Charles University

Faculty of Pharmacy in Hradec Králové

Department of Pharmaceutical Chemistry and Pharmaceutical Analysis

Diploma Thesis

Stability evaluation of magnesium complexes of phthalocyanines and azaphthalocyanines under acidic conditions

Supervisor: Assoc. Prof. Petr Zimčík, Ph.D

Hradec Králové, 2018 **Anita Mulaku**

This is to declare that this diploma thesis in my own work. I have cited all the appropriate literature sources on the reference list.

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The work was supported by Charles University SVV 260 401.

ABSTRACT

Charles University, Faculty of Pharmacy in Hradec Králové

Department of Pharmaceutical Chemistry and Pharmaceutical Analysis

Candidate: Anita Mulaku

Supervisor: Assoc. Prof. Petr Zimčík, Ph.D.

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Phthalocyanines are categorized as analogues of porphyrin where the structures is based on four isoindoline units condensed via a nitrogen atom. Alongside subphthalocyanines, these compounds are attracting attention as useful dyes that find application in photodynamic therapy, organic electronic devices, organic solar cells and other areas. Phthalocyanines are capable of forming complexes with different types of metal and can be used in fluorescence probing due to their suitable absorption and emission in the red region of the visible spectrum. Red or near-infrared excitation and emission is important for biological applications since longer wavelength light penetrates deeper into tissues, it is less scattered and the autofluorescence of endogenous chromophores is limited. The photophysical and photochemical properties of phthalocyanines and their analogues depend highly on the central metal and peripheral substitution. The

magnesium complexes are highly suitable for the diagnostic purposes as they emit strongly fluorescence and are characterized by rather low singlet oxygen quantum yields.

In my thesis, we have conducted numerous experiments to determine the stability of four magnesium phthalocyanines in water, organic solvent (tetrahydrofuran), liposomes, microemulsions and silica nanoparticles. The transition of a metal complex to a metal -free ligand can be observed in the absorption spectra by the decrease of the Q-band followed by its splitting. From our experimental data, we may conclude that phthalocyanine complexes of magnesium with bulky substituents serve as a better protection of the macrocyclic core from demetallation compared to non-bulky substituents. In the organic solvents, protonation of the phthalocyanine complex occurs first and afterwards it is followed by the formation of a metal-free complex unlike in water where the demetallation occurs directly without presence of the detectable protonated form. In the case of delivery systems, we have observed that liposomes serve as a better protection system from the acidity of the surrounding system compared with the microemulsions. In the case of silica nanoparticles, we have observed a decrease of the emission spectra that is independent of the pH value. The results of this experimental project can serve as a valuable foundation for future studies.

ABSTRAKT

Univerzita Karlova, Farmaceutická fakulta v Hradci Králové

Katedra farmaceutické chemie a farmaceutické analýzy

Kandidát: Anita Mulaku

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Název diplomové práce: Hodnocení stability hořečnatých komplexů ftalocyaninů a azaftalocyaninů v kyselém prostředí

Ftalocyaniny patří mezi látky odvozené od porfyrinů, u kterých je struktura založena na čtyřech isoindolinových jednotkách spojených přes dusíkový můstek. Podobně jako subftalocyaniny, tyto látky získávají v poslední době výraznou pozornost jako fotosensitizéry ve fotodynamické terapii, organické elektronické přístroje, solární články, a jiné. Ftalocyaniny jsou schopné tvořit komplexy s celou řadou kationtů kovů a lze je využít také jako fluorescenční sondy vzhledem k jejich vhodné absorpci a emisi světla v červené oblasti spektra. Právě excitace a emise v červené a blízké infračervené oblasti je důležité pro biologickéaplikace, vzhledem k tomu, že světlo těchto vlnových délek proniká hlouběji do tkání, je méně rozptylováno a fluorescence endogenních chromoforů je potlačena. Fotofyzikální a fotochemické vlastnosti ftalocyaninů silně závisí na centrálním kationtu a na periferní substituci. Z hlediska fluorescence jsou hořečnaté komplexy nejvhodnější pro diagnostické účely, protože silně emitují fluorescenci a produkce singletového kyslíku je potlačena.

Ve své práci jsem se zaměřila na určení stability čtyř hořečnatých komplexů ve vodě, v organickém rozpouštědle (tetrahydrofuranu), liposomech, mikroemulzi a nanočásticích. Změnu a z hořečnatého komplexu na bezkovový derivát lze pozorovat v absorpčních spektrech jako pokles intensity Q-pásu s jeho následným rozštěpením. Z experimentálních dat vyplynulo, že v případě substituce ftalocyaninů objemnými substituenty lze zvýšit stabilitu v kyselém prostředí. V organickém rozpouštědle dochází nejprve k protonaci ftalocyaninového makrocyklu, která je následována odkovením. Toto je v kontrastu s chováním ve vodě, kde k odkovení dochází přímo bez detekovatelné přítomnosti protonované formy. V přítomnosti „delivery" systémů lze konstatovat, že liposomy chrání hořečnaté komplexy před kyselým prostředím daleko lépe než mikroemulze. V případě křemičitých nanočástic jsme pozorovali snížení fluorescenční intenzity bez závislosti na pH prostředí. Výsledky těchto experimentů mohou sloužit jako cenný základ pro další studie.

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ACKNOWLEDGMENT

I would like to thank my supervisor Assoc. Prof. Petr Zimčík, Ph.D. for his extensive help, guidance and patience during the completion of this diploma thesis.

ABBREVIATIONS

AlPc	Aluminium phthalocyanine
AzaPc	Azaphthalocyanine
CDDs	Charge-coupled device detectors
DOPC	Dioleoylphosphatidylcholine
ICT	Intramolecular charge transfer
LUVETs	Large unilamellar vesicles
MLVs	Multilamellar vesicels
MRI	Magnetic resonance imaging
Nc	Naphthalocyanine
PDT	Photodynamic therapy
PET	Photoinduced electron transfer
TFA	Trifluoroacetic acid
TFEO-Pc	Trifluoroethoxy-substituted phthalocyanine
THF	Tetrahydrofuran
TPyzPzs	Tetrapyrazionoporphyrazines
ZnPc	Zinc phthalocyanine

AIM OF RESEARCH

The azaphthalocyanine group within the department of Pharmaceutical Chemistry and Pharmaceutical Analysis is conducting research of the different complexes of phthalocyanines and their applications in different areas. However, the application of phthalocyanines is limited due to their tendency to demetallate in acidic environment. Hence, we decided to perform several experiments to study the stability of magnesium phthalocyanine complexes in water and organic solvent and in three different delivery systems (liposomes, microemulsions and nanoparticles).

INTRODUCTION

FLUORESCENCE AND ITS APPLICATIONS

Fluorescence is an important technology used in different areas of research such as genetic analysis, biotechnology, flow cytometry and/or sequencing medical diagnostics.[1] Research from twenty years ago have proven that fluorescence spectroscopy can be used for the assessment of waste water quality as a tool for the detection of discharge in natural water systems and for process control in waste water treatment plants. This technique has been proposed because it is cheap, fast, reagentless, requires little sample preparation, is highly sensitive and non-invasive. Fluorescence monitoring can contribute to high spatial and temporal resolution studies. In the last decades, fluorescence spectroscopy has been used successfully as a monitoring and detection tool in natural and engineered systems.[2] The technology of fluorescence is based on the principle of luminescence. The phenomena of luminescence is known as the emission of light from any substance, and occurs from electronically excited states. During this process, the electron in the excited orbital is paired with the electron in the ground state orbital. As a consequence, the return to the ground state is spin allowed and occurs rapidly by emission of the photon. The rate of an emission of a fluorescence photon is 10^8 s^{-1}, so the typical lifetime of a fluorescence is near 10 ns.[1]

Fluorescence measurements can be classified into two types: steady-state and time-resolved. The most common type of measurements are steady-state, and are performed with constant illumination and observation. While the sample is illuminated with a continuous beam of light, the intensity or emission spectrum is recorded. Due to the nanosecond timescale of fluorescence, most measurements are steady-state measurements. The steady state is reached immediately, when the sample is exposed to light.[1]

The second type of fluorescence measurements is time-resolved measurements. This type of measurement is used to measure intensity decays or anisotropy decays. In time-resolved measurements, the sample is exposed to a pulse of light, where the pulse width is shorter than the decay time of the sample. A high-speed detection speed is used to record the data of the intensity decay, hence the intensity or anisotropy are measured in the ns timescale. The time-resolved measurement require expensive and complicated equipment.[1]

Time-resolved fluorescence spectroscopy has the capacity to differentiate between tumor and normal tissue in real time surgical excision. Recently, a new TR-FS device has been designed that has the ability to quantify the relative concentration of fluorescence dyes in a mixture by unmixing of lifetime decays. This instrument has the ability to acquire near real-lifetime fluorescence lifetime in multiple spectral brands and analysis.[1]

Continuous and/or time-resolved fluorescence spectroscopy can provide optical contrast from the morphological alterations occurring in the damaged tissue where emission and absorption of light by biological tissue has been studied extensively. However, time-resolved fluorescence spectroscopy offers more advantages than steady state spectral analysis such as fluorescence decay parameter analysis. The fluorescence decay is a vigorous measure of tissue characteristics and it is not affected by fluorescence intensity fluctuations that are found in intraoperative data. There exists a correlation between the steady-state and time-resolved measurements. A steady-state measurement is the average of the time-resolved phenomena over the intensity decay of the sample.[1]

One distinct attribute of the fluorescence is the detection with high sensitivity. In 1877, through the feature of fluorescence, it was proven that the rivers Danube and Rhine were connected by underground streams. The connection was proven by placing fluorescein into the Danube. Sixty hours later, its characteristic green fluorescence appeared in a small river that lead to the Rhine. In present times, fluorescein can be used as an emergency marker to locate individuals at sea, and it was also present on the landing of space capsules in the Atlantic Ocean.[1]

The spectral data of fluorescence is presented as emission spectra. A fluorescence emission spectrum is defined as the plot of the fluorescence intensity versus wavelength (nanometers) or wavenumber (cm^{-1}). A variety of emission spectra exist, and are dependent upon the chemical structure of the fluorophore, and the solvent in which the fluorophore is dissolved. Certain compounds such as perylene, show significant structure due to their individual vibrational energy levels, but other compounds such as quinine, show fluorescence spectra that lacks a vibrational structure (Fig. 1).[1]

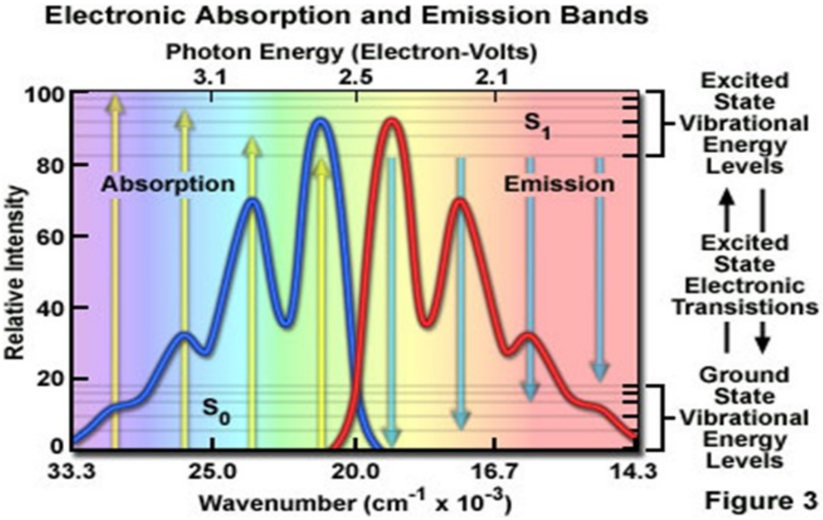


Figure 1 – Absorption and emission spectra of quinine.[3]

Typical fluorescent substances are defined as fluorophores. The process of fluorescence occurs in aromatic molecules. A typical example of a fluorophore is quinine, which is found in tonic water. If a glass of tonic water is displayed to the sunlight, a blue color is evident at the surface. Furthermore, other types of fluorophores are found in daily life. In antifreeze, trace quantities of rhodamine are present and hence the green or red-orange glow is sometimes present. Other types of fluorescent molecules include polynuclear aromatic hydrocarbons, such as anthracene and perylene, which are used for environmental monitoring of air pollution. Another type of fluorescent molecules include certain types of substituted organic compounds, such as 1,4-bis(5-phenyloxazol-2-yl)benzene (POPOP) and acridine orange. POPOP is used in scintillation counting, and acridine orange is used as a DNA stain. Certain fluorophores such as pyridine 1 and rhodamine find application as dye lasers.[1]

Aromatic organic molecules are fluorescent, but in contrast the atoms are nonfluorescent in condensed phases. An exception to this rule include the group of elements known as lanthanides. In the elements of europium and terbium ions, the fluorescence results from electronic transitions between the f orbitals. Furthermore, these orbitals are shielded from the solvent by higher filled orbitals. Due to this shielding effect, the lanthanides have long decay times, and the emission rates are low.[1]

Jablonski diagram is a diagram that illustrates the processes that happen between the absorption and emission. The Jablonski diagrams are used in a variety of forms, to discuss several molecular processes that occur in excited states. The Jablonski diagram is named after Professor Alexander Jablonski, who is regarded as the father of fluorescence spectroscopy, because of his many accomplishments such as describing the concentration depolarization and defining the term "anisotropy" to describe the polarized emission from solutions. In a typical Jablonski diagram, the singlet ground, first and second electronic states are depicted as S_0, S_1 and S_2 respectively (Fig. 2). At these electronic energy levels, the fluorophores can exist in a number of vibrational energy levels depicted by 0, 1, 2 etc.[1]

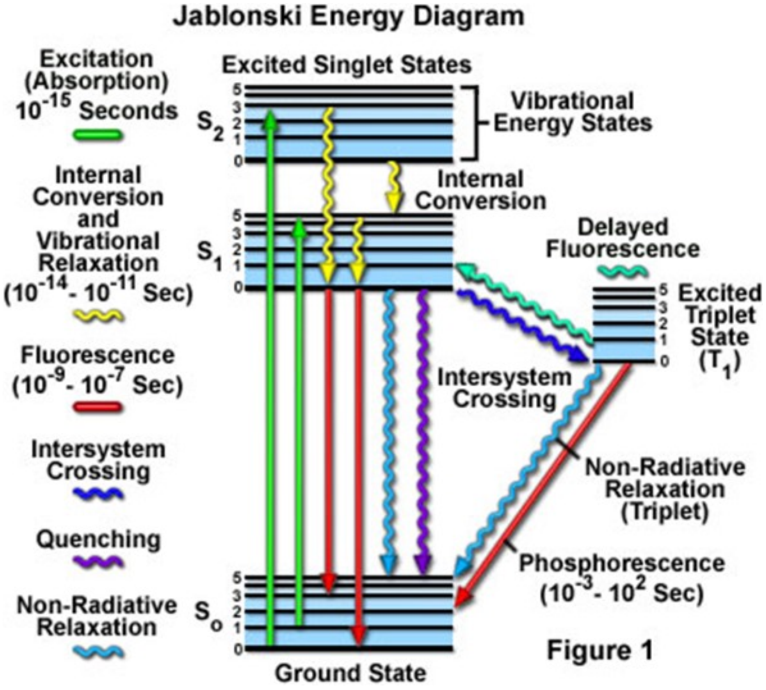


Figure 2 - Jablonski diagram.[3]

The phenomena of fluorescence exhibit several characteristics such as Stokes shift, mirror image rule and independence of the emission spectra from the excitation wavelength. If any of the general characteristics are not exhibited by a fluorophore, the compound is showing some special behavior.

The Jablonski Diagram shows that the energy of emission is generally lower than the energy of absorption. Fluorescence occurs usually at lower energies or longer wavelengths. Fluorescent molecules in a solution display energy losses between excitation and emission. A casual reason of the Stokes shift is the fast decay to the lowest vibrational level of S_1. Fluorophores generally decay to higher vibrational levels of S_0, which leads to further loss of excitation energy by thermalization of the excess vibrational energy. Stokes shift can occur due to solvent effects, excited-state reactions, complex formation and/or energy transfer.[1]

Another general property of fluorescence is the independence of emission spectra from the excitation wavelength, which is known as Kasha's rule. The fluorophore reaches the lowest vibrational level of S_1, upon excitation into higher electronic and vibrational level, because the energy is quickly dissipated from higher vibrational levels of S_1. This relaxation occurs at 10^{-12} s, and comes as a result of a strong overlap among several states of nearly equal energy. Due to this rapid relaxation, the emission spectra are usually independent of the excitation wavelength. Several fluorophores don't obey this rule, such as fluorophores that exist in two ionization states that display distinct absorption and emission spectra. Furthermore, certain molecules emit from the S_2 level, but this emission is rare and generally not observed in biological molecules.[1]

In order for fluorescence experiments to succeed, the researcher must pay attention to experimental details and thoroughly understand the instrumentation. There are many potential situations when the data can be twisted. There are times when light can be detected with high sensitivity, and as a result the amplification of instruments is increased to obtain observable signals, even when the sample is nearly non fluorescent. Hence, the signals seen at high amplification may not originate from the fluorophore of interest. Other interference sources include background fluorescence from the solvents, light leaks in the instrumentation, emission from the optical components, stray light passing from the optics, light scattered by turbid solutions etc.[1]

THE COMPONENTS OF A SPECTROFLUOROMETER

There is no ideal spectrofluorometer to conduct the measurements. The available instruments don't give the actual emission or excitation spectra, because of the nonuniform spectral output of the light sources and the wavelength-dependent efficiency of monochromators and detector tubes. The measured fluorescence intensities can be affected by the polarization or anisotropy of emitted light, hence it is important to control numerous factors in order for experiments to succeed.[1]

Most spectrofluorometers can record both emission and excitation spectra. Emission spectrum is the wavelength distribution of an emission measures at single constant excitation wavelength. On the other hand, an excitation spectrum depends of the emission intensity, measured as a single emission wavelength, after scanning the excitation wavelength. These kinds of spectra can be presented on a wavelength scale or wavenumber scale. Light of a given energy can be described in terms of wavelength, frequency or wavenumber. The unit for wavelength is nanometers and the unit for wavenumber is cm^{-1}. These two units can be interconnected by taking the reciprocal of each value.

The individual components of a spectrofluorometer are: light sources, monochromators, optical filters and photomultiplier tubes (Figure 3).[1]

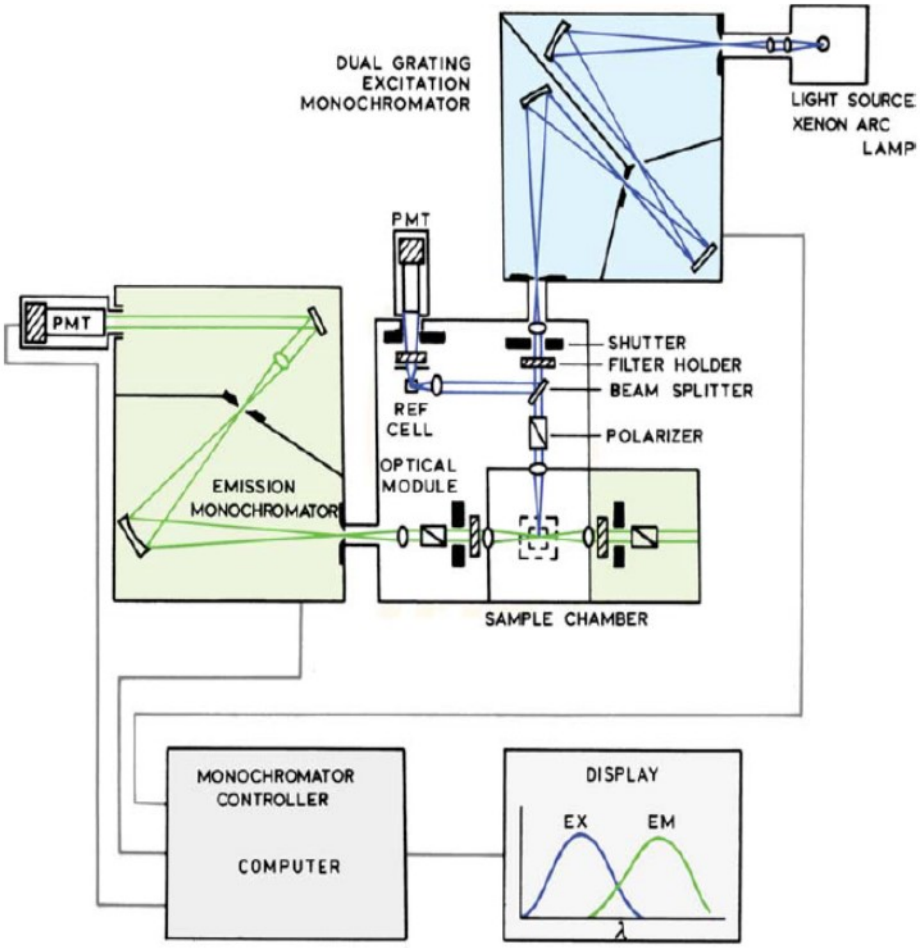


Figure 3 – The scheme of the spectrofluorometer.[1]

LIGHT SOURCES

The different types of light sources used in spectrofluorometers include the following:

- *Arc and Incandescent Xenon Lamps*

- *Pulsed Xenon Lamps*

- *High-Pressure Mercury (Hg) Lamps*

- *Xe-Hg Arc Lamps*

- *Quartz-Tungsten Halogen (QTH) Lamps*
- *Low-Pressure Hg and Hg-Ar Lamps*
- *LED Light Sources*
- *Laser Diodes*[1]

Arc and Incandescent Xenon Lamps

The most functional source of light for a steady-state spectrofluorometer is high-pressure xenon arc lamp. These lamps emit light continuously as a result of recombining electrons with ionized Xe atoms.[1]

Pulsed Xenon Lamps

Xenon flash lamps are often used in compact fluorometeres and plate readers. The output of a xenon flash lamp is more structured than of a continuous lamp. The output of a flash lamp is higher in the UV. These flash lamps expend less power and generate less heat.[1]

High-Pressure Mercury (Hg) Lamps

This kind of lamps have a higher intensity than Xe lamps, but the intensity is concentrated in lines. These lamps are only useful if the Hg lines are at suitable wavelengths for excitation.[1]

Xe-Hg Arc Lamps

This type of lamps have higher intensities in the ultraviolet than Xe lamps, and the presence of Xe broadens the spectral output. The Hg lines dominate the output of these lamps. The output is higher between the Hg lines, because when it is first started the Hg-Xe lamp output is mostly due to Xe.[1]

Quartz-Tungsten Halogen (QTH) Lamps

This kind of the lamp provides a continuous output in the visible and IR regions of the spectrum. These days the interest about the QTH lamps has increased because there is increasing interest in fluorophores absorbing in the read and near infrared regions.

Low-Pressure Hg and Hg-Ar Lamps

These lamps are used for calibration purposes because they yield very sharp line spectra.[1]

LED Light Sources

LEDs can be used to obtain a wide range of wavelengths. The advantage of LEDS is low power consumption and long life. This kind of light source can be placed close to the samples, and if needed the excitation wavelength can be better defined by the use of an excitation filter.[1]

Laser Diodes

Laser diodes are available with wavelengths ranging from 405 to 1500 nm. This light source is useful because the output is easily focused and manipulated. The amplitude can be modulated to several GHz.[1]

MONOCHROMATORS

Monochromators can be used to disperse polychromatic or white lights into different colours or wavelengths. The dispersion can be conducted using prisms or diffraction gratings. In most spectrofluorometers, the monochromators use diffraction gratings. The specifications for the performance of a monochromator include dispersion, efficiency and stray light levels. Monochromators also possess high efficiency to maximize the ability to detect low light levels. A typical monochromator has an entrance and an exit slit. If photobleaching of the sample is a problem, then this factor can be minimized by decreasing the excitation intensity. Simple and gentle stirring of the sample can also decrease photobleaching. Only a portion of the sample is illuminated, and the bleached portion of the sample is continuously replaced by fresh solution.[1]

OPTICAL FILTERS

The optical filters which can be used in fluorescence spectroscopy include:

- *Colored Filters*
- *Thin-Film Filters*
- *Neutral-Density Filters*
- *Filters for fluorescence microscopy*[1]

Colored filters

In addition to monochromators, optical filters are used to reimburse for the less than ideal behavior of monochromators. Excitation and emission filters can be used in all experiements, even when monochromators are used in order to reduce the possibility of undesired wavelengths corrupting the data. A wide range of filters are available, and the transmission spectra is of filters is provided by the manufacturer.[1]

Thin-film filters

Thin-Film filters are being designed for specific applications, and are used to obtain any desired transmission curve. Thin-film filters can also specifically transmit or reject laser lines.[1]

Filter combinations

Even though the development of technology allows the design of specific filters for specific experiments, this development is usually not convenient. Hence, sometimes it is adequate that for a given experiment to combine two or more bandpass filters to obtain the desired spectral properties.[1]

Neutral-density filters

Neutral density filters are used to attenuate light equally at all wavelengths, and are composed of sheets of glass or quartz coated with a metal to obtain the desired optical density.

Filters for fluorescence microscopy

Fluorescence microscopy uses filters rather than monochromators for wavelength selection. Most of the fluorescence microscopy is done using epifluorescence configuration. Epifluorescence is defined as excitation and emission passing through the same objective. The advantage of this configuration is that most of the excitation travels away from the detector. Observation of the fluorescence image requires a specific set of filters, which contains an excitation filter, an emission filter and a dichroic beam splitter. The excitation filter selects wavelengths from a broad source to excite the sample. The emission filter transmits the emission and rejects the excitation wavelengths.[1]

PHOTOMULTIPLIER TUBES

The majority of fluorometers use photomultiplier tubes as detectors. A photomultiplier tube serves as a current source, and the current is proportional to the light intensity. A PMT responds to individual photons, and the pulses can be detected as an average signal or can be counted as individual photons.[1]

Spectral response of PMTs

The spectral response of a photomultiplier tube is based on the type of the transparent material used for the window and the chemical composition of the photocathode. Only the light that enters the PMT can generate photocurrent. The input windows should be transparent to the desired wavelengths. Another important factor is the material used for the photocathode. Most photocathodes are sensitive in the UV, blue, green regions of the spectrum. The differences in the photocathode material are mostly important for wavelengths above 600nm.[1]

Time response of photomultiplier tubes

The PMT time response is important for lifetime measurements, but not for steady-state measurements. The three main timing characteristics of PMT are the transit time, rise time and transit time spread. The transit time of PMT is defined as the time interval between the arrival of a photon at the cathode, and the arrival of the amplified pulse at the anode. The transit time usually ranges from 20 to 50 ns. The rise time is defined as the time required for the PMT anode signal to rise from 10 to 90% on its final level. The rise time is determined by the variations of the transit time in the PMT. The most important specification for time-resolved measurements is the transit time spread. These time variations occur due to different geometric paths that the electrons can take from the photocathode to the anode.[1]

CHARGE-COUPLED DEVICE DETECTORS

Charge-coupled device detectors (CDDs) are imaging detectors that contain remarkable sensitivity and linear dynamic range. CCDs usually contain one million or more pixels. Each pixel behaves as an accumulating detector, where charge accumulates in proportion to total light exposure. CCDs are used widely in fluorescence spectroscopy. Small spectrofluorometers using CCDs are commercially available, and these devices are interfaced via a USB and have no moving parts. Through the use of a fiber-optic cable, the signal is easily brought to the device. When the instrument is combined with a LED light source, the entire instrument becomes a solid device. These spectrofluorometers are useful for bringing the instrument to the experiment.[1]

THE ANALOGUES, PROPERTIES AND APPLICATIONS OF PHTHALOCYANINES

THE PROPERTIES OF SEVERAL PHTHALOCYANINE COMPLEXES

Based on the chemical structure, phthalocyanines (Pcs) are classified as analogues of porphyrins condensed with four isoindoline units *via* a nitrogen atom, and display a deep blue color due to their wide 18π electron conjugated system.

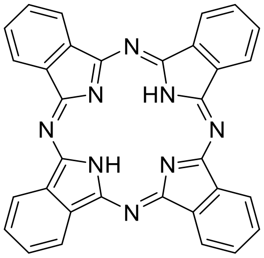


Figure 4 - Chemical structure of phthalocyanine

Along with subphthalocyanines, these compounds are attracting attention as useful dyes that are applicable to organic solar cells, photodynamic therapy, organic electronic devices and other appliances. One of the most essential phthalocyanine complexes is the phthalocyanine copper complex, also known as the blue organic pigment or phthalocyanine blue.

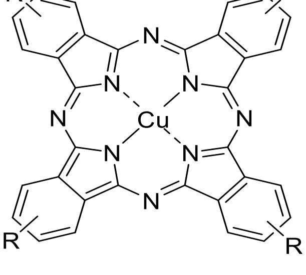


Figure 5 –The chemical structure of copper phthalocyanine complex

For a long time, phthalocyanine has been used as a blue pigment for road signs and bullet trains, since it can be manufactured easily, are very robust and difficult to discolor.[4]

Phthalocyanines are capable of forming complexes with different types of metals. Pcs can be used in fluorescence probing due to their suitable absorption and emission in the red region of the visible spectrum. Red or near-infrared excitation and emission is important for biological applications since longer wavelength light penetrates deeper into tissues, it is less scattered and the autofluorescence of endogenous chromophores is limited. The photophysical and photochemical properties of Pcs and their analogues depend highly on the central metal and peripheral substitution. There are two positions where the substituent can be introduced into the phthalocyanine, at the alfa position near the center of the macrocycle, and in the beta position on the outer side of the macrocycle.[5]

The presence of heavy metals increases the chances of molecular relaxation via intersystem crossing, which can cause high quantum yields of singlet oxygen formation and low quantum yield of fluorescence. Heavy atom effect is defined as the increase of the spit-orbit coupling in compounds substituted or complexed with heavy atoms.[5]

Magnesium is the lightest cations that is stable in the center of these macrocycles, and it is also the metal of choice for porphyrinoid compounds used for fluorescence probing in the red region. Lithium, beryllium and sodium are only weakly bound to these macrocycles and can easily be replaced by hydrogen atoms in weakly acidic media. If boron is used as the central metal, it leads to the formation of subphthalocyanines that absorb and emit at lower wavelengths.[5]

Certain phthalocyanine derivatives may be used as sensors, and as materials for photovoltaic, photoelectronic and optical properties. In order to acquire conducting compounds, phthalocyanines should be partially oxidized to introduce an unpaired electron into the phthalocyanine macrocycle. Semiconductivity or almost-one-dimensional metallic conductivity can be obtained when this type of macrocycles have a columnar or layered arrangement together with effective π - π stacking. If paramagnetic atoms are placed in the center of the phthalocyanine macrocycle, an enormous magnetoresistance may be induced. The oxidation of metal phthalocyanines can lead to the formation of manganese (III) phthalocyanine or substituted napthalocyanine radical anions. These types of compounds are found in molecular magnets.[6]

Phthalocyanines are difficult to handle due to their strong ability to aggregate, so this property should be controlled for further application of phthalocyanines. Trifluoroethoxy-substituted phthalocyanines (TFEO-Pc) also possess the ability to suppress aggregation due to the repulsion of the trifluoroethoxy group. Nevertheless, the electronic characteristics of phthalocyanines are changed due to the strong electronegativity of fluorine.[4]

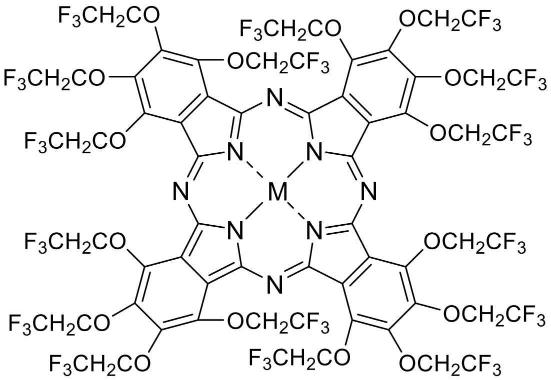


Figure 6 – The chemical structure of trifluoroethoxy phthalocyanine (TFEO-Pc)

When a fluorine atom is introduced to the structure of phthalocyanines, the molecule is expected to become a new functional material that reflects the exact properties of the fluorine atom.

The introduction of a trifluoroethoxy group leads to non-aggregation properties and high solubility and also an electron-deficient π space and thermal or chemical stability to the phthalocyanine.

The number of substituted trifluoroethoxy groups and their position in the molecules can define the properties of the trifluoroethoxylated phthalocyanine.[4]

TFEO-Pcs are highly soluble so chemical modifications and purifications are easily conducted. Di or trinuclear phtahlocyanines may be functionalized compared with the mononuclear phthalocyanines by chemically modifying each unit by an interaction between each unit. In dimer-type pthalocyanines it is possible to separate charges, so these types of compounds may be applied to new electron or energy transfer systems such as solar cell materials as well as photocatalysts for organic reactions.[4]

Several phthalocyanine complexes containing Group 16 (S, Se and Te) elements and Group 15 (P, As and Sb) elements as peripheral and central cations, respectively were developed. These elements have notable properties such as diversity of coordination, effective orbital interactions and large electronegativity, so their introduction into Pcs can change the spectrum significantly.

The presence of group 16 elements into a free-base Pc, resulted in a red-shift of the Q-band due to the electron-donating ability of the group 16 elements particularly at α positions.[4]

Additionally, when an element from group 15 was introduced into the center of the Pc ring, the resulting Pcs displayed a single Q-band peak above 1000 nm.

The combination of electrochemical, spectral and theoretical considerations revealed that all of the central group 15 elements, peripheral group 16 elements and their positions are necessary to shift the Q-band beyond 1000 nm, leading to the conclusion that the substitution effects of group 15 and 16 elements act synergistically.[4]

APPLICATIONS OF PHTHALOCYANINES

Due to their excellent spectroscopic properties, phthalocyanines are likely to be applied to medical fields such as biological image probes and as agents for photodynamic therapy, allowing phthalocyanine to be helpful medical dye because it absorbs light of long wavelengths with high tissue transparency. Conversely a common problems encountered among phthalocyanines is the formation of aggregates, which leads to the deterioration of solubility and spectroscopic properties. Hence it is necessary to suppress aggregation, and the ability of the TFEO group to suppress the aggregation of phthalocyanines is outstanding. Hence, a medical dye can be developed by using the effect of suppressing aggregation. In the case of phthalocyanines, the improvement of biocompatibility is as significant as suppressing the aggregation properties. Furthermore to increase the bioavailability, it is also essential to increase the water solubility of the lipophilic phthalocyanines. A frequent method that is used to increase the water solubility of lipophilic phthalocyanines is the combination with a biomolecule such as sugar or a peptide.[4]

One form of cancer treatment is photodynamic therapy, and it involves the introduction of organic dyes into cancer cells and kills them by reactive oxygen species with cytotoxicity formed after light irradiation. The advantages of this type of therapy include reduced physiological burden of the patients because this process doesn’t require surgery and the damage to normal cells can be minimized by irradiating the laser only to target the tumor cells. An ideal PDT agents would have the following properties: strong absorption in the red wavelength region, non-aggregation property, high biocompability, good selectivity for cancer cells and high quantum yield of singlet oxygen.[4]

Numerous Pc compounds have reached clinical trials such as water soluble sulfonated aluminium phthalocyanines (AlPc). Water soluble sulfonated AlPc can be dissolved in water and their use has been tested in various types of cancer such as skin, breast, cervical, larynx, lung oropharyngeal cancers.

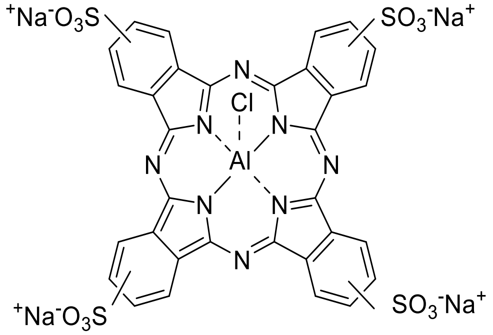


Figure 7 - The sulfonated derivative of aluminium phthalocyanine that is being tested in multiple types of cancer such as skin, breast, cervical, larynx, lung and oropharyngeal cancers.

Zinc phthalocyanine (ZnPc) was encapsulated in liposomes made of palmitoyl-oleolyl-phosphatidylcholine and dioleoyl phosphatidylserine, which were used for the treatment of carcinoma of the upper aerodigestive tract.[7]

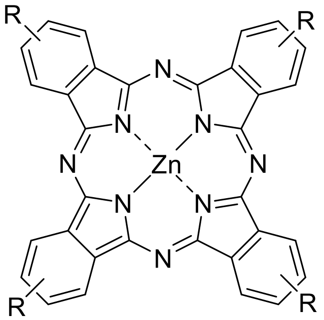


Figure 8 – The chemical structure of unsubstituted zinc phthalocyanine

Silicon-based phthalocyanine formulations dissolved in propylene glycol (for topical administration) or in Cremophor EL (for intravenous administration) together with ethanol were tested and reached clinical trials for different skin diseases and cancers.

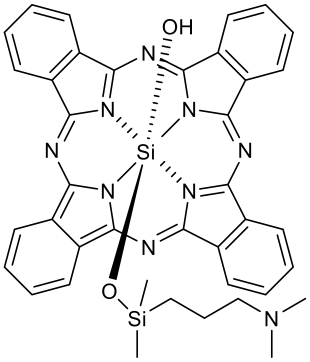


Figure 9 – The chemical structure of silica – based phthalocyanine

Disulfonic diphthalimidomethyl ZnPc based cremophor EL formulation has also been tested in phase I clinical trials for the treatment of skin or esophageal cancer.[7]

Phthalocyanines (Pcs) and naphthalocyanines (Ncs) have been used as contrast imaging agents in several techniques such as magnetic resonance imagining (MRI), fluorescence imaging, positron emission tomography (PET), photoacoustic imaging and other different types. Pcs and Ncs are expected to produce better contrast for photoacoustic imaging than the other types of porphyrins due to their higher extinction coefficients compared on those of porphyrins. Pcs and Ncs based contrast agents may be used as well for fluorescence imaging. Optical fluorescence imaging provides the advantages of high sensitivity, high speed and low cost, but its use may be limited by the fluorescence quenching within Pcs and Ncs. However, a number of strategies such as hydrophilic modifications have been developed to restore the fluorescence. It has been determined that modified graphene nanosheets have the ability to prevent the aggregation of Pc and enable fluorescence imaging guidance for phototherapy.[7]

Most Pcs that contain a flat aromatic macrocycle aren't soluble in water, so liposomes are a commonly used carries system to improve their solubility. Liposome cargo has the capacity to exchange with serum lipoproteins following systemic administration. Receptor mediated transport mechanisms allow low-density lipoproteins to bind to tumor cells, so liposomes can facilitate targeting of photosensitizers efficiently *in vivo* even at low doses.[7]

THE PROPERTIES AND APPLICATIONS OF AZAPHTHALOCYANINES

The aza analogs of phthalocyanines (azaphthalocyanines, AzaPcs) have similar application as phthalocyanines, but the presence of nitrogen atoms can add new specific properties to these compounds compared to phthalocyanines. High photostability is one of the aspects that decides the suitability of a compound as a fluorescence probe. Photoproduced singlet oxygen is believed to be an important factor that is responsible for the photodecomposition of Pcs and AzaPcs. Photostability as evaluated by the quantum yields of photodecomposition was determined, and according to the measurements there is a disappearance of all absorption bands that is common for the destruction of the macrocycle. According to the values of photodecomposition, the AzaPc derivatives are more stable than the corresponding Pcs.[5]

The magnesium AzaPcs are promising red-emitting fluorescent probes. Based on the peripheral substitution, the alkylsulfanyl derivatives are the compounds of choice for several reasons:

- The magnesium AzaPc the alkylsulfanyl substituents displays an absorption in the Q-band region approximately two times greater than the magnesium AzaPc with the aryloxy substitution

- Another positive feature of the alkylsulfanyl derivative is the high photostability

- Simpler synthetic methods are applied to yield the alkylsulfanyl AzaPcs compared to the corresponding aryloxy AzaPcs.[5]

- Furthermore, alkylsulfanyl AzaPcs absorb and emit in the area that overlaps the red cyanine Cy5 fluorophore. Red cyanine Cy5 fluorophore is considered a fluorescence standard for the red portion of the spectrum. In comparison to red cyanine Cy5 fluorophore AzaPcs have additionally strong absorption band at around 380 nm, which extends up to 500nm that can be used as the alternative band for excitation.

- AzaPcs can be incorporated into biomembranes and not lose fluorescence.

- The alkylsulfanyl derivatives can be modified to obtain water-soluble fluorescent derivatives

- The alkylsulfanyl AzaPcs show a fluorescence quantum yields value greater than 0.50, absorption molar coefficient of approximately 3×10^5 M^{-1} cm^{-1} and considerable photostability, which makes them suitable for use as potential fluorescence probes in aqueous media, organic solvents and bio membranes. In the future, alkylsulfanyl AzaPcs may find application as outstanding red fluorophores with properties better than those of commercially used Cy5.[5]

Tetrapyrazinoporphyrazines (TPyzPzs) are the AzaPcs that have been studied the most. This group has received a lot of attention due to their promising fluorescent, optical limiting, liquid and catalytic crystalline properties, and the ability to form nanoporous materials.

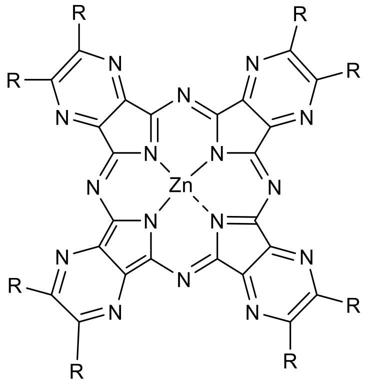


Figure 10 – The chemical structure of tetrapyrazinoporphyrazines

Furthermore TPyzPzs are capable of producing singlet oxygen upon irradiation. Due to this property, TPyzPzs have been investigated as photosensitizers in photodynamic therapy (PDT), which has also been confirmed *in vitro* on cells. Aza substitution in benzene rings of Pcs gives the TPyPzs important electron deficient properties, that leads to a macrocycle core which can undergo intramolecular charge transfer (ICT) or photoinduced electron transfer (PET) after combination with a suitable donor moiety. ICT and PET can be used in sensing because these are competitive relaxation pathways to fluorescence and can easily be switched ON and OFF after binding to an analyte.[8]

An interesting approach that may lead to higher selectivity at certain pH values is the development of sensors that function on the OFF-ON-OFF principle, using pH indicators that are turned on only at certain pH values. Thus far, only a few indicators have been published that work based on the presence of two different donors for PET or on changing the overall charge using several micelles.

Nevertheless, the published molecular switches so far are limited only to emission in the UV region. Several pH-driven OFF-ON-OFF indicators for an acidic pH range have been designed. These type of pH indicators work on two completely different switching mechanisms available in TPyzPzs, which are the ICT switching at the peripheral donor amine and protonation of the azomethine nitrogen atoms. This design is based on the idea that the fluorescence is switched ON when the proton concentration is increased, but is switched OFF when the acidity is increased. Hence, strong fluorescence is expected to occur only at intermediate pH values, leading to a narrow pH-sensing window (Figure 11).[9]

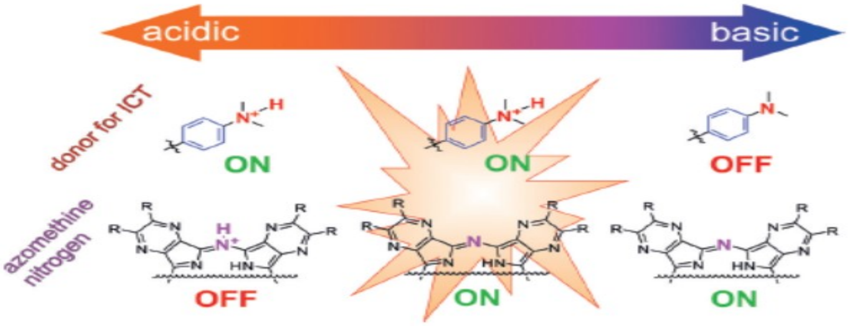


Figure 11 – A short description of the structures that may be used for the principle of OFF-ON-OFF fluorescence.[9]

Several red fluorescent TPyPz sensors for pH or cation detection have recently been reported. These sensors possess several amines that serve as donors in ICT, and the fluorescence was switched ON upon protonation in acidic pH or upon binding to a cation (Figure 12). Furthermore, amines can be used as donors in Pcs for the development of pH sensitive photosensitizers in PDT.[8]

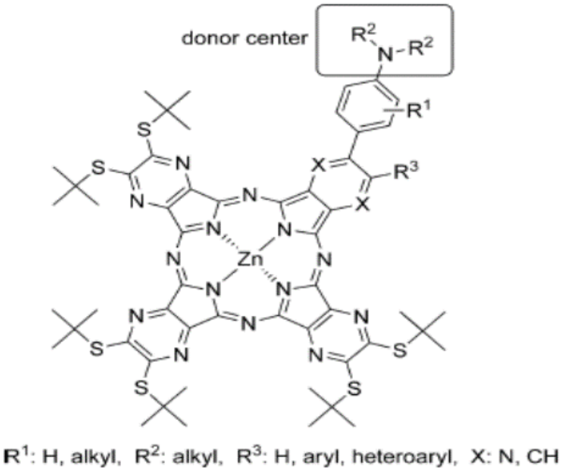


Figure 12 – The chemical structure of TPyzPzs and the substituents in the amine that can be used as donor center in intramolecular charge transfer.[8]

EXPERIMENTAL PART

GENERAL

The dyes that were tested were prepared in our laboratory before and published.[5, 10, 11] The chemicals used during the project were bought from the following suppliers: dioleoylphosphatidylcholine (Lipoid GmbH, Germany), Cremophor® EL (BASF, Germany), medium-chain triacylglycerides (Ecogreen oleochemicals, meeting specifications of Ph. Eur.), trifluoroacetic acid (Fluorochem, UK). The absorption spectra were measured on Shimadzu UV-2600 spectrophotometer (Shimadzu), the fluorescence spectra were obtained on the FS5 fluorimeter (Edinburgh Instruments).

THE BUFFER PREPARATION

The Britton-Robinson buffers with selected pH were prepared by mixing appropriate volumes of 0.2M solution of NaOH, and the solution cointaing 0.04M of phosphoric acid, 0.04M acetic acid and 0.04M phosphorous acid. Six buffers with different pH values were prepared: 1.14, 1.49, 2.00, 2.98, 4.01 and 7.01.

TESTING IN WATER

Stock solutions (100 μM) of compounds **ZIP65Mg-Et**, **ZIP65Zn-Et** or **9Mg-MeI** were prepared in water. Subsequently, the stock solution (20 μl) was added to the tested buffer solution (2 mL) in the quartz cuvette and the absorption spectra were collected immediately after mixing the solutions and then in selected time intervals to monitor the potential changes.

TESTING IN ORGANIC SOLVENT

A THF solution of compound **ZIP21Mg** with concentration of 1 µM was prepared and 2 ml of this solution were transferred into quartz cuvette. The absorption spectra were measured immediately after addition of specific volume (typically 50-100 µl) of trifluoroacetic acid (TFA) up a total volume of 1.2 ml of TFA in solution. In another experiment, the 1 µM THF solution of **ZIP21Mg** (2 ml) in quartz cuvette was added with 500 µl of TFA and the absorption spectra were collected in selected time intervals.

THE LIPOSOME PREPARATION

Using the suspension of multilamellar vesicles (MLVs), large unilamellar vesicles were formed by extrusion using the following procedure. The lipids (dioleoylphosphatidylcholine, DOPC, 19.7 mg, 25 µmol) were dissolved in chloroform, 0.25 mL of 100 µM THF solution of **ZIP21Mg** or **P9-0Mg** were added and the solution was evaporated in a 100 mL round bottom flask on a water bath at a temperature of 37 °C. In order to remove all the traces of organic solvents, the thin lipid film was left on the water bath for 30 minutes at a pressure of 5 mbar. The Britton-Robinson buffer of pH 7.01 (1.0 ml) was added and the lipids were removed from the flask walls by gentle hand shaking. Subesequently, the suspension was vortexed for 5 min to from MLVs and was left to stand for 4 h at room temperature in order to allow complete swelling. Using a small hand extruder LiposoFast Basic, LUVETs were prepared from this MLV suspension. The suspension was passed back and forth 21 times through two stacked polycarbonate filters (pore size 100 µm) at a room temperature. The final suspension of liposomes contained the dye at concentration of 25 µM incorporated in unilamellar DOPC vesicles with lipid concentration of 25 mM, i.e. the lipid-to-dye ratio was 1000:1. Liposomes of such composition have been already characterized before using dynamic light scattering with the approximate size about 128.1 ± 0.6 nm.[12]

Subsequently, the stock liposomal suspension (40 µl) was added to the tested buffer solution (2 mL) in the quartz cuvette and the absorption spectra were collected immediately after mixing the solutions and then in selected time intervals to monitor the potential changes. The spectra were collected seven times for approximately 24 hours.

THE NANOPARTICLE PREPARATION

The silica nanoparticles were prepared by colleagues from Institute of Inorganic Chemistry, Czech Academy of Sciences (dr. Kirakci, dr. Lang). In a typical procedure, 180 mg of Tween 80, 0.4 mL of 1-butanol, and 10 ml of deionized water were added to a glass vial (40 mL) and vigorously stirred for 15 minutes. Then, a solution containing 0.1 mg of **ZIP21Mg** or **P9-0Mg** and 0.1 mL of pyridine in 0.1 mL of DMF was added dropwise to the micellar solution. After one hour, 0.03 mL of triethoxyvinylsilane was added dropwise and the emulsion was left to stir for 3 days. Residual DMF, pyridine, 1-butanol, and Tween 80 were removed by dialysing the nanoparticle dispersion against deionised water in a 12–14 kDa cutoff cellulose membrane (Spectrum Laboratories,Inc.) for 96 h with changing of the water every 24 hours. The following codes were used for the prepared nanoparticles: **KK792** (containing **ZIP21Mg**) and **KK795** (containing **P9-0Mg**). Typical size of the nanoparticles was 13 nm as determined by dynamic light scattering

During the testing, the nanoparticle suspension (12 µl) was added to the tested buffer solution (2 mL) in the quartz cuvette and the fluorescence emission spectra (λ_{ex}= 370 nm for both dyes; λ_{em} =

666 nm or 712 nm for **ZIP21Mg** and **P9-0Mg**, respectively) were collected immediately after mixing the solutions and then in selected time intervals to monitor the potential changes.

THE MICROEMULSION PREPARATION

The medium chain triacylglycerides (29 mg) and Cremophor® EL (72 mg) were dissolved in chloroform (ca 4 mL) and mixed with 100 μM THF solution (1.25 ml) of **ZIP21Mg** or **P9-0Mg**. The solvent was evaporated under reduced pressure and the flask was kept under deep vacuum (5 mbar) for 30 min at 40 °C in order to remove all traces of solvent. Afterwards, the Britton-Robinson buffer of pH 7.01 was added to a total volume of 5 ml, and the mixture was shaken well on a vortex and orbital shaker. The microemulsions prepared in this way contained the dye at concentration of 25 μM incorporated in the oil particles. Microemulsions of such composition have been already characterized before using dynamic light scattering with the approximate size about 300 nm.[8]

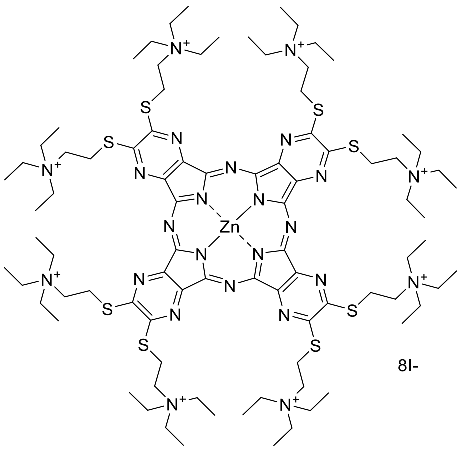
During the measurements, the stock microemulsion (40 μl) was added to the tested buffer solution (2 mL) in the quartz cuvette and the absorption spectra were collected immediately after mixing the solutions and then in selected time intervals to monitor the potential changes. The absorption spectra were collected eight times for approximately 22 hours.

DISCUSSION PART

We have conducted numerous experiments to determine the stability of phthalocyanine complexes of magnesium and zinc in water, organic solvent, liposomes, microemulsions and silica nanoparticles. Seven buffers ranging from pH 1.14 to 7.00 were prepared. The stock solutions of the samples were added to buffer and the changes in the absorption and fluorescence spectra of the tested dyes were monitored for a period of time, usually for up to 24 hours. The potential transition from a metal complex to a metal-free ligand can be observed in the absorption spectra by the decrease of the Q-band followed by its splitting. In the case of nanoparticles, due to its low concentration fluorescence spectra, I was able to collect only their fluorescence spectra since fluorescence is much more sensitive method.

WATER SOLUBLE DERIVATIVES

ZIP65Zn-Et



ZIP65Zn-Et

Figure 13 – The chemical structure of the compound with the code ZIP65Zn-Et

It is generally known that the zinc complexes of the phthalocyanines are stable in acidic environment. We decided to confirm this by measurement of highly water-soluble **ZIP65Zn-Et**. The absorption spectra of this compound did not change in time even at the most acidic pH tested (Figure 14). We may conclude that even at the most acidic pH (1.14) and after 21 hours and 41 minutes, there were no changes of its absorption spectra and the central metal is stable. No demetallation occured.

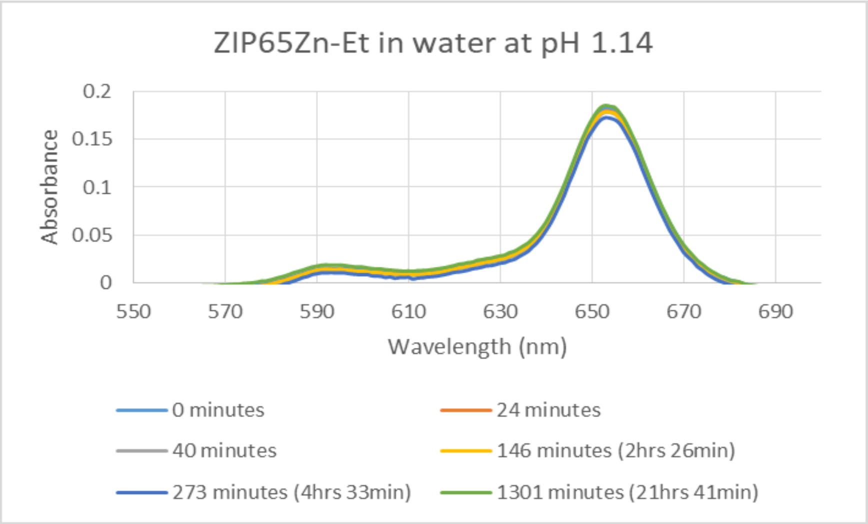


Figure 14 - The absorption spectra of ZIP65Zn-Et at pH 1.14 measured at selected times

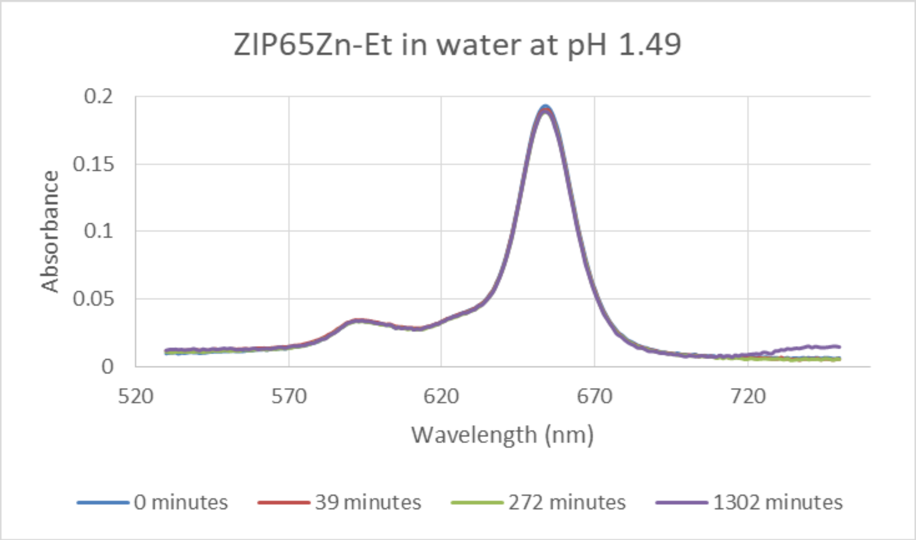


Figure 15 – The absorption spectra of ZIP65Zn-Et at pH 1.49 measured at selected times

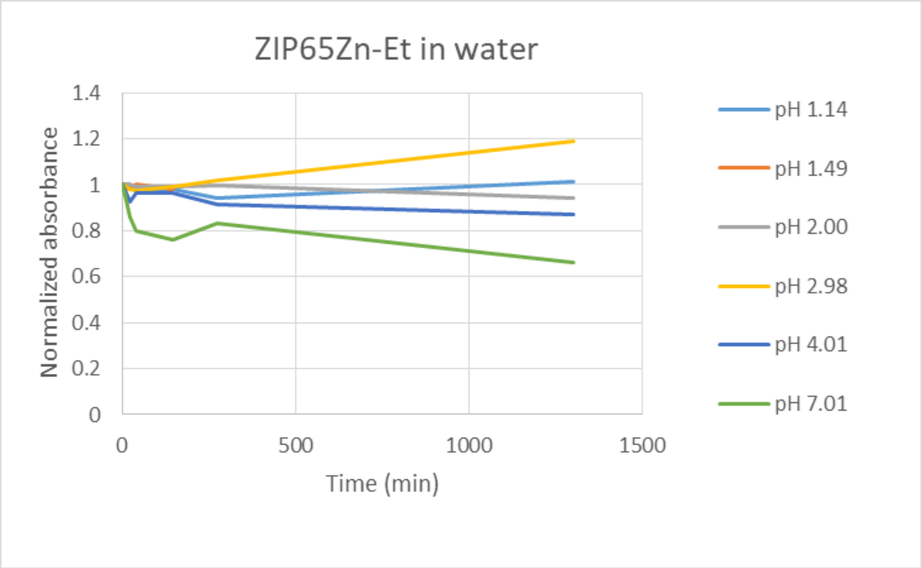
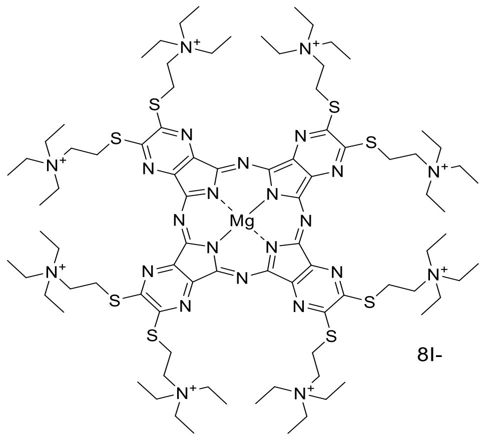


Figure 16 – Time-lapse graph of ZIP65Zn-Et

ZIP65Mg-Et



ZIP65Mg-Et

Figure 17 – The chemical structure of the compound with the code ZIP65Mg-Et

Subsequently we tested the magnesium complex of the same ligand. The experimental procedure was the same as for **ZIP65Zn-Et**. In the two most acidic buffers (pH 1.14 and 1.48), the spectra after dilution of the sample into the buffer indicated immediate demetallation as deduced from splitting of the Q-band. The spectra did not change further suggesting that the demetallation is complete just few seconds after mixing the solvents.

A different situation occurred at pH 2.00 where the changes were observable on the minute time-scale (Figure 18). In this case, the spectra were collected every 30 s and the full demetallation occurred just after 4 minutes after mixing the solution (Figure 18). Similar changes were observed also for the higher pH (pH 2.98, Figure 19) but here the full demetallation occurred after about 20 minutes indicating much slower reaction.

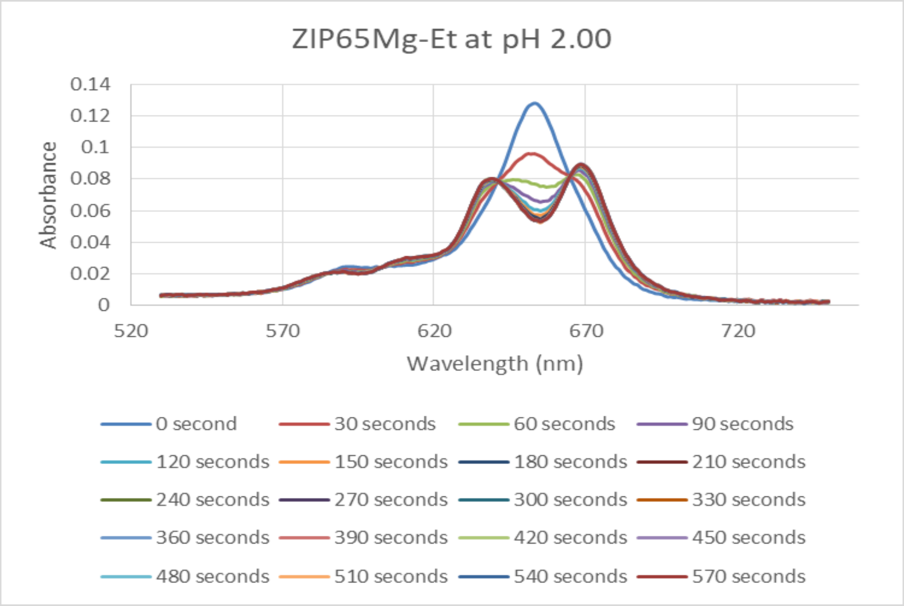


Figure 18 – The absorption spectra of ZIP65Mg-Et at pH 2.00 at selected times

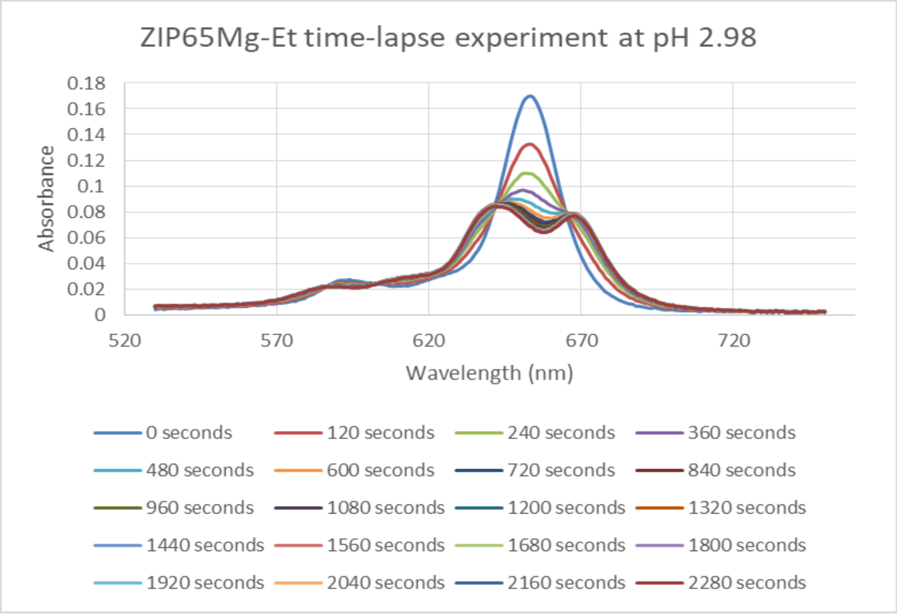


Figure 19 – The absorption spectra of ZIP65Mg-Et at pH 2.09 at selected times

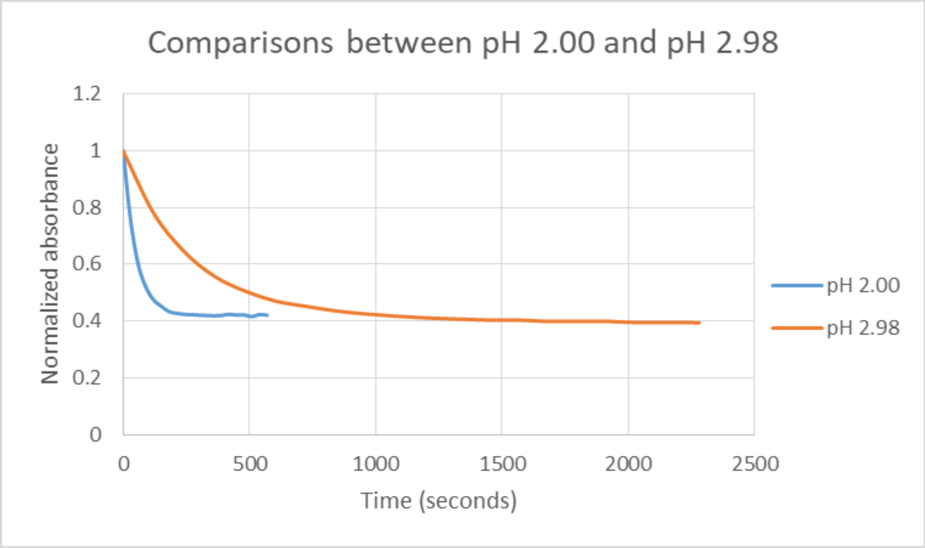


Figure 20 – Time-lapse graph of ZIP65Mg-Et at pH 2.00 (blue) and pH 2.98 (orange) collected at different times

At pH 7, we observed a small decrease in the absorption spectra during the first measurements. However there were no changes of the absorption spectra when we collected it after 5hrs 30mins (330 minutes). The absorption spectra remained the same even after longer time periods (Figure 21).

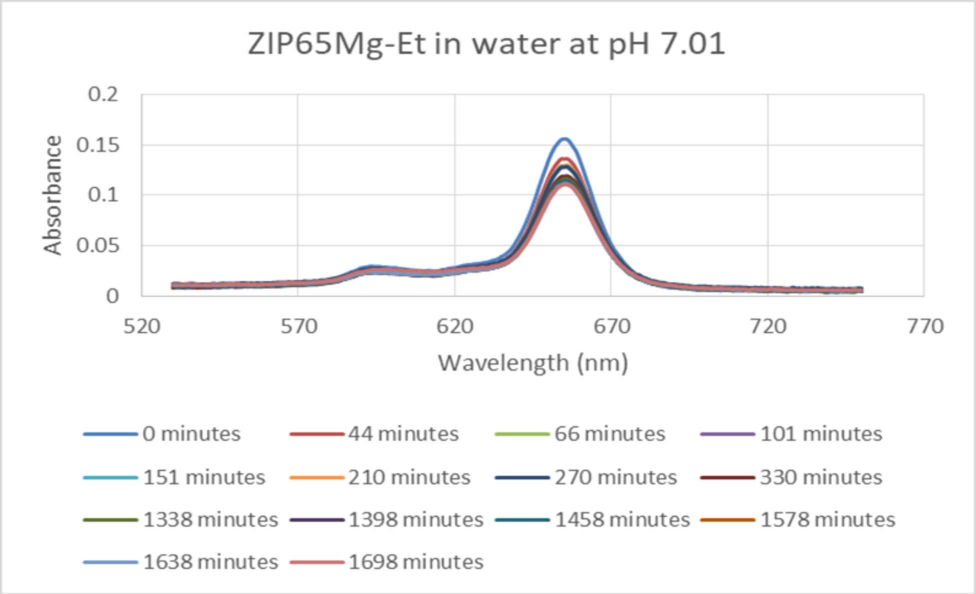
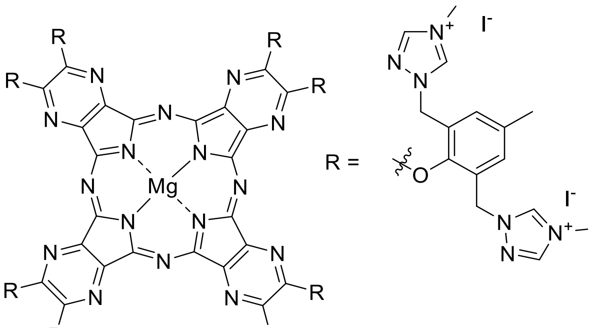


Figure 21 – The absorption spectra of ZIP65Mg at pH 7.01 measured in selected times

<u>9Mg-MeI</u>



9Mg-MeI

Figure 22 – The chemical structure of magnesium phthalocyanine and its bulky substituent

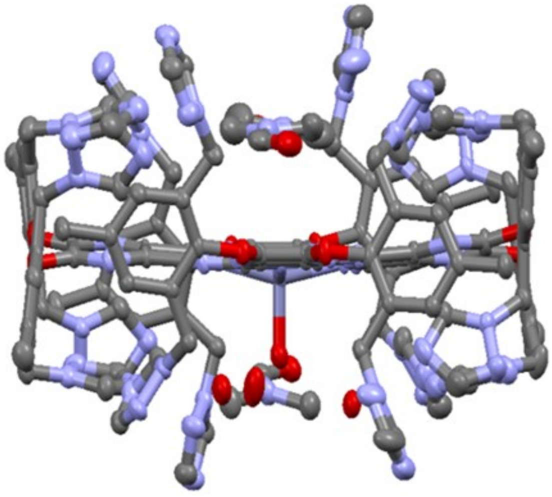


Figure 23 – Crystal structure (view in the plane of macrocyclic core) of 9Mg-MeI (CCDC 1530165). Color code: grey-carbon, blue-nitrogen, red-oxygen. Hydrogens were hidden for clarity.[10]

Regarding the compounds soluble in water, we also tested the compound with the code **9Mg-MeI**, whose chemical structure is distinct compared to the previous ones. The main difference is in bulky peripheral substituents that are located above and below the macrocyclic core as confirmed also by its X-ray crystallographic structures published recently. The behavior of **9Mg-MeI** is different from the **ZIP65Mg-Et**. The data of the absorption spectra indicates the formation of a metal-free complex of **9Mg-MeI** but the changes were not so fast as in the case of previous sample. Even at the lowest pH 1.14, we were able to observe not fully demetallated complex (Figure 24) just after mixing the solutions. At pH 1.14, the progress of the changes (Figure 24) was much slower than in the case of **ZIP65Mg-Et** and the complete demetallation was observed after 59 minutes that is significantly longer time than 20 min for the previous one. The bulky substituents are located perpendicularly to the central core, hence there is protection against attack of protons. From these experiments, we may conclude that the formation of a metal-free ligand occurs, but the rate of formation is much slower due to the protection by the bulky substituents.

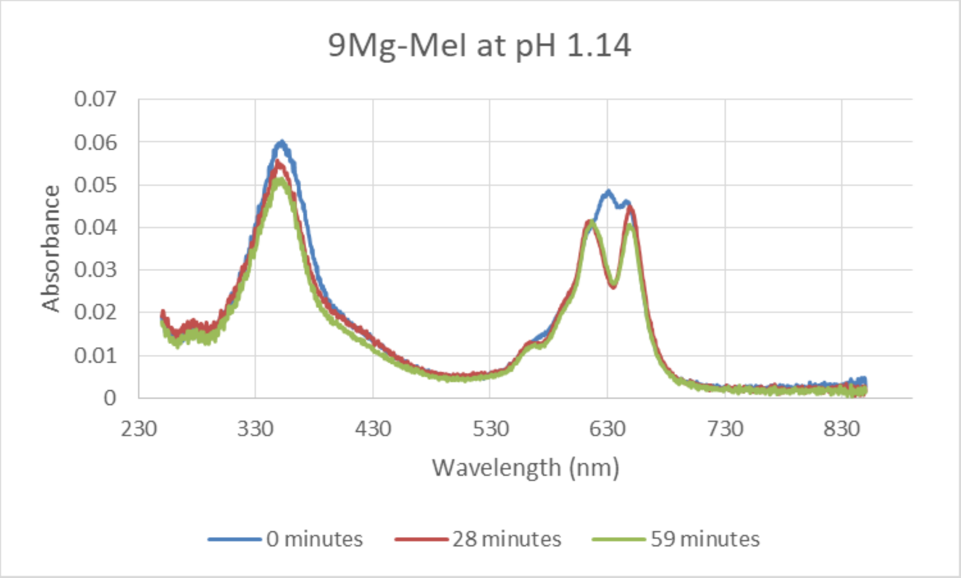
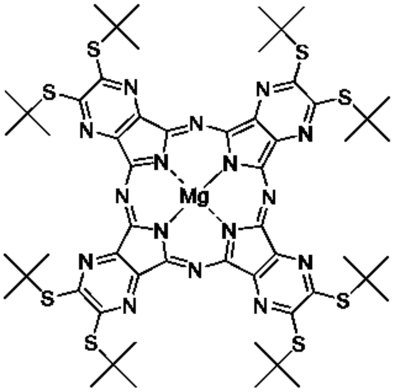


Figure 24 – The absorption spectra of 9Mg-MeI at pH 1.14 measured at selected times

ORGANIC SOLVENTS

ZIP21Mg in THF



ZIP21Mg

Figure 25– The chemical structure of the magnesium complex of phthalocyanine that we tested in tetrahydrofuran (THF)

Compound **ZIP21Mg** is not soluble in water but well dissolved in organic solvent. Its demetallation was therefore monitored in tetrahydrofuran (THF). First, the 1 μM solution of this compound was titrated by trifluoroacetic acid (TFA) (Figures 26 and 27). Upon the increase of the amount of acid certain changes ensued in the absorption spectra. First, there was a decrease in the Q - band maximum, accompanied with an increase of the red shifted band at 695 nm. These kind of spectral changes are typical for protonation of one of the azomethine nitrogens. This has occurred up to 600 μL of acid in solution. Further increase of the amount of acid leads to a decrease of the intensity of the new red-shifted band, and splitting of the Q band that are typical for formation of metal-free ligand. In this case, we suggest that demetallation occurs in two steps (Figure 28). First, the azomethine nitrogen is protonated and afterwards a metal-free ligand is formed. The absorption spectra of the metal-free form indicated that the metal-free ligand is not protonated on the azomethine nitrogen (the spectrum is typical for neutral metal-free derivative). It suggests that the basicity of the azomethine nitrogen is higher in the magnesium complex than in metal-free ligand.

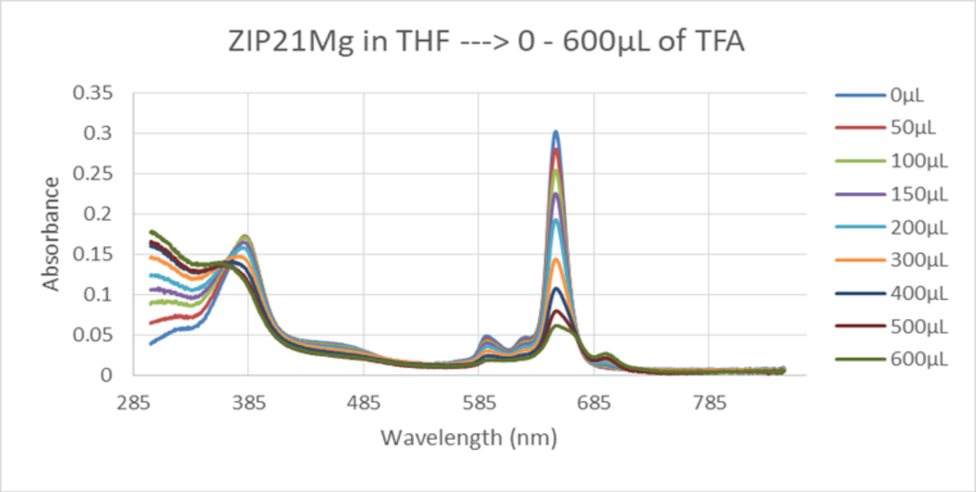


Figure 26 – The absorption spectra of ZIP21Mg in THF, up to the addition of 600 μL of TFA

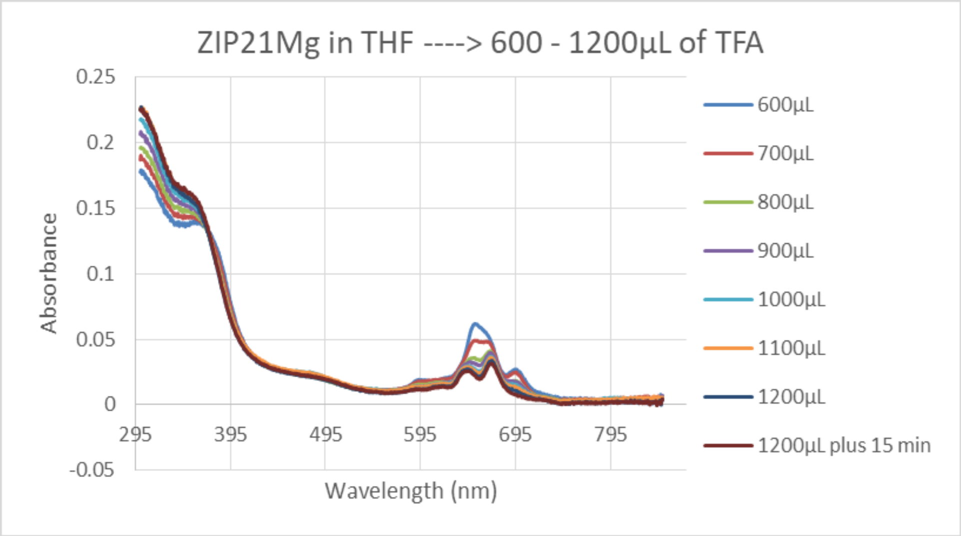


Figure 27 - The absorption spectra of ZIP21Mg in THF, up to the addition of 1200 µL of TFA

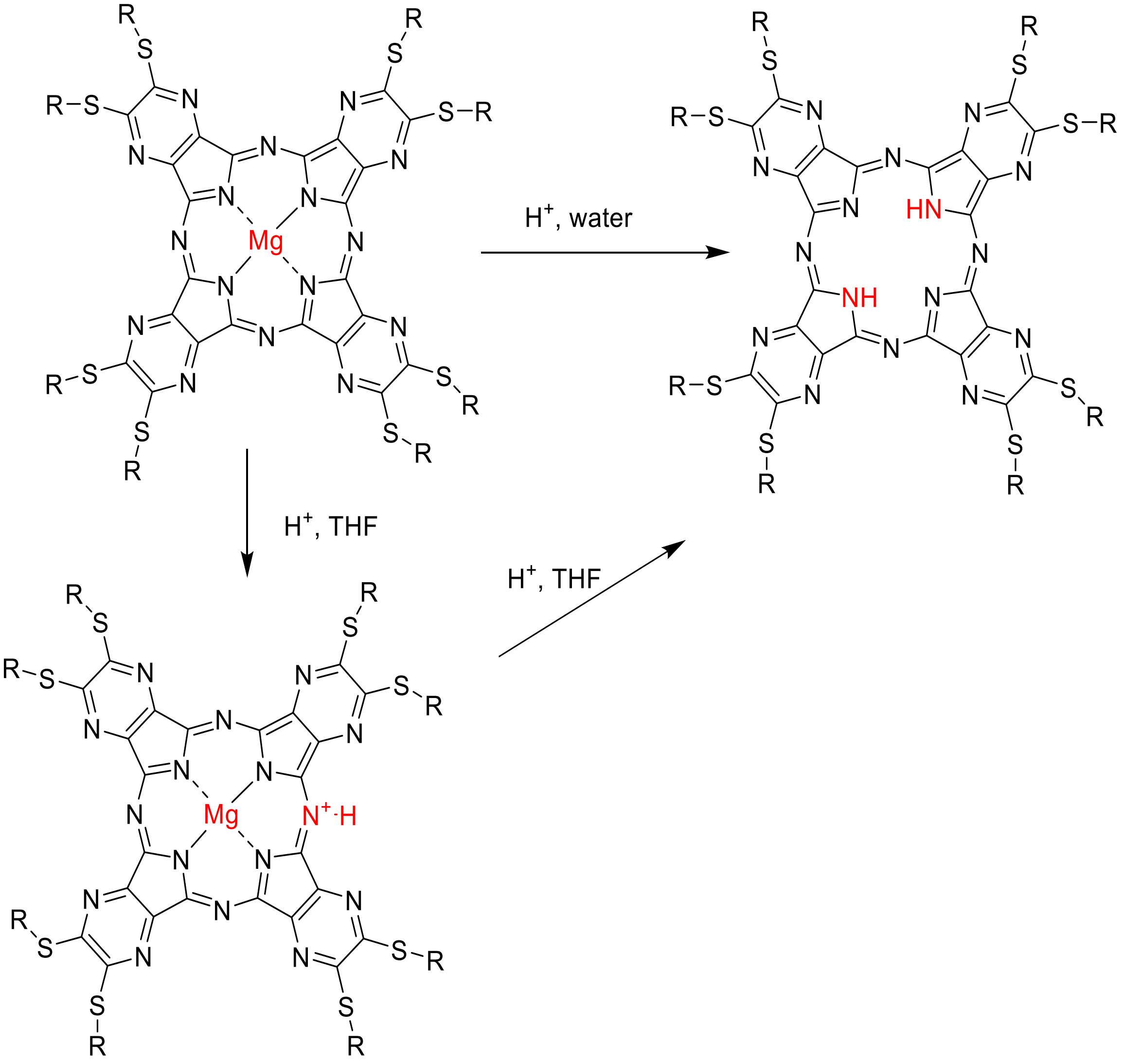


Figure 28 – Two different chemical reaction that refer to the formation of the metal-free ligand of the same compound in water and organic solvent. When the compound is dissolved in organic solvent, firstly it is protonated and then the formation of a metal-free ligand occurs. On the other hand, when the compound is dissolved in water, the formation of the metal-free ligand occurs immediately.

Another experiment was performed to see the changes in time.

Upon the addition of a fixed amount of acid, protonation ensues and it is indicated by the red shift band at 665 nm. After protonation, there is slow demetallation that is indicated by an increase in the red-shifted band and occurrence of typical splitting of the Q-band (Figure 27). It leads to conclusion that the demetallation is a function of both acid concentration and time. At low acid concentration (e.g. addition of 100 µL of TFA into 2.5 mL of THF), the demetallation is very slow and typically we detected only the spectrum of protonated species and the demetallation was almost undetectable even after a long time. On the other hand, full demetallation occurred almost immediately at higher acid concentration (addition of 1000 µL of TFA into 2.5 mL of THF). The amount selected on Figure 29 (addition of 500 µL of TFA into 2.5 mL of THF) allowed monitoring of the changes on a reasonable time-scale (Figure 30).

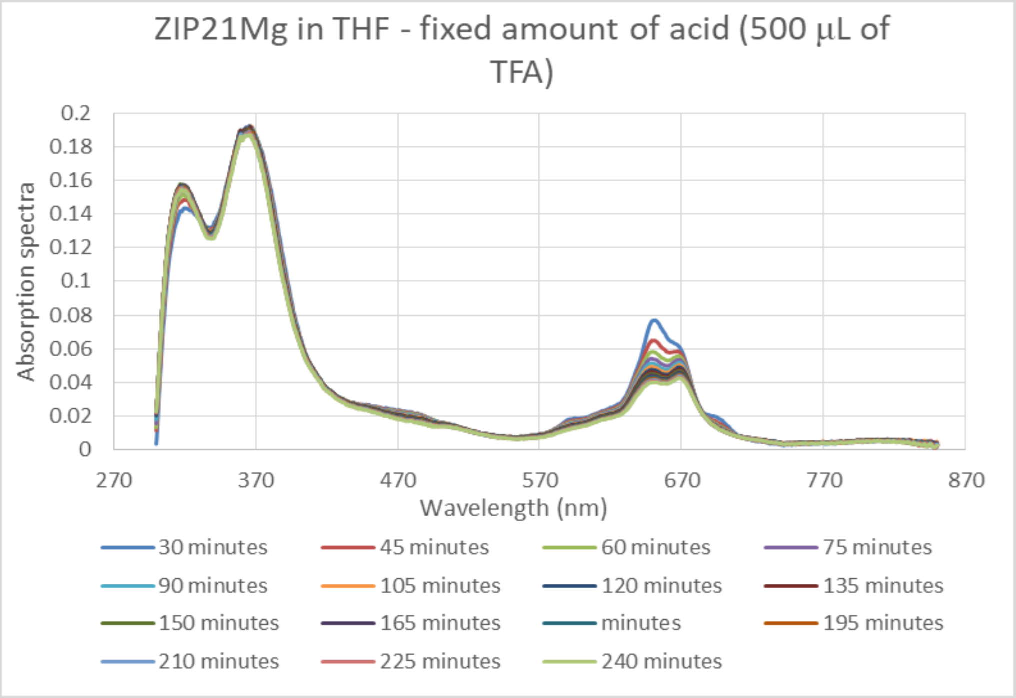


Figure 29 – The absorption spectra of ZIP21Mg monitored in time after dissolution in THF and addition of 500 µL of TFA

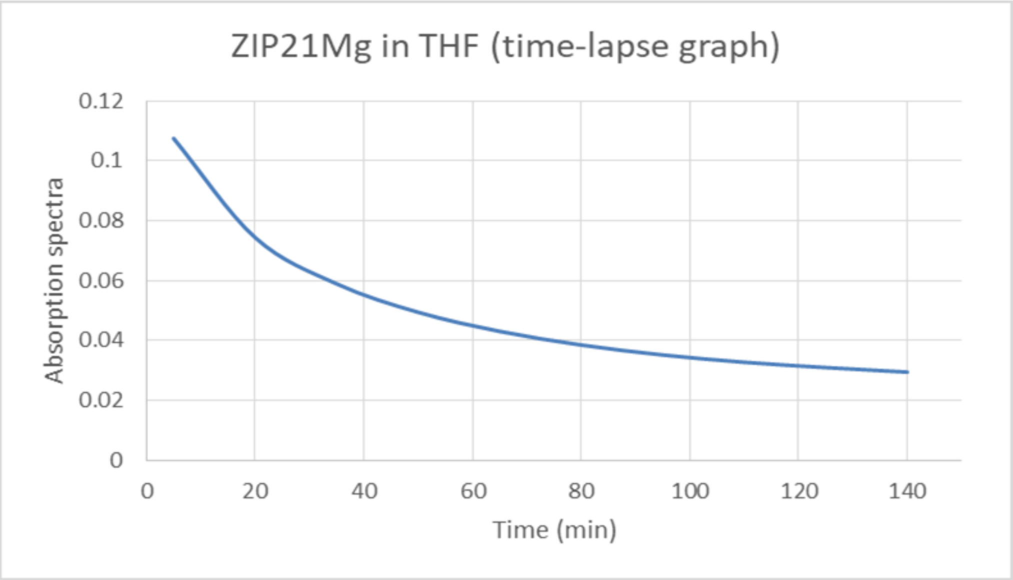


Figure 30 – The time lapse representation of the absorbance of ZIP21Mg in a fixed amount of TFA

DELIVERY SYSTEMS

The main idea of testing the compounds in the delivery systems, was to determine whether these delivery systems can serve as protection of the magnesium complexes against the acidity of the surrounding environment.

LIPOSOMES

ZIP21Mg in liposomes

ZIP21Mg is a lipophilic azapthalocyanine that is not soluble in water but dissolves well in lipidic bilayer of liposomes (or microemulsions, see below). The data collected from the absorption spectra shows that this magnesium complex is stable at the lowest pH and even after a longer time period such as 25 hours. The absorption spectra of **ZIP21Mg** at pH 1.14 in liposomes (Figure 31) did not change in time and were the same as the absorption spectra of **ZIP21Mg** in liposomes at pH 7.00. (Figure 32) or at other pH (Figure 33).

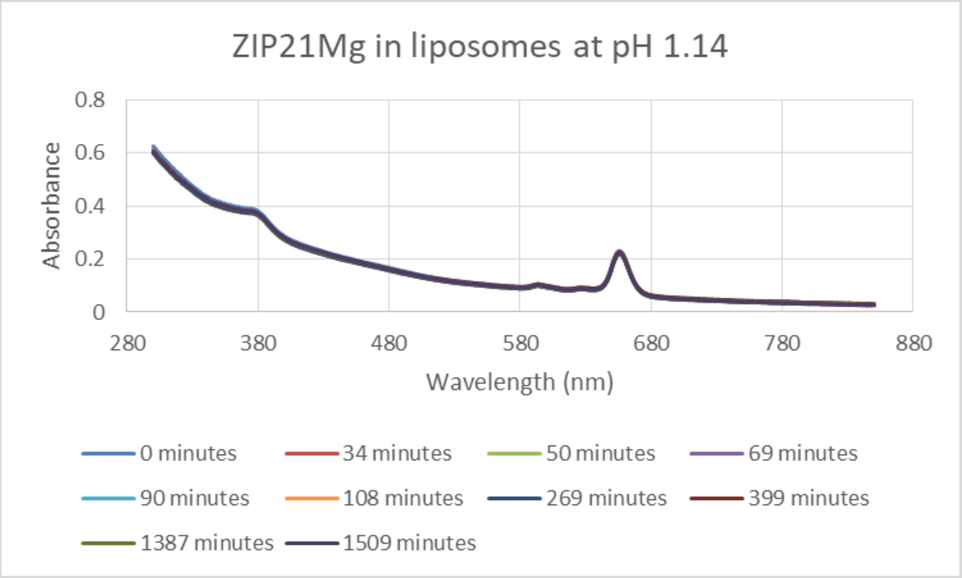


Figure 31 – The absorption spectra of ZIP21Mg in liposomes at pH 1.14 collected during a certain period of time

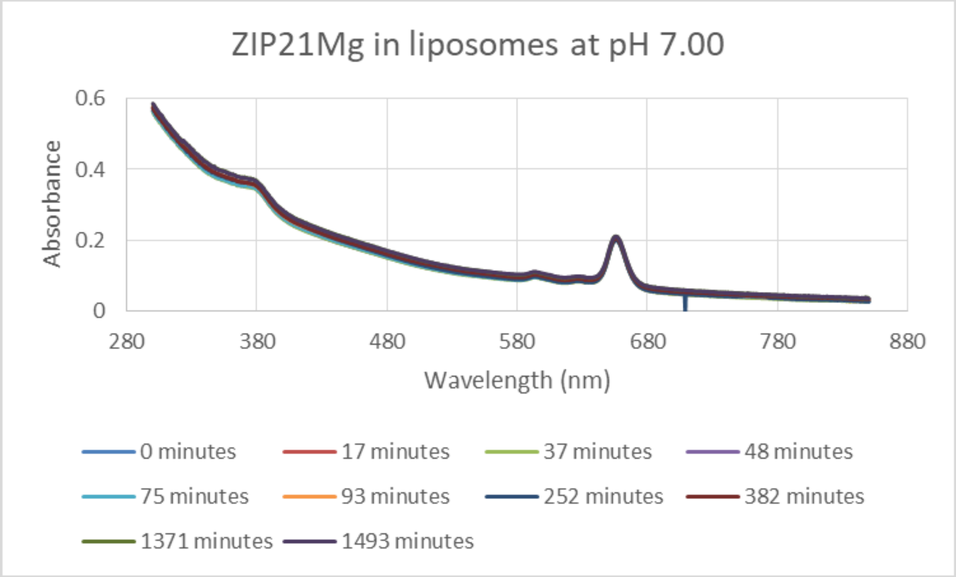


Figure 32 – The absorption spectra of ZIP21Mg in liposomes at pH 7.00 collected during a certain period of time

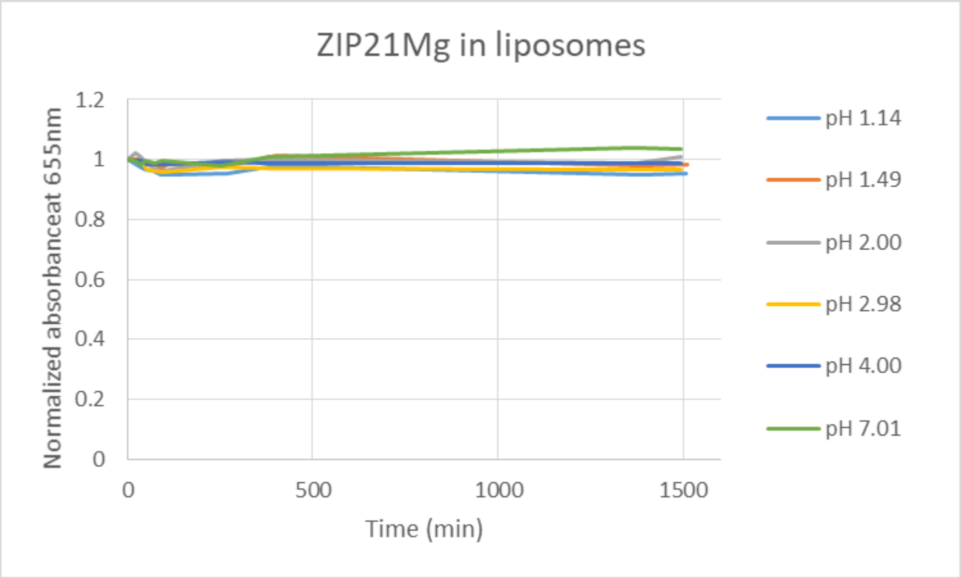


Figure 33 – Time-lapse graph of ZIP21Mg in liposomes measured at six buffers with different pH values. It represents the correlation of absorbance against the time interval

MICROEMULSIONS

We tested two different compounds in microemulsions (MEs) with the following codes: **ZIP21Mg** and **P9-0 Mg** (Figure 34). The only difference in these compounds was in their macrocyclic core, the peripheral substitution remained the same.

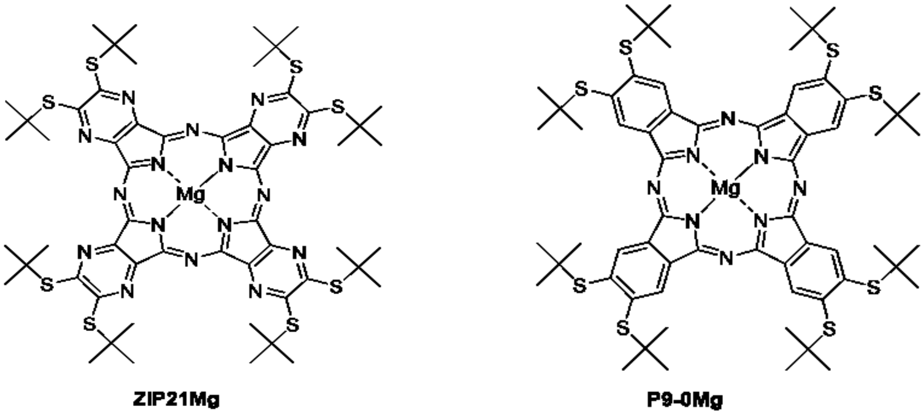


Figure 34 – The chemical structures of ZIP21Mg and P9-0Mg

ZIP21Mg in microemulsions

In the case of **ZIP21Mg**, microemulsions don't show a very good protection (compared to liposomes) from acidity of the surrounding environment. The absorption spectra of **ZIP21Mg** at pH1.14 show decrease of the Q-band indicating that the formation of a metal-free ligand occurs immediately after the second measurement in time of 11:57 min (Figure 35). Some small changes were observed at the absorption spectra of **ZIP21Mg** at pH7.00 (Figure 36), and at the other pH conditions (Figure 37).

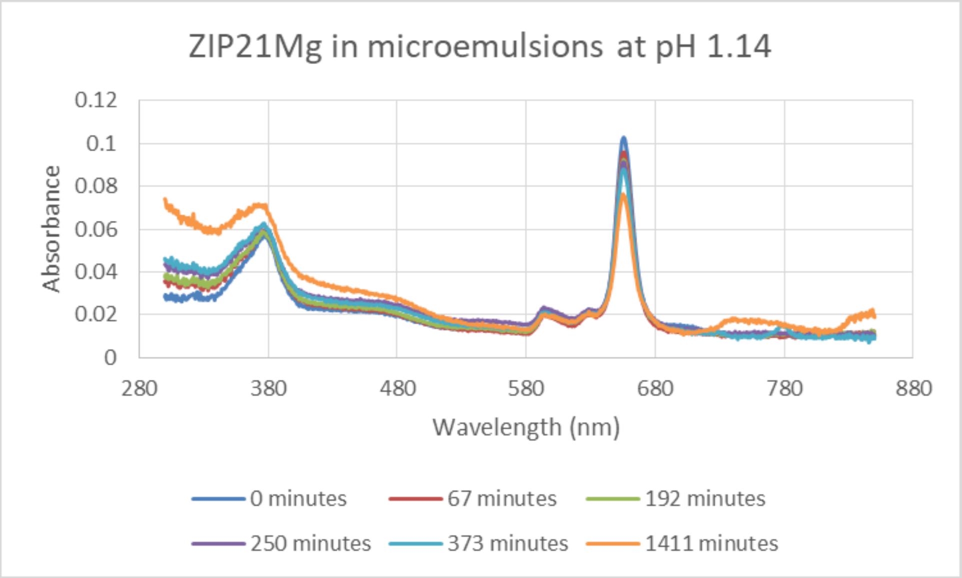


Figure 35 – The absorption spectra of ZIP21Mg in microemulsions at pH 1.14 collected at certain intervals of time

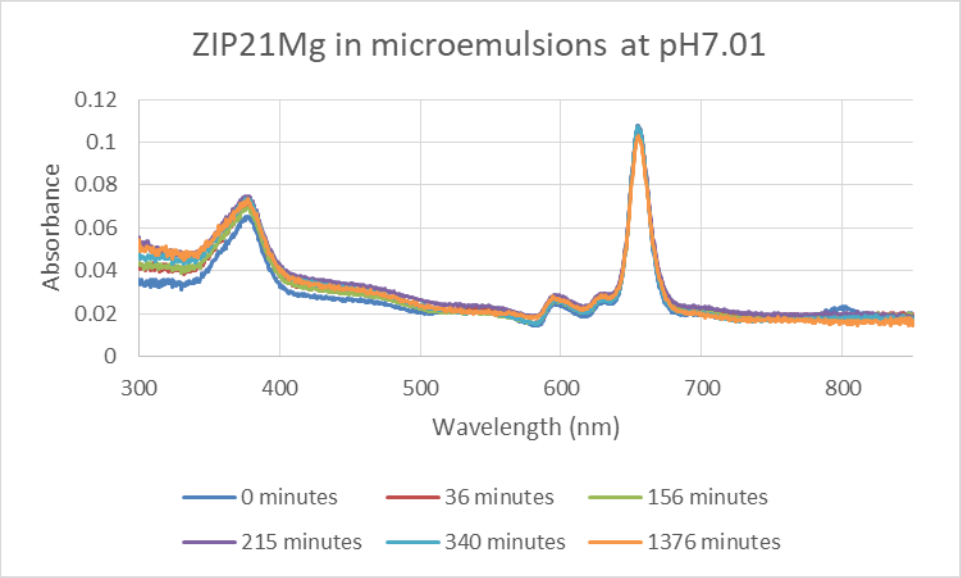


Figure 36 – The absorption spectra of ZIP21Mg in microemulsions at pH 7.01 collected at certain intervals of time

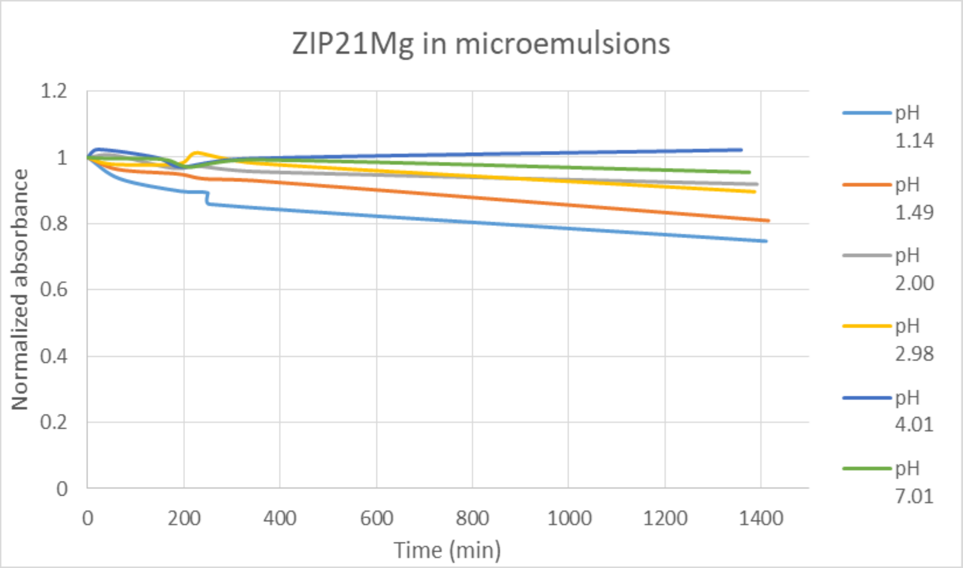


Figure 37 – Time-lapse graph of ZIP21Mg in microemulsions. It represents the correlation between normalized absorbance and time for all the samples tested in buffers in different pH values

P9-0 Mg in microemulsions

A similar scenario occurs for **P9-0Mg** in microemulsions. After the second measurement at pH 1.14, a significant decrease of the absorption spectra is observed that further progresses in time with occurrence of splitting of the Q-band typical for metal-free derivative (Figure 38). At higher pH, the changes were similar but the progress was much slower (Figure 39). No changes were detected at pH 7. These results may be compared to those obtained for **ZIP21Mg** indicating that the stability of the previous complex was substantially higher than for **P9-0 Mg**.

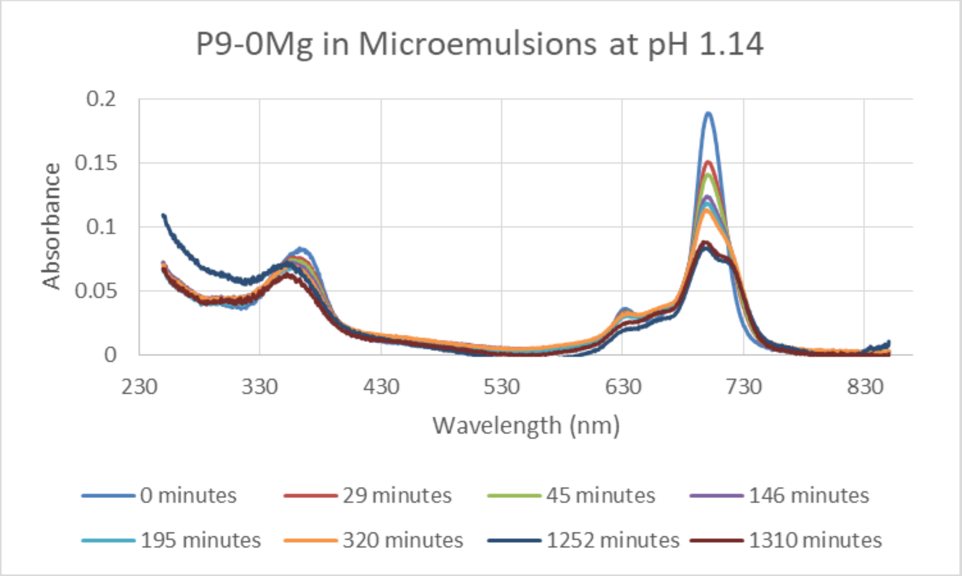


Figure 38 – The absorption spectra of P9-0Mg in microemulsions at pH 1.14 collected at certain intervals of time

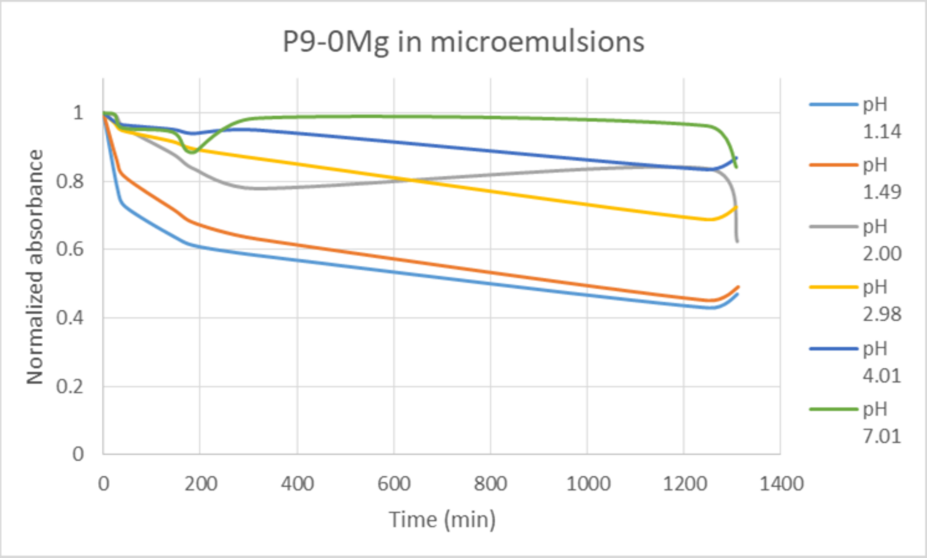


Figure 39 – Time-lapse graph of P9-0Mg in microemulsions collected at buffers with different pH values. The graph represent a correlation between normalized absorbance and the time interval

NANOPARTICLES

In the case of silica nanoparticles, the concentration of the compound was too low to see any differences in the absorption spectra. For this reason, we monitored the changes using the fluorescence spectra. Metal-free derivatives of both **ZIP21Mg** and **P9-0Mg** have much lower fluorescence and that is why the potential decrease in the fluorescence intensity might be due to demetallation (Figure 40 and 41). Indeed, we observed a decrease in the emission intensity during the experiment. However, the changes were observed independently of the pH that may lead to other explanation than demetallaton. The nanoparticles were most likely aggregating. On the other hand we cannot exclude also the possibility of potential demetallation of the magnesium complexes.

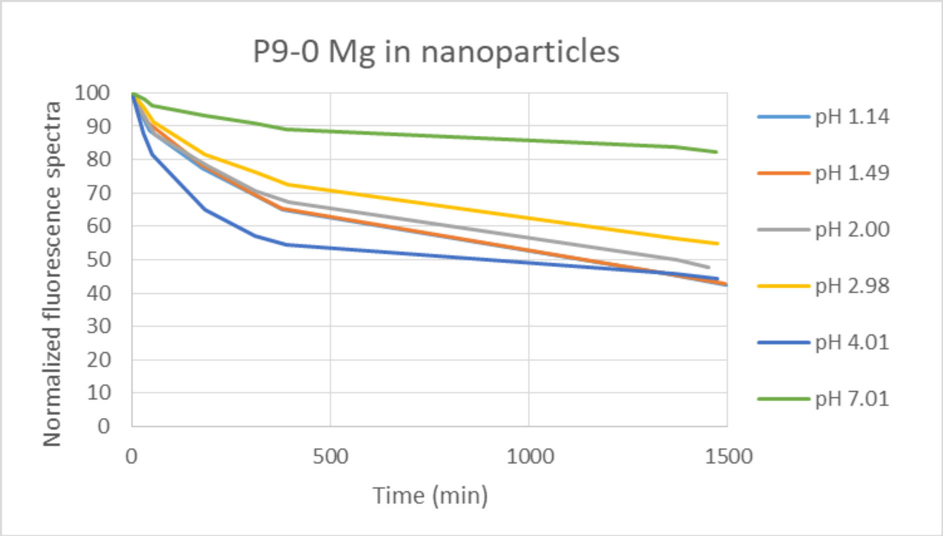


Figure 40 – Time-lapse graph of P9-0Mg in nanoparticles. The graph represent a correlation between normalized fluorescence spectra and time interval

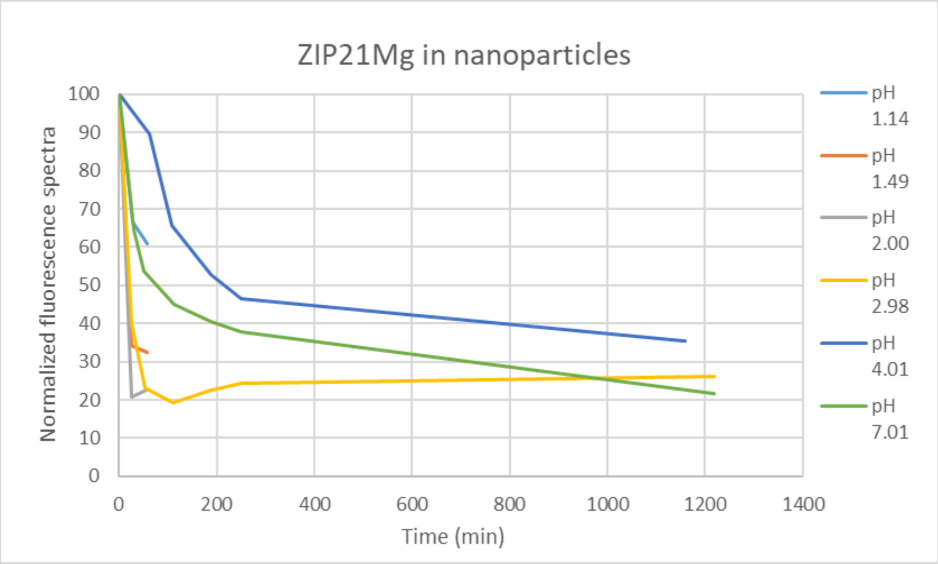


Figure 41 – Time-lapse graph of ZIP21Mg in nanoparticles. The graph represents a coorelation between normalized fluorescence spectra and the time interval

CONCLUSION

The purpose of our work was to test the stability of magnesium phthalocyanines in water and organic solvent (tetrahydrofuran) and in three different delivery systems (liposomes, microemulsions and nanoparticles).

According to the results that we have obtained, **ZIP65Mg-Et** is not very stable in water compared to the zinc complex of the same ligand. Immediate demetallation of **ZIP65Mg-Et** occurred after the stock solution of the sample was added the most acidic buffers (pH 1.14 and 1.49). Nevertheless, the process of demetallation was detectable in buffer with pH 2.00 and the absorption spectra was collected every 30 s. A similar situation occurred at pH 2.98, but full demetallation occurred after 20 minutes, indicating a much slower reaction rate.

We also tested another compound soluble in water with the code **9Mg-MeI**, whose chemical structure is similar to the previous compound. The main difference is that **9Mg-MeI** contains bulky peripheral substituents, which protects the macrocyclic core from the acidity of the surrounding environment. Hence, from the absorption spectra of **9Mg-MeI** it was detectable that the formation of a metal-free ligand occurs, but in a much slower rate.

When the compound with the code **ZIP21Mg** was dissolved in tetrahydrofuran, protonation preceded to the formation of a metal-free ligand. The formation of a metal-free ligand was proportional to an increase amount of acid.

When it comes to delivery systems, we incorporated phthalocyanine complexes in three different delivery systems (liposomes, microemulsions and nanoparticles). We may conclude from our results that liposomes offer the best protection against acidity from the three delivery systems that we have tested. The absorption spectra of **ZIP21Mg** at pH 1.14 did not change in time and it was the same as the absorption spectra of **ZIP21Mg** at pH 7.00. On the other hand, when the same compound was incorporated in microemulsions, the formation of a metal-free ligand occurred after approximately 30 minutes. From the following experimental results we may also conclude that liposomes offer better protection against acidity compared to microemulsions.

In the case of silica nanoparticles, the concentration of the compound was too low to collect the absorption spectra, so we were able to collect only the fluorescence spectra. We observed the decrease of the fluorescence for both **ZIP21Mg** and **P9-0Mg** that might occur due to demetallation. However, it is not certain whether the decrease of fluorescence is occurring due to the aggregation of nanoparticles or the demetallation of the magnesium complexes.

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