles.

6.3 Results and Discussion

6.3.1 Phase Transition

One of the most prominent effects of the PEGylation was the shift in the phase transition temperatures (Fig. 22). The rise in the phase transition temperature stems from the decrease in the overall lateral pressure as the fraction of DSPE-PEG increases.^{166, 167} In addition to increments in phase transition temperature,

broad phase transition peaks below 41 °C are obtained in the liposomal samples. This is caused by the existence of liposomes smaller than 80 nm in diameter.¹⁶⁸

Figure 22. Change in heat capacity versus temperature in DSC scanned in A) upward (1 °C min⁻¹) and B) downwards (0.5 °C min⁻¹).

The total number subgroups in the main phase transitions was ten for both DSC scan directions. Between the upward scan (Fig. 22A) and the downward scan (Fig. 22B), the phase transition temperatures are shifted because of the differences in the kinetics of freezing and melting. Thus, the “liquidus” and the “solidus” are separated (Fig. 23A). In principle, this gap is the drug release window. Hence, this type of system could be used for partial drug release applications. The peak emerging near 45 °C represents bicelles.¹⁴⁸ This information was also used to determine the bicelle fraction of the laurdanC data (Fig. 23B).

Figure 23. A) Phase transition temperatures of Fig. 22. B) The deconvolution of $100 \cdot \partial GP / \partial T$. The red and blue fits count as bicellar and liposomal, respectively.

The WAXS measurements (see Fig. 4A in Publication IV) showed no major difference between the PEG samples. The lipid bilayers are in the gel phase at 25 °C,¹⁶⁹ in the ripple phase at 41 °C, and in the fluid phase at 50 °C.

6.3.2 Shape Transformation

Fig. 24A is a typical cryo-TEM image of the PEG3 sample. As expected from DSC, only liposomal particles (Fig. 24B) were observed, whereas bicellar particles with the face orientation (Fig. 24C) and the edge orientation (Fig. 24D) (as in Refs. 149-152) are found in the PEG24 sample (Fig. 24E). The fraction of bicelles in Fig. 24F shows a rapid increase after PEG9.

The shape transformation was also verified with MD simulations. Fig. 24G shows that liposomes are formed in PEG2.2 system. PEG10.5 system composed of bicelles (Fig. 24H), whereas PEG50 system produced slightly elongated micelles (Fig. 24I).

Figure 24. Cryo-TEM image of A) PEG3 sample containing B) liposomes, while C) bicellar faces, and D) bicellar edges are found in E) PEG24 sample. The scale bar is 200 nm. The fractions of bicelles in cryo-TEM samples is shown in F. Snapshots of G) PEG2.2, G) PEG10.5, and I) PEG50 were taken from the MD simulations after 0.5 μ s.

6.3.3 Small-Angle X-ray Scattering

The SAXS patterns are presented in Fig. 25A with their respective fits (gray lines). The particle shape and radius (Fig. 25B) matched well with the cryo-TEM results (black line). In addition, it was showed that the bilayer thickness was increased when the liposome-to-bicelle transformation took place (Fig. 25C), and that the PEG-lipids were shifted towards the bicelle edge (inset in Fig. 25B).

Figure 25. A) SAXS measurements (black dots) and their fits (gray lines) for PEG3, PEG6, PEG12, PEG15, and PEG24 samples ($k = -2, -1, 0, 1$, and 2 , respectively). Fit parameters for liposomes (dots) and bicelles (crosses): B) radius (the line is the cryo-TEM average) and C) bilayer thickness.

The intermediate q range ($q = 0.1 \dots 2 \text{ nm}^{-1}$ in Fig. 25A) signifies the response of a particle shape and size. The SAXS fits were accurate in this range. Thus, the deviations in PEG3 and PEG6 fits in $q < 0.1 \text{ nm}^{-1}$ are not significant, and the presence of spherical and discoid structures were consolidated with SAXS.

In the high q range, the fits deviate from the measurements. This is due to the lack of a background parameter in the model, not included for the sake of simplicity. This makes the fits decay faster than the experimental data. However, the fits capture the form factor oscillations, proving that the thicknesses in Fig. 25C are in the correct size range.

6.3.4 Shape-Recognition with LaurdanC

Based on cryo-TEM alone, the bicellation took place between PEG fractions 9 and 12 mol%. According to the LaurdanC measurements, this transition is quite sharp. The comparison of $x_{\text{bicelle}}^{\text{LC}}$ and $x_{\text{bicelle}}^{\text{TEM}}$ shows a discontinuity at $x_{\text{PEG}}^{\text{C}} = 0.096$ (Fig. 26A). This is the critical bicellation fraction (CBF).

LaurdanC was also utilized to determine the aspect ratios of the particles. The aspect ratio can be directly calculated from Eq. (31). In Fig. 26B, the aspect ratios from cryo-TEM correlate superbly with the laurdanC data. This data thus confirms the shape and the size of the aggregates, as the radius of the flat surface is $R_{\text{flat}} = d_{\text{HH}}(\eta_{\text{limit}} - 1)$. With η^{LC} , this radius goes to $R_{\text{flat limit}}^{\text{LC}} = 16.2 \text{ nm}$ at the complete bicellation. This is equivalent with a disc of radius $R_{\text{flat limit}}^{\text{TEM}} = 13.7 \text{ nm}$ in cryo-TEM.

Figure 26. A) $x_{\text{bicelle}}^{\text{TEM}}$ vs. x_{PEG} : the dotted lines mark the fraction where the discontinuity occurs between $x_{\text{bicelle}}^{\text{TEM}}$ and $x_{\text{bicelle}}^{\text{LC}}$. This is the CBF. B) Aspect ratio η^{TEM} vs. η^{LC} . η^{LC} values are obtained from the measured $x_{\text{bicelle}}^{\text{LC}}$ by using the Eq. (31).

Another way to view bicellation is through the curvature energies that were introduced in Chapter 2.1.1. The shape of a lipid particulate comes from the condition of minimum curvature energy or surface stress^{8, 9}

$$f = \frac{1}{2}\kappa(K_{12} - K_0)^2 + \gamma. \quad (39)$$

Together with the surface tension γ , the difference $K_1 + K_2 - K_0$ is minimized throughout the bilayer resulting to a minimum energy shape. One can therefore obtain a prediction for the shape-transformation (see *e.g.* Ref. 170).

If the spontaneous total curvature of the mixture $K_{0,\text{mix}}$ follows a simple additivity with a constant surface tension, the transition from liposomes to bicelles occurs at the concentration in which the lipid mixture can produce bicelle edges. This is the case when the spontaneous total curvature^{171, xvii}

$$K_{0,\text{mix}} = x_{\text{PEG}}K_{0,\text{DSPE-PEG}} + (1 - x_{\text{PEG}})K_{0,\text{DPPC}} \quad (40)$$

matches with the curvature of the bicelle edge ($K_{12} = 1/d_{\text{HH}}$). For $d_{\text{HH}} = 4.4(5)$, the minimum of Eq. (39) is directly at CBF, $x_{\text{PEG}} = 0.096$. The next transition of elongated micelles can occur at ca. 34 mol% ($K_{12} = 2/d_{\text{HH}}$). However, this transition is not clearly defined, because the lipids in bicelles are segregated, unlike in liposomes. Finally, the system produces spherical micelles at ca. 84 mol% ($K_{12} = 4/d_{\text{HH}}$). This is in perfect agreement with Ashok *et al.*¹⁰, who report 85 mol% at the final transition point, although their lipids, Egg PC, slightly differ from DPPC.

^{xvii} $K_{0,\text{DPPC}} = 0.136 \text{ nm}^{-1}$,¹⁷¹ and $K_{0,\text{DSPE-PEG}} = 1.06 \text{ nm}^{-1}$.¹⁷²

7. Conclusions

This compendium started by describing the key features of the liposomal drug delivery systems (DDSs) and the photothermal agents used therein to provide the reader with a fundamental understanding of the field of photothermal liposomal DDSs. This thesis sums up four featured articles, Publications I-IV, that cover a small portion of the whole topic.

The broad objectives of this work are the physicochemical aspects in liposomal DDSs with photothermal agents and the mechanisms of the light triggered contents release, in addition to some novel characterization methods. The main effect is the photothermal heating and the phase transition in the lipid bilayer, studied in Publications I, II and IV.

In Publication I, the effects of photothermal heating of spherical gold nanoparticles (GNPs) were studied with QCM. The method showed a significant increase in the relaxation time and thickness of the layer (of adsorbed liposomes) with encapsulated GNPs when compared with control samples. Thus, a clear proof-of-concept of the functionality of the light-to-heat conversive lipid systems was obtained. Unfortunately, the QCM data was not able to provide more information of the actions taking place during the illumination, although the temperature rise was later obtained via thermocouple measurement (see Fig. 10).

In Publication II, the heating effect was triggered with gold nanorods (GNRs) and indocyanine green (ICG); a novel candidate to act as a photothermal agent in liposomes. The drug release is connected with the phase transition of the lipid bilayer. Publication II provided a method to detect this phase transition while measuring the temperature of the system with two fluorescent thermometers: laurdan and CdSe quantum dots. The temperature of the GNR surface reached temperatures of ca. 65 °C, causing a phase transition in the lipid bilayer with luminous power of 3 W. Same effect was detected with ICG (3W), releasing the contents via a photothermal effect. The method described in Publication II can be used to determine the luminous power and concentration of the photothermal agent needed to release the contents. For example, ICG concentrations as low as 1 μ M can be used with light power of 3 W, as shown in Fig. 17.

In addition to photothermal effects and phase transitions, portrayed in Publications I, II and IV, a novel method to characterize liposomal carriers was introduced in Publication III. The drug-bearing liposomes must be limited to a certain size range, because many target cells sieve larger particulates out off the circulation (discussed in Chapter 2.1). The nanosized liposomes can be hard to detect due to their low contrast to the many baselines of different techniques. Especially the real-time detection with very low concentrations can be difficult. This is possible, however, with the SPRI microscopy technique. The SPRI microscope was successfully used to detect the adsorbing liposomes of the size range below 200 nm. In addition, the number of GNP-encapsulated liposomes

were determined based on the intensity and the diffraction pattern fitting with a novel analysis method. The SPRI microscopy is a promising technique that can be used to detect soft nanoparticulates in room temperature, in real time, and with no additional probes.

Throughout this thesis, we have emphasized the importance of the phase transition mechanisms as the enabling factor for drug release. For the future DDSs, it is thus important to understand the basic physical phenomena taking place on the drug-protecting boundary. A simple addition of a second component to a single component lipid bilayer can alter the drug release properties. This was shown in Publication IV, where the DPPC lipids were coupled with PEGylated DSPE-PEG(2000) lipids. The effects of PEGylation and other lipid functionalizations could be used to develop smarter DDSs by utilizing the extended drug release window and by triggering the shape transitions via polymer moieties on the bilayer. Publication IV also introduced a new method to detect shape transformations in the lipid systems. This was done with laurdanC using a regular fluorescence spectrometer.

This thesis provides a pervasive viewpoint of the physicochemical effects taking place in the photothermal lipid systems and encompasses some research tools that can be used to approach topics related to liposomes and similar nanomaterials in the future. We have dealt with the phase transition mechanisms in the lipid bilayer and connected them with drug release. We have obtained information of the encapsulated material inside the liposomes with novel approaches. We have also studied the effects of small changes in the lipid composition that can have a grand effect in the functionality of the lipid carrier that may be taken into account in the design of future drug delivery systems. Perhaps these DDSs will include lipid-polymer aggregates that could be triggered to change their shape via shifts in the spontaneous curvature, enhancing their chances of reaching a specific target, or maybe they bear more than one triggering mechanisms. One of the most interesting example of the present is to combine pH-sensitive and temperature-sensitive lipids together, and thus weaken the bilayer in the cytosol prior to the photothermal release.

The possibilities of lipid formulations are almost limitless. PEGylation is one of the standard practices at the moment, but for instance, hyaluronic acid targets the PEG moieties in blood, and prolongs the drug circulation times in the same way with less effect from the accelerated blood clearance phenomenon. Targeting ligands set another topic of research with both physicochemical and pharmaceutical interest. In order to understand how these formulations work, it is important to develop more sensitive detection and characterization methods, such as the advanced SPR methods, that account to the adsorption events of the particulates on a functional surfaces. The work towards the new DDSs is multidisciplinary. ICG is a promising photothermal agent because it is nontoxic and has already acquired an FDA status thanks to the research conducted in the field of pharmacy and clinical imaging. As a final remark of this thesis, I hope that my research has illuminated the concepts of liposomal DDSs and provided information that can be used in the development of new methods and DDSs in the future. I thank you for your interest to my work.

8. References

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Appendix A: Optical Properties of Plasmonic Nanoparticles

Lorentz Model

In the classical treatise of surface plasmons, usually referred as the Lorentz model, the plasmonic oscillation is influenced by three forces:

- 1) Hooke's law $\vec{F}_H = -k\vec{r}_e$,
- 2) Newton's second law $\vec{F}_N = m_e\vec{a}$, and
- 3) The damping force $\vec{F}_D = -m_e\gamma\vec{v}$.

Here, k is the spring constant of an oscillator, m_e is the mass of an electron, γ is the damping constant, and $\vec{r}_e$, $\vec{v}$, and $\vec{a}$ are the displacement, velocity, and acceleration of the charge. These forces combine as the driving force

$$\vec{F}_{\text{driving}} = -q_e\vec{E} = m_e\partial_t^2\vec{r}_e + m_e\gamma\partial_t\vec{r}_e + m_e\omega_0^2\vec{r}_e, \quad (\text{A2})$$

where $\omega_0^2 = k/m_e$. Since the electric polarization is also $\vec{P} = -Nq_e\vec{r}_e$ (N is the number of charges), we can write

$$\epsilon_0\omega_p^2\vec{E} = \partial_t^2\vec{P} + \gamma\partial_t\vec{P} + \omega_0^2\vec{P}, \quad (\text{A3})$$

where $\omega_p^2 = Nq_e^2/m_e\epsilon_0$ is the plasma frequency. With sinusoidal field terms

$$\vec{P} = \vec{p}(\omega)e^{i\omega t} \text{ and } \vec{E} = \vec{e}(\omega)e^{i\omega t}, \quad (\text{A4})$$

the polarization gets a familiar form

$$\vec{P} = \frac{\omega_p^2}{\omega_0^2 - \omega^2 + i\gamma\omega} \epsilon_0\vec{E} = \epsilon_0\chi_e\vec{E}. \quad (\text{A5})$$

Here, χ_e is the electric susceptibility that can be also expressed with the relative permittivity ϵ_r as

$$\chi_e = \epsilon_r - 1 = \frac{\omega_p^2}{\omega_0^2 - \omega^2 + i\gamma\omega}. \quad (\text{A6})$$

In the case of a conductor, such as gold, $\omega_0 = 0$ and we obtain so-called Drude solution.

Excitation of Nanoparticles

When the diameter of a particle ($2R$) is smaller than the wavelength of light λ , the collision frequency is reduced:⁸⁷⁻⁹⁰

$$\Gamma(R) = \gamma - a\frac{v_f}{R}. \quad (\text{A7})$$

Here, a is a geometrical factor, usually close to unity, and v_f is the Fermi velocity ($1.395 \cdot 10^6$ m/s). This can be taken into account by correcting the relative permittivity of the bulk as follows:⁹⁰

$$\epsilon_r = \epsilon_{r,\text{bulk}} + \frac{\omega_p^2}{\omega^2 - i\Gamma(R)\omega} - \frac{\omega_p^2}{\omega^2 - i\gamma\omega}. \quad (\text{A8})$$

Here, the relative permittivity is usually given relative to solvent ($\epsilon_r = (\epsilon_1 + i\epsilon_2)/\epsilon_m$). The geometry of the particle plays a role as well. The internal field inside the nanoparticle is of the form¹⁷³

$$\vec{E}_p = \vec{E} + \vec{E}_d = \vec{E} - \frac{1}{\epsilon_m} \sum_j^N \vec{L}_j \vec{p}_j = \vec{E} - \frac{1}{\epsilon_m} \vec{L} \vec{P}, \quad (\text{A9})$$

where $\vec{E}$ is the applied field and $\vec{E}_d$ is the depolarization field, $\vec{L}_j$ is the diagonal depolarization matrix and $\vec{p}_j$ is the polarization of the j^{th} dipole, with $\vec{L}$ as the average depolarization matrix. If we now combine Eq. (A9) with the polarization of the nanoparticle in an isotropic host medium ($\vec{P} = \epsilon_m \vec{\chi}_e \vec{E}_p$), we obtain

$$\vec{P} = \epsilon_m \vec{\chi}_e (\vec{I} + \vec{\chi}_e \vec{L})^{-1} \vec{E}, \quad (\text{A10})$$

which gives us the permittivity matrix of the particle

$$\vec{\epsilon}_p = \epsilon_m (\vec{I} + \vec{\chi}_e (\vec{I} + \vec{\chi}_e \vec{L})^{-1}). \quad (\text{A11})$$

In terms of optical properties, the excitation coefficient of the system, containing averagely oriented small particles, is given by summing the imaginary parts of the permittivity values together⁸⁸

$$\sigma_{\text{ext}} = \frac{2\pi}{3\lambda\sqrt{\epsilon_m}} \Im\{\text{tr}(\vec{\epsilon}_p)\}. \quad (\text{A12})$$

In the case of gold nanosphere, the depolarization factor is $\vec{L} = \frac{1}{3}\vec{I}$, following the rule

$$L_x + L_y + L_z = 1, \quad (\text{A13})$$

and the dielectric constant is

$$\begin{aligned} \vec{\epsilon}_p &= \epsilon_m \left(1 + \frac{3\chi_e}{3+\chi_e}\right) \vec{I} = \epsilon_m \left(1 + \frac{3(\epsilon_1+i\epsilon_2-\epsilon_m)}{3\epsilon_m+\epsilon_1+i\epsilon_2-\epsilon_m}\right) \vec{I} \\ \Leftrightarrow \Im\{\text{tr}(\vec{\epsilon}_p)\} &= \frac{27\epsilon_2\epsilon_m^2}{(2\epsilon_m+\epsilon_1)^2+\epsilon_2^2}. \end{aligned} \quad (\text{A14})$$

Combining this result with the Eq. (A12) gives us the excitation coefficient

$$\sigma_{\text{ext}} = \frac{18\pi}{\lambda} \frac{\epsilon_2\epsilon_m^{3/2}}{(2\epsilon_m+\epsilon_1)^2+\epsilon_2^2}. \quad (\text{A15})$$

Similarly, nanorods can be regarded as elliptic particles that follow prolate ($r_x > r_y = r_z$) geometry. The depolarization in the x axis is given by

$$\begin{aligned} L_1 &= \frac{r_x r_y r_z}{2} \int_0^\infty (r_x + q)^{-\frac{1}{2}} \{(r_x + q)(r_y + q)(r_z + q)\}^{-\frac{1}{2}} = \\ &= \frac{1-e^2}{e^2} \left\{ \frac{1}{2e} \ln \frac{1+e}{1-e} - 1 \right\}, \text{ where } e^2 = 1 - \left(\frac{r_y}{r_x}\right)^2. \end{aligned} \quad (\text{A16})$$

The shorter axis depolarization is given by Eq. (A13), and

$$\Im\{\text{tr}(\vec{\epsilon}_p)\} = \frac{1}{L_1^2} \frac{\epsilon_{2,1}\epsilon_m^2}{\left(\epsilon_{1,1} + \frac{1-L_1}{L_1}\epsilon_m\right)^2 + \epsilon_{2,1}^2} + \frac{2}{L_2^2} \frac{\epsilon_{2,2}\epsilon_m^2}{\left(\epsilon_{1,2} + \frac{1-L_2}{L_2}\epsilon_m\right)^2 + \epsilon_{2,2}^2}. \quad (\text{A17})$$

Or in the other words,

$$\sigma_{\text{ext}} = \frac{2\pi}{3\lambda} \epsilon_m^{3/2} \sum_j^3 \frac{\epsilon_{r2,j}}{L_j^2} \left[\left(\epsilon_{r1,j} + \frac{1-L_j}{L_j} \epsilon_m \right)^2 + \epsilon_{r2,j}^2 \right]^{-1}, \quad (\text{A18})$$

which is the well-known Mie-Gans result for a spherical particle.

Appendix B: SPR modelling in Matlab

This function was written by Lauri Viitala in USA during 11/2015-12/2015. The matlab code is based on Mathematica script in Aaron Halpern's dissertation. The theoretical basis is defined in Ghatak and Thyagarajan¹⁴⁵, and discussed in Chapter 5.2. Fig. 18C is one of the outputs of this function.^{xviii}

```
function main()
close all; clear all; clc
im = sqrt(-1);
% Initial refractive indexes
n10 = 1.515;
n20 = 0.1726 - 3.422*im;
n30 = 1.45;
n40 = 1;
% Initial thicknesses [m]
d20 = 45*1e-9;
d30 = 1.5*1e-9;
d40 = 1*1e-9;
% Initial wavelength [m]
lambda0 = 632.8*1e-9;
% Initial limits of incident angles (degrees)
theta0 = [39 55];

h_info_text = uicontrol('FontSize',12.0,'Position',[0 300 500 90],'style','text','string',{'This function is based on a mathematica code that can be found in the attachments of Aaron Halpern's dissertation. The original calculations are from Ghatak, A.K.; Thyagarajan, K.; Optical Electronics; Cambridge university Press: Cambridge, 1989.});
h_n1_text = uicontrol('FontSize',12.0,'Position',[0 270 125 30],'style','text','string',{'n1 (prism)'});
h_n1 = uicontrol('FontSize',12.0,'Position',[0 240 125 30],'style','edit','string,num2str(n10),'callback',@changes);
h_n2_text = uicontrol('FontSize',12.0,'Position',[125 270 125 30],'style','text','string',{'n2 (gold)'});
h_n2 = uicontrol('FontSize',12.0,'Position',[125 240 125 30],'style','edit','string,num2str(n20),'callback',@changes);
h_n3_text = uicontrol('FontSize',12.0,'Position',[250 270 125 30],'style','text','string',{'n3 (film)'});
h_n3 = uicontrol('FontSize',12.0,'Position',[250 240 125 30],'style','edit','string,num2str(n30),'callback',@changes);
h_n4_text = uicontrol('FontSize',12.0,'Position',[375 270 125 30],'style','text','string',{'n4 (water)'});
h_n4 = uicontrol('FontSize',12.0,'Position',[375 240 125 30],'style','edit','string,num2str(n40),'callback',@changes);
h_n5_text = uicontrol('FontSize',12.0,'Position',[500 270 125 30],'style','text','string',{'n5 (exterior)'});
h_n5 = uicontrol('FontSize',12.0,'Position',[500 240 125 30],'style','edit','string,num2str(n50),'callback',@changes);
h_d2_text = uicontrol('FontSize',12.0,'Position',[125 180 125 30],'style','text','string',{'d2'});
h_d2 = uicontrol('FontSize',12.0,'Position',[125 150 125 30],'style','edit','string,num2str(d20),'callback',@changes);
h_d3_text = uicontrol('FontSize',12.0,'Position',[250 180 125 30],'style','text','string',{'d3'});
h_d3 = uicontrol('FontSize',12.0,'Position',[250 150 125 30],'style','edit','string,num2str(d30),'callback',@changes);
h_d4_text = uicontrol('FontSize',12.0,'Position',[375 180 125 30],'style','text','string',{'d4'});
h_d4 = uicontrol('FontSize',12.0,'Position',[375 150 125 30],'style','edit','string,num2str(d40),'callback',@changes);
h_lambda_text = uicontrol('FontSize',12.0,'Position',[125 120 125 30],'style','text','string',{'lambda'});
h_lambda = uicontrol('FontSize',12.0,'Position',[125 90 125 30],'style','edit','string,num2str(lambda0),'callback',@changes);
h_theta_text = uicontrol('FontSize',12.0,'Position',[250 120 125 30],'style','text','string',{'theta'});
h_theta = uicontrol('FontSize',12.0,'Position',[250 90 125 30],'style','edit','string,num2str(theta0),'callback',@changes);

function draw(n,d,lambda,theta)
np = [n(1:2) 1 1]; % pure sensor
kw = 2*pi(i)/lambda; % wavevec
kwp = 2*pi(i)/lambda; % wavevec for pure sensor
for k = 1:length(theta);
% sensor with a film
p = n(1)/n1;
cos2 = (sqrt(1-(p*sin(theta(k)))^2));
cos2 = cos2 - 2*(imag(cos2)-0); %cos2;% imag(cos2) < 0 due to n = n - k*i formalism!
% pure sensor
np = n(1)/np;
cos2p = (sqrt(1-(np*sin(theta(k)))^2));
cos2p = cos2p - 2*(imag(cos2p)-0); %cos2p;% imag(cos2) < 0 due to n = n - k*i formalism!
rp = [1;res1];
for j = 1:3
% sensor with a film
rp(j) = (n(j)*cos2(j)-n(j-1)*cos2(j))/((n(j)*cos2(j)+n(j-1)*cos2(j)));
rs(j) = (n(j)*cos2(j)-n(j-1)*cos2(j-1))/((n(j)*cos2(j)+n(j-1)*cos2(j-1)));
% pure sensor
rpp(j) = (np*cos2p(j)-np(j-1)*cos2p(j-1))/(np(j)*cos2p(j)+np(j-1)*cos2p(j));
rsp(j) = (np(j)*cos2p(j)-np(j-1)*cos2p(j-1))/(np(j)*cos2p(j)+np(j-1)*cos2p(j-1));
end
% sensor with a film
a2 = exp(2*im*cos2(2)*d(1)*kw(2));
b2 = exp(2*im*cos2(2)*d(2)*kw(2));
fp = ((a2*b2*rp(1)+b2*rp(2)+rp(3)+a2*rp(1)*rp(2)+rp(3))/(a2*b2+b2*rp(1)*rp(2)+rp(3)+a2*rp(2)*rp(3)));
fs = ((a2*b2*rp(1)+b2*rp(2)+rp(3)+a2*rp(1)*rp(2)+rp(3))/(a2*b2+b2*rp(1)*rp(2)+rp(3)+a2*rp(2)*rp(3)));
Ref4(k,:) = [abs(fp)^2 abs(fs)^2];
Phase4(k,:) = [2*atan(imag(fp)/(sqrt(imag(fp)+2*real(fp)+2*real(fp)))/sqrt(imag(fp)+2*real(fp)+2*real(fp)))];% Aro(fp)
Phase4(k,:) = Phase4(k,:)-[Phase4(k,:)-2]*2*pi(i);% 2pi phase jump
% pure sensor
a2 = exp(2*im*cos2p(2)*d(1)*kwp(2));
b2 = exp(2*im*cos2p(2)*d(2)*kwp(2));
fp = ((a2*b2*rpp(1)+b2*rpp(2)+rpp(3)+a2*rpp(1)*rpp(2)+rpp(3))/(a2*b2+b2*rpp(1)*rpp(2)+rpp(3)+a2*rpp(2)*rpp(3)));
fs = ((a2*b2*rpp(1)+b2*rpp(2)+rpp(3)+a2*rpp(1)*rpp(2)+rpp(3))/(a2*b2+b2*rpp(1)*rpp(2)+rpp(3)+a2*rpp(2)*rpp(3)));
Ref4p(k,:) = [abs(fp)^2 abs(fs)^2];
Phase4p(k,:) = [2*atan(imag(fp)/(sqrt(imag(fp)+2*real(fp)+2*real(fp)))/sqrt(imag(fp)+2*real(fp)+2*real(fp)))];% Arg(fp)
dR(k,:) = abs(Ref4(k,:)-Ref4p(k,:)); %deltar
dphi(k,:) = abs(Phase4(k,:)-Phase4p(k,:)); % phase shift deltaphi
end
figure(2) % Plot refractions and refraction separation deltar
[ax,p1,p2] = ploty(theta*180/pi(0):theta*180/pi(1),[Ref4(:,1) Ref4p(:,1)],theta*180/pi(0),dR(:,1));
xlim([theta(1)*180/pi(0) theta(end)*180/pi(1)])
ylim([floor(min(dR(:,1))) ceil(max(dR(:,1)))/10]);
xlabel('angle','FontSize',30)
ylabel(ax(2),'Reflectivity','FontSize',30) % left y-axis
ylabel(ax(2),'deltar','FontSize',30,'color','k') % right y-axis
set(ax(1),'FontSize',24,'PlotBoxAspectRatio',[1 1 1],'ylim',[0 1],'ytick',[0:0.1:1])
set(ax(2),'ycolor','k','FontSize',24,'PlotBoxAspectRatio',[1 1 1],'ylim',[1m2,'ytick',1:m2(2)-1:m2(1)]/(2*ceil(10*max(dR(:,1)))/10):1:m2(2))
p1(1).Linewidth = 2; p1(2).Linewidth = 2; p2.Linewidth = 2;
p1(2).Linewidth = 2; p2.Linewidth = 2;
p2.Color = 'black';
figure(3) % Plot phase and phase separation deltaphi
[ax,p1,p2] = ploty(theta*180/pi(0):theta*180/pi(1),[Phase4(:,1) Phase4p(:,1)],theta*180/pi(0),dphi(:,1));
xlim([theta(1)*180/pi(0) theta(end)*180/pi(1)])
ylim([floor(min(Phase4(:,1))) ceil(max(Phase4(:,1)))]);
LIM1 = [floor(min(dphi(:,1))) ceil(max(dphi(:,1)))]);
LIM2 = [floor(min(dphi(:,1))) ceil(max(dphi(:,1)))]);
xlabel('angle','FontSize',30)
ylabel(ax(1),'Phase','FontSize',30) % left y-axis
ylabel(ax(2),'deltaphi','FontSize',30,'color','k') % right y-axis
set(ax(1),'FontSize',24,'PlotBoxAspectRatio',[1 1 1],'ylim',[1m1,'ytick',1:m1(2)-1:m1(1)]);
set(ax(2),'ycolor','k','FontSize',24,'PlotBoxAspectRatio',[1 1 1],'ylim',[1m2,'ytick',1:m2(2)-1:m2(1)]/(2*ceil(max(dphi(:,1)))/10):1:m2(2))
p1(1).Linewidth = 2; p1(2).Linewidth = 2; p2.Linewidth = 2;
p2.Linewidth = 2;
p2.Color = 'k';
end
function changes(hObject,eventdata)
if ~isempty(hObject)
str = get(hObject,'String'); set(h_n1,'String',str);
elseif (hobject == h_n2)
str = get(hObject,'String'); set(h_n2,'String',str);
elseif (hobject == h_n3)
str = get(hObject,'String'); set(h_n3,'String',str);
elseif (hobject == h_n4)
str = get(hObject,'String'); set(h_n4,'String',str);
elseif (hobject == h_d2)
str = get(hObject,'String'); set(h_d2,'String',str);
elseif (hobject == h_d3)
str = get(hObject,'String'); set(h_d3,'String',str);
elseif (hobject == h_lambda)
str = get(hObject,'String'); set(h_lambda,'String',str);
elseif (hobject == h_theta)
str = get(hObject,'String'); set(h_theta,'String',str);
end
n1 = str2num(get(h_n1,'String'));
n2 = str2num(get(h_n2,'String'));
n3 = str2num(get(h_n3,'String'));
n4 = str2num(get(h_n4,'String'));
d2 = str2num(get(h_d2,'String'));
d3 = str2num(get(h_d3,'String'));
lambda = str2num(get(h_lambda,'String'));
theta = str2num(get(h_theta,'String'));
theta = (theta(0):0.01:theta(2))*pi(0)/180;
n = [n1 n2 n3 n4]; d = [d2 d3];
draw(n,d,lambda,theta)
end
end
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```

^{xviii} This code is provided mainly for the readers of the electric version. Hence the small font in the code.

Appendix C: Bicelle-Liposome SAXS model

This function was written by Lauri Viitala in Finland in 9/2018. The Matlab code is based on the theory presented in Publication IV. This implementation provides a user interface (Fig. C1) that is used to fit SAXS data of systems containing liposomes and/or bicelles. It was used in Publication IV to fit DPPC:DSPE-PEG(2000) systems.^{xix}

Figure C1. The user interface.

```

function main()
{
    local paramset = all(ric::set(0,'defaultControlFontName','Georgia','defaultAxesFontName','Georgia','defaultTextFontName','Georgia');
    local parameters = data_set Fig p filename data D K background;
    % external parameters
    D = 0;K=constant that is subtracted from the measured data
    C = 10/1000's concentration
    fig = 0;parameters.set = [data,set = []];
    D = 0;% index of the plot in a data file (Default: 1)
    D = ones(24,1);% keeps track on which parameters are in use. note: the length is 24, but only 22 are used in this code.
    File name = userfile('Fig','%d','%s').image,'MultiSelect','on'; % Selecting an image
    run = cdf(ruta);filename = 'fig_parameters.mat';
    if(iscell(Filename,{c}));cellmat=num2cell(File);file = G:endp = length(File);
    h_general = uicontrol('FontSize',12.0,'Position',[0 330 100 30],'Style','pushbutton','String','Next sample','Callback,@next');
    h_next = uicontrol('FontSize',12.0,'Position',[0 330 100 30],'Style','pushbutton','String','Fit','Callback,@fitter');
    h_xpeg_text = uicontrol('FontSize',12.0,'Position',[0 300 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>x</i>-sub-PEG','Callback',@xpeg);
    h_xbic_text = uicontrol('FontSize',12.0,'Position',[0 270 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>b</i>-sub-bicelles','Callback',@xbict);
    h_xbicelles = uicontrol('FontSize',12.0,'Position',[0 270 80 30],'Style','edit','String,num2str(0.6),'Callback',@bicelle);
    h_sol_text = uicontrol('FontSize',12.0,'Position',[0 240 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>s</i>-sub-core</i>-sub-PEG</i>-sub-solv</i>-sub-PEG','Callback',@hsolve);
    h_solve_text = uicontrol('FontSize',12.0,'Position',[0 240 80 30],'Style','edit','String,num2str(9.47),'Callback',@hsolve);
    h_PEG_text = uicontrol('FontSize',14.0,'Position',[0 210 100 30],'Style','text','String','PEG',@di);
    h_di_text = uicontrol('FontSize',12.0,'Position',[0 210 80 30],'Style','edit','String,num2str(0.25),'Callback',@di);
    h_fit_text = uicontrol('FontSize',14.0,'Position',[0 135 100 60],'Style','text','String','Fit region:');
    h_region_text = uicontrol('FontSize',12.0,'Position',[0 135 80 60],'Style','edit','String,num2str(1),'Callback',@region1);
    h_region2_text = uicontrol('FontSize',12.0,'Position',[0 150 100 60],'Style','edit','String,num2str(1),'Callback',@region2);
    % bicelle
    h_core_shell_bicelle = uicontrol('FontSize',14.0,'Position',[200 330 200 30],'Style','text','String','Core shell bicelle:');
    h_core_text = uicontrol('FontSize',12.0,'Position',[200 300 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>c</i>-sub-core','Callback',@dcore);
    h_shell_text = uicontrol('FontSize',12.0,'Position',[200 270 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>s</i>-sub-core','Callback',@dshe);
    h_c_bic_text = uicontrol('FontSize',12.0,'Position',[200 270 100 30],'Style','edit','String,num2str(4.5),'Callback',@drim);
    h_drim_text = uicontrol('FontSize',12.0,'Position',[200 240 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>d</i>-sub-face','Callback',@dfacet);
    h_dfacet_text = uicontrol('FontSize',12.0,'Position',[200 210 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>d</i>-sub-rim','Callback',@drim);
    h_rim_text = uicontrol('FontSize',12.0,'Position',[200 210 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>r</i>-sub-core','Callback',@dcoret);
    h_core_text = uicontrol('FontSize',12.0,'Position',[200 180 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>c</i>-sub-face','Callback',@dfacet);
    h_core_text = uicontrol('FontSize',12.0,'Position',[200 180 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>c</i>-sub-face','Callback',@dfacet);
    h_bicellface = uicontrol('FontSize',12.0,'Position',[200 150 100 30],'Style','edit','String,num2str(12.0),'Callback',@oface);
    h_oface_text = uicontrol('FontSize',12.0,'Position',[200 150 80 30],'Style','edit','String,num2str(10.4),'Callback',@rim);
    % lipid
    h_liposome_PEG = uicontrol('FontSize',14.0,'Position',[200 90 200 30],'Style','text','String','Liposome PEG:');
    h_x_rim_text = uicontrol('FontSize',12.0,'Position',[200 60 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>x</i>-sub-rim','Callback',@xrimit);
    h_rim_text = uicontrol('FontSize',12.0,'Position',[200 60 100 30],'Style','edit','String,num2str(0.20),'Callback',@drim);
    h_G_L1_text = uicontrol('FontSize',12.0,'Position',[200 30 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>l</i>-sub-G','Callback',@lback);
    h_L1_text = uicontrol('FontSize',12.0,'Position',[200 30 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>l</i>-sub-PEG','Callback',@lback);
    h_PEG1_text = uicontrol('FontSize',12.0,'Position',[200 0 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>p</i>-sub-PEG','Callback',@PEGB1);
    h_PEG2_text = uicontrol('FontSize',12.0,'Position',[200 0 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>p</i>-sub-PEG','Callback',@PEGB2);
    % parameter 20 is unused
    h_o_text = uicontrol('FontSize',12.0,'Position',[200 0 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>o</i>-sub-head','Callback',@ohot);
    h_o_text = uicontrol('FontSize',12.0,'Position',[200 0 100 30],'Style','edit','String,num2str(0),'Callback',@ho);
    set(h_o_text,'visible','off');set(h_o,'visible','off');D(20) = 0;
    % liposome
    h_core_shell_liposome = uicontrol('FontSize',14.0,'Position',[400 330 200 30],'Style','text','String','Core-shell liposome:');
    h_core_text = uicontrol('FontSize',12.0,'Position',[400 300 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>c</i>-sub-tail','Callback',@dtailt);
    h_shell_text = uicontrol('FontSize',12.0,'Position',[400 270 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>s</i>-sub-core','Callback',@dlipot);
    h_t_tail_text = uicontrol('FontSize',12.0,'Position',[400 240 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>t</i>-sub-head,in','Callback',@tint);
    h_t_in_text = uicontrol('FontSize',12.0,'Position',[400 210 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>t</i>-sub-head,out','Callback',@tint);
    h_h_out_text = uicontrol('FontSize',12.0,'Position',[500 210 100 30],'Style','edit','String,num2str(9.7),'Callback',@tout);
    h_tout_text = uicontrol('FontSize',12.0,'Position',[500 180 100 30],'Style','edit','String,num2str(7.2),'Callback',@tailt);
    h_lipidtail = uicontrol('FontSize',12.0,'Position',[500 150 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>l</i>-sub-head','Callback',@thead);
    h_head_text = uicontrol('FontSize',12.0,'Position',[500 150 80 30],'Style','edit','String,num2str(10.4),'Callback',@thead);
    % Liposome PEG
    h_x_out_text = uicontrol('FontSize',12.0,'Position',[400 60 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>x</i>-sub-out','Callback',@xout);
    h_out_text = uicontrol('FontSize',12.0,'Position',[400 60 100 30],'Style','edit','String,num2str(0.2),'Callback',@drack);
    h_r_g2_text = uicontrol('FontSize',12.0,'Position',[400 30 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>r</i>-sub-PEG','Callback',@RG1);
    h_RG2_text = uicontrol('FontSize',12.0,'Position',[400 30 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>r</i>-sub-PEG','Callback',@RG1);
    h_PEG2_text = uicontrol('FontSize',12.0,'Position',[400 0 100 30],'Style','edit','String,num2str(17.5),'Callback',@RG1);
    h_PEG2_text = uicontrol('FontSize',12.0,'Position',[400 0 100 30],'Style','edit','String,num2str(10.8),'Callback',@OPEG1);
    % parameter 24 is unused
    h_o_o_text = uicontrol('FontSize',12.0,'Position',[400 30 100 30],'Style','pushbutton','Backgroundcolor','w','String','<HTML><i>o</i>-sub-head','Callback',@ohead);
    h_o_text = uicontrol('FontSize',12.0,'Position',[400 30 100 30],'Style','edit','String,num2str(0),'Callback',@oo);
    set(h_o_text,'visible','off');set(h_o,'visible','off');D(24) = 0;
    % units
    h_units = uicontrol('FontSize',14.0,'Position',[600 330 100 30],'Style','text','String','Units:');uicontrol('FontSize',12.0,'Position',[600 300 100 30],'Style','pushbutton','Backgroun-color','w','String','A:');uicontrol('FontSize',12.0,'Position',[600 270 100 30],'Style','pushbutton','Backgroundcolor','w','String','B:');uicontrol('FontSize',12.0,'Position',[600 240 100 30],'Style','pushbutton','Backgroundcolor','w','String','C:');uicontrol('FontSize',12.0,'Position',[600 210 100 30],'Style','pushbutton','Backgroundcolor','w','String','D:');uicontrol('FontSize',12.0,'Position',[600 180 100 30],'Style','pushbutton','Backgroundcolor','w','String','E:');uicontrol('FontSize',12.0,'Position',[600 150 100 30],'Style','pushbutton','Backgroundcolor','w','String','F:');uicontrol('FontSize',12.0,'Position',[600 120 100 30],'Style','pushbutton','Backgroundcolor','w','String','G:');uicontrol('FontSize',12.0,'Position',[600 90 100 30],'Style','pushbutton','Backgroundcolor','w','String','H:');uicontrol('FontSize',12.0,'Position',[600 60 100 30],'Style','pushbutton','Backgroundcolor','w','String','I:');uicontrol('FontSize',12.0,'Position',[600 30 100 30],'Style','pushbutton','Backgroundcolor','w','String','J:');uicontrol('FontSize',12.0,'Position',[600 0 100 30],'Style','pushbutton','Backgroundcolor','w','String','K:');uicontrol('FontSize',12.0,'Position',[600 0 100 30],'Style','pushbutton','Backgroundcolor','w','String','L:');uicontrol('FontSize',12.0,'Position',[600 0 100 30],'Style','pushbutton','Backgroundcolor','w','String','M:');uicontrol('FontSize',12.0,'Position',[600 0 100 30],'Style','pushbutton','Backgroundcolor','w','String','N:');uicontrol('FontSize',12.0,'Position',[600 0 100 30],'Style','pushbutton','Backgroundcolor','w','String','O:');uicontrol('FontSize',12.0,'Position',[600 0 100 30],'Style','pushbutton','Backgroundcolor','w','String','P:');uicontrol('FontSize',12.0,'Position',[600 0 100 30],'Style','pushbutton','Backgroundcolor','w','String','Q:');uicontrol('FontSize',12.0,'Position',[600 0 100 30],'Style','pushbutton','Backgroundcolor','w','String','R:');uicontrol('FontSize',12.0,'Position',[600 0 
```

^{xix} This code is provided mainly for the readers of the electric version. Hence the small font in the code.

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Errata

Publication I

Page 21397: The light output power was ca. 2.8 W cm^{-2} instead of 0.7 W cm^{-2} .

Publication III

Page 25959: The vector $\vec{r}_n = \vec{r} - \vec{r}_n$ in Eq. (5) should be $\vec{r}_n = \vec{r} - \vec{r}_n$, where $\vec{r}_n$ is the adsorption site of the n^{th} dipole. Similarly, Eqs. (6), (7), (8), (12), and (14) should be dependent on the variable r instead of r , and Eqs. (11) and (15) should have $x = |x - x_0|$ and $y = |y - y_0|$ instead of $x = |x - x_0|$ and $y = |y - y_0|$

Page 25960: The buffer solution contained 137 mM of NaCl instead of 13.7 mM.

Page 25963: Eq. (25) should read $f_x = \lim_{y \rightarrow 0} \text{Re} \left\{ -iH_0^{(2)}(\tilde{a}\sqrt{x^2 + y^2}(-i\tilde{a}e^{-i\tilde{a}x})^*) \right\}$ without the extra s.

Page 25964: Figure 5 should contain the variable n_c instead of n .

Page 25965: Figure 6 should contain the variable n_c instead of n .

Photothermal liposomal drug delivery systems (DDSs) contain two key components. The first is the lipid drug carrier, usually liposome. The second is the photothermal agent that converts light into heat. As the heat transports to the surrounding lipid bilayer, the lipids undergo a phase transition that increases the permeability of the membrane. The contents are thus released.

This thesis examines the general physical chemistry of photothermal liposomes. The scopes of this work can be divided in three parts. First, the photothermal effect is studied in connection with the phase behavior of the lipid aggregates. Second, novel methods to characterize photothermal liposomes are examined. For instance, an SPRI microscope is used to determine the encapsulation efficiency. Third, the effects of lipid formulations are examined. In particular, PEGylation and the resulting extended phase transition regions and shape transitions are being discussed as potential phenomena for new DDSs.

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