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SIGRID SELBERG

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Author´s contribution

Paper I. Lead author in preparing the manuscript. Performed all the synthesis and most of the measurement experiments.

Paper II. Lead author in preparing the manuscript. Performed all the experiments.

Paper III. Lead author in preparing the manuscript. Performed all the synthesis and most of the measurement experiments.

ABBREVIATIONS

λ_{abs}	Wavelength of absorbance maximum
λ_{em}	Wavelength of emission maximum
ΔpK_a	Relative pK_a value
A^-	Counterion
ATR-FT-IR	Attenuated total reflection Fourier-transform infrared
HPLC	High performance liquid chromatography
HRMS	High-resolution mass spectra
IFE	Inner filter effect
ISFET	Ion-sensitive field-effect transistor
log*D*	Logarithm of distribution ratio of a compound between the water-immiscible and aqueous phases
log*P*	Logarithm of partition coefficient of the neutral form for ionizable compounds
MeCN	Acetonitrile
NMR	Nuclear magnetic resonance
Ph	Phenyl group
pK_a	Negative logarithm of the dissociation constant
pK_a^{ow}	Biphasic pK_a value in 1-octanol/water system
pK_{ac}^{ow}	Concentration based pK_a^{ow} value
pyrr	Pyrrolidino group

INTRODUCTION

The pH value is among the few chemical parameters that are widely measured and are highly important in many fields. In recent years there are more and more applications (real time pH monitoring in human tissue[1] or blood[2], pH effects on viruses[3], in geology[4] and oceanography[5]) that require improved methods for pH sensing. Among the possible approaches optical sensing is increasingly receiving attention. The design of an optical pH sensor is not trivial. There are a number of important properties of the pH indicator molecule and the matrix that need to be considered for a certain application which sets restrictions on the characteristics of the sensor. The key features of the indicator molecule that need to be considered are pK_a value, spectral properties (high extinction coefficient, absorption/excitation and emission band in the visible region, a large Stokes shift), lipophilicity, chemical and photo-stability. In specific applications polarity/hydrophilicity may be essential, since the matrix is usually a non-polar proton-permeable polymer membrane.[6,7]

Although a large number of acid-base indicators have been reported[6,8], the majority of available indicators have well-defined charged groups in at least one of their charge states. Therefore the charged form tends to be hydrophilic and is prone to leaching out from the non-polar membrane into the aqueous test solution. This reduces the lifetime of the sensor and contaminates the test solution. There is a need to design indicator molecules that are hydrophobic in both neutral and charged forms. Such molecules would not be easily leached out of non-polar membranes. Additionally, they could act as "silent spectators" in systems where indicators are directly added, because they would have limited capability of specific interactions.[6,9] In addition, such molecules should be photochemically and chemically stable with pK_a in the basic region at pH > 7.[6,7] Currently there is also lack of such molecules.

In order to be able to measure pH in an extended range we could in principle use indicator molecules with several pK_a values. However, that would immediately lead to multiply charged species, which are very likely to be highly hydrophilic. An alternative approach taken in this thesis is using a group ("family") of similar indicator molecules with different pK_a values.[6,7,10]

The main aim of this thesis was to design and synthesize a family of novel lipophilic indicators – active both via visible absorption and fluorescence. A major requirement was a high lipophilicity of both neutral and charged forms, thus lack of localized charges in the cationic forms. In addition, these compounds should have absorbance maxima in the visible spectral range upon protonation/deprotonation or even more preferably have a strong fluorescence. Also, the spectral properties have to change upon protonation/deprotonation. An advantageous feature would be easily tunable range of pK_a values which depends on used substituents. Phosphazene (iminophosphorane) structure – known for its basicity and lipophilicity – is proposed in this work as the basicity center. This structure is then modified by adding an absorbing and/or

fluorescing moiety. Such structures are expected to allow the synthesis of 0/+1 charge type indicators which satisfy the above requirements.[11,12] Phosphazenes are well-known as strong bases but have not been used in design of indicators.

An additional aim was to create an original method for quantifying acidity/basicity of lipophilic molecules in practically water-immiscible solvents (biphasic pK_a values).

1. LITERATURE OVERVIEW

1.1. Phosphazenes

Phosphazenes (iminophosphoranes or phosphine imides) are compounds of general structure $RN{=}PR'_3$ in which a nitrogen atom is covalently linked to a tetracoordinate phosphorus atom by a (formally) double bond. The nitrogen atom is linked by single bond with the R, which in this work is an aromatic ring with different substituents. Additionally, the phosphorus atom is linked with three other groups (R′) by single bonds. This work is mainly focused on the pyrrolidino and phenyl groups. Although they are structurally similar to phosphorus ylides ($RHC{=}PR'_3$), the analogous phosphazenes are appreciably more stable.[12–14] Phosphazenes are chemically stable to oxidation and hydrolysis and are easily available which is why phosphazenes are widely used in organic synthesis.[12,15–17]

The nature of the phosphorus–nitrogen double bond in phosphazenes is important for understanding the basicity and spectral data of these compounds.[13] Phosphazenes behave similarly to phosphorus ylides, where the phosphorus – carbon bond can be regarded either as a formal double bond (ylenic) or a formal single bond between two oppositely charged centers (ylidic), *i.e.* a zwitterionic structure.[11,13] The ylidic structure is generally considered to have higher contribution, as evidenced by computational and experimental results. It has been shown that in phosphazenes there is a strong contribution of the zwitterionic (ylidic) structure.[13] This means that the nitrogen atom in the P=N fragment should rather be presented as $P^+–N^-$. The N^- center is largely isoelectronic with the oxygen in a phenolate anion. This causes a significant delocalization of the electrons into the aromatic ring if attached to the imino group, which in turn is expressed by significantly lowered basicity, as compared to alkyl substituents on the imino nitrogen. This has been confirmed by basicity measurements of both phosphazenes[13] and phosphorus ylides.[18]

Phosphazenes are well known as strong bases, since the ylidic structure dominates the ylenic structure. Therefore the imino nitrogen owns a significant negative partial charge and serves the site for protonation. Basicity of phosphazenes is easily predictable and tunable by substituents that are bonded with the phosphorus or the nitrogen atom. Therefore it is easy to design a phosphazene structure with desirable pK_a value [11,12,14,18–20]. For example unsubstituted $PhN{=}P(pyrr)_3$ is a strong base (pK_a = 22.6 in MeCN), adding substituents to phenyl ring it is possible to modify pK_a value over a wide range (pK_a = 14.12 2,6-$(NO_2)_2$-$PhN{=}P(pyrr)_3$, pK_a = 23.9 4-$(CH_3)_2N$-$PhN{=}P(pyrr)_3$).[20] Alkyl phosphazenes tend to be stronger bases than aryl phosphazenes ($EtN{=}P(pyrr)_3$ pK_a value is 28.89[20] and $PhN{=}P(pyrr)_3$ pK_a value is 22.17[20]).

1.1.1. Preparation of phosphazenes

There are two different approaches to prepare phosphazenes, the direct coupling of nitrogen and phosphorus atoms or the indirect approach of substituting on a previously prepared phosphazene.[14] For the direct preparation of N-substituted phosphazenes two main and generally applicable routes are used, the Staudinger reaction of alkyl or aryl azides with tertiary phosphanes and the Kirsanov reaction of amines with PCl_5 or tertiary phosphane dibromides.[12,14,20] The Kirsanov reaction is known to work better with simpler phosphazenes.[12,20]

1.2. Optical pH sensing

A large number of chemical reactions and processes are pH dependent, therefore a rapid and continuous pH determination is important in many fields.[6,9]

Electrochemical, using a glass electrode or the ISFET electrode, pH determination method is the most commonly used.[10,21] The electrochemical pH determination is a fast and reliable method, however there are some bottlenecks and problems using the electrochemical sensing. Especially when the aim is to measure the pH value in small systems like continuous pH determination in cells (pH-sensitive dyes that could be loaded into the cytosol) or *in situ* measurement in blood.[2,3,22] Potentiometric pH electrodes are too sizeable for described applications. In addition, measuring pH with potentiometric electrodes in environments with a low ionic-strength, in non-aqueous solvents is rather problematic and the electrodes show a rather poor performance in both extremes of the pH scale, especially in the highly alkaline region.[10,21,23] The alternative way to get over these bottlenecks is an optical pH sensing.[2,6] Optical sensors operate through changes in their optical properties such as absorption, fluorescence intensity and decay time, or reflectivity upon variations in pH value of the environment.[6]

Some oldest optical pH sensing methods involve an indicator compound simply in a test solution or bound on a paper (pH stripes).[6] Current trends are using different optrodes or indicator molecules loaded into the tissue for pH determination.[1,2] Designs of the optical sensors can be very miniature (down to sub micrometer dimensions and do not require a separate reference sensor), flexible and durable, and therefore offer a competition for the electrochemical pH electrodes.[6] However, the optical pH sensing also has some bottlenecks. Since the indicator molecule is generally bound into a low polarity medium (polymer membrane[8], sol-gel films[24], hydrogels[25]) and the pH is measured in aqueous solution we need to consider a diffusion and according to this a response time. If the diffusion is slow, then the response time can reach up to several minutes. Other problem is a narrow working range, in the ideal case the same sensor can be used to measure pH value in the range of 0–14. Typically one indicator molecule can be used in a narrow range, sensing 2–3 pH units, to measure the whole range (0–14) we need to use several different indicator molecules.[6,10,25]

1.2.1. Design of an optical pH sensor

As mentioned before, different materials have been used to design an optical pH sensor. The suitable indicator molecule is usually bound into the solid matrix (polymer, sol-gel and hydrogel membranes) using adsorption, physical entrapment or covalent binding. Such matrices provide permeability, flexibility, as well as mechanical and chemical stability of the sensor. The target analyte is able to diffuse into the matrix and react with the entrapped indicator molecule. It is important to consider that the properties of the indicator molecule may change when it is bound into the matrix, pK_a shift can occur or spectral properties may be altered.[6,7]

There are many different indicator molecules that can be engaged in the sensor application. Most common absorption-based pH sensor operation is based on a color change of a sensor layer in which a pH indicator dye is encapsulated. Examples of such dyes are phenolphthalein, phenol red, bromocresol green. Emission-based pH indicator molecules generally offer better selectivity and sensitivity than absorption based pH indicators. The best known and used fluorescent indicator molecules are 1-hydroxypyrene-3,6,8-trisulfonic acid (HPTS), fluorescein and their derivatives.[6,7,26] In addition, emission-based sensing enables a wide range of measurement techniques: based on fluorescence intensity, lifetime, energy transfer, and polarization.

Adsorption is not very reliable as the binding method, since the indicator molecule may easily leach out from the matrix. Therefore the adsorption method is not widely used in sensor design.[6,7]

In the case of covalent binding the indicator molecule is covalently bound into the polymer membrane or is bound on the surface of the membrane. Therefore the preparation of the senor is more complex and time-consuming and requires appropriate indicators and matrices with functional groups suitable for the immobilization, but there are less problems with the indicator molecule leaching out, low diffusion and the sensor has a longer lifetime. The design is even more sophisticated when binding several different indicator molecules.[6] In addition, the working range have to be specified, since the covalent bond may be pH dependent (C-O-C bond is suitable for sensing neutral pH, but is labile in acidic and basic solutions[27]).

A relatively easy method is mechanical binding (physical entrapment): the indicator molecule is non-covalently bound into vacancies in a polymer membrane. Generally the method presumes an indicator molecule with a higher molecular weight and a small analyte molecule that can migrate into the polymer layer. It is a rapid and straightforward method, the indicator molecule is dissolved into a solution of the polymer and then thin films are prepared. In particular, it is useful when there is a need to bind several different indicator molecules.[6] Still, there are some bottlenecks like potentially short lifetime, when binding is not strong enough and the indicator molecule can leach out of the polymer membrane.[24,28,29] The leaching out can be avoided by using highly lipophilic indicator molecules and polymer with small pores.[6] Both neutral and

charged forms of such indicators are highly lipophilic, thus lack localized charges in the cationic/ anionic forms, and therefore, exhibit poor water solubility

1.3. Lipophilicity

The most common measures of lipophilicity are the logarithms of partition (log*P*) or distribution (log*D*) coefficients.[30] Lipophilicity is an important property in biochemistry, toxicology and medicinal chemistry[31] as well as in chemistry related to sensors, receptors and indicator molecules embedded into polymer matrix. Reliable estimates of lipophilicity can be used to increase an efficiency of technological processes involving liquid extractions[32], predict physicochemical and physiological properties[33] of compounds and designing sensors with a longer lifetime[24,34–36].

The most widely used and important solvent combination is the n-octanol/ water system. The solvation properties of octanol saturated with water are similar to the solvation properties of cell membranes[37–39]. Therefore logP_{ow} and logD_{ow} are useful descriptors for properties related to distribution, absorption, metabolism, and excretion properties. Out of these two parameters logP_{ow} is more commonly used. There have also been studies measuring partition coefficients between other organic solvents like toluene or alkanes and water.[33,37,40] The partition coefficient (P) is a ratio of equilibrium concentrations of the unionized form of a compound in organic solvent and water which are mutually saturated. The partition coefficient describes the lipophilicity of the unionized form of the compound and is independent of pH. The distribution coefficient (D) is a ratio of total concentrations of all forms of an ionizable solute in the system and therefore is dependent on pH value. In this work the D values were directly measured, because it was impossible to measure the P values directly due to the very low solubilities of the phosphazene bases in water.

1.4. Methods for pK_a determination

The acid dissociation constant (pK_a) is one of the most frequently used physicochemical parameter, and determination of pK_a is beneficial to a wide range of research fields. In this chapter we discuss pK_a measurement methods that are suitable for implementation in solutions. In general, several different methods are known to be used for the determination of pK_a values for example potentiometry, spectrometry, liquid chromatography, nuclear magnetic resonance spectroscopy and calorimetry.[13,41–43] Generally any method which allows the quantitative measurement of a certain pH dependent parameter can be used for pK_a determination.[41]

The pKa determination methods can be divided into two groups – absolute and relative methods. The solvated proton activity is required to determine

absolute pK_a value, which is easy to measure in water using a pH-meter. The relative pK_a determination method can be used in non-aqueous solvents, since in the non-aqueous solvent the measurement of pH is frequently problematic.[44] In the case of relative pK_a methods it is possible to find the difference of dissociation constants of two compounds without measuring pH values directly.[13,45]

One of the most used method for pK_a determination is spectrophotometric method for relative pK_a measurements.[41] A requirement for the spectrophotometric measurement is the presence of a chromophore close to the ionization site in the molecule. Therefore, the spectra of the protonated and the deprotonated form can be expected to differ. In principle any wavelength can be used for the determination of pK_a, except at the isosbestic point at which wavelength of both forms have the same molar absorptivity. The best choice is a wavelength range where there is the largest difference between the absorbances of the protonated and deprotonated forms.[41] In addition, two bases are needed for determination of the relative pK_a value. One with previously known pK_a value (reference base) and a compound of interest with an unknown pK_a value.[11,13,46] The relative spectrophotometric method and calculations are described in detail in refs 13,45–47.

Over the years, a large number of data collections of pK_a values have been published in various solvents and their mixtures.[43] These pK_a values work really well when using pure solvents or their mixtures. Despite that, these pK_a values are not adequate for description of the solute properties in biphasic systems. Biphasic system here stands for a water-immiscible medium that is at equilibrium with water, and contains a lipophilic acid or base. Due to the low aqueous solubility the acid or base resides mainly in the water-immiscible medium. It can be envisaged that biphasic pK_a values will be useful in different fields[48]: in catalysis where biphasic systems or emulsions are used,[49] in sensors based on polymeric membranes,[6,50] in any processes with involvement of cell membranes)[23].

In this work an original method for determination of biphasic pK_a values is presented for the first time.

2. EXPERIMENTAL SECTION

2.1. General procedures

The UV-Vis spectra (from 190 to 1100 nm, with 1 nm steps) of compounds, spectra for measurements of biphasic pK_a values and for correcting the inner filter effects (IFEs) to fluorescence spectra were measured with an Evolution 300 UV-Vis spectrophotometer (Thermo Nicolet) in the double-beam mode. The instrumental settings included 2 nm resolution and intelliscan (scan speed 120 – 1200 nm/min). Concentrations of compounds for measuring absorption spectra were in the order of $n \cdot 10^{-5}$ mol L^{-1}. Concentrations in the case of measurements for IFE corrections were in the order of $n \cdot 10^{-6}$ mol L^{-1}. The pK_a values in MeCN were measured using different UV-Vis spectrophotometers from Perkin-Elmer (Lambda 12 or Lambda 40) and Agilent (Cary 60). The spectrophotometers were connected to a glovebox using a fiber-optics accessory. The measurement method consisted in determining ΔpK_a values of two compounds and has been described in refs 13,15,46,51.

The fluorescence emission spectra were measured using a Horiba FluoroMax-4 spectrofluorometer and were corrected for IFE. Lifetime measurements were carried out with a portable filter-based phase shift fluorimeter (Tau Theta, Boulder, CO-MFPF-1 M model). The LED modulated light source of 405 nm was operated at 1 MHz. Detection was performed by an on-board avalanche photodiode. The lifetime instrument was calibrated against coumarin 6 known lifetime (2.39 ns[52]).[53] The ultrasound deoxygenation was used as described in ref 54.

Methods to verify the compound molecular structures (HRMS, ATR-FT-IR and NMR) and detailed information of measurements are presented mainly in work I.

Analysis of the aqueous, octanol and toluene phases obtained during lipophilicity measurements were made with an Agilent 1200 liquid chromatograph equipped with a 5-wavelength UV-Vis detector. The stationary phase was Zorbax Eclipse XDC-C18 (Agilent) with column dimensions 4.6 × 250 mm and average particle size 5 μm.

The origin and quality of the used chemicals are presented in works I, II and III.

In this work two different methods for pK_a determination are involved: pK_a measurement in MeCN and biphasic pK_a measurement in the octanol/water system. Due to the high lipophilicity and very low aqueous solubility of the synthesized phosphazenes **1a**–**4d** (Scheme 1), it was impossible to measure their pK_a values directly in water. The pK_a values were directly measured in MeCN and in an octanol/water system (biphasic pK_a values). The latter measurement method is novel and was created in the framework of this thesis (work II).

The pK_a values in MeCN were measured by Märt Lõkov, Sahil Chhabra and Juhan Luik using a spectrophotometric titration method that has been described previously[13,15,46,51]. Detailed information is presented in work I and III.

Due to the very low aqueous solubility of the compounds their aqueous pK_a values were estimated from pK_a values in MeCN via correlation analysis. The correlation equation is based on experimental MeCN and aqueous pK_a values (data from ref 11) of compounds having related molecular structures. A number of phenylphosphazenes were involved, some of them are also highly lipophilic in nature. The standard uncertainties of the aqueous pK_a values estimated this way are evaluated as 0.5 pK_a units[I,III].

2.2. Biphasic pK_a determination

2.2.1. Principle of the method

Proton transfer processes in two-phase systems – an organic water-immiscible solvent at equilibrium with water – are often encountered: biphasic catalysis[49], sensors with polymeric membranes[50] and processes in cell membranes[23]. Direct pK_a measurement cannot be easily done in low-polarity media because of the difficulties with quantifying the activity of H^+ in them.[55] The involved compounds have often low aqueous solubility. Therefore, it is impossible or impractical to use aqueous pK_a values.

In work II, for the first time, an experimental pK_a measurement method of lipophilic molecules in essentially water-immiscible solvents in equilibrium with aqueous solution is presented. The term biphasic pK_a values – denoted as pK_a^{ow} – is used for this approach. Similar approach has been envisaged before[48] but a respective measurement method has not been developed. The main principle of the method is that a lipophilic acid or base is dissolved in the organic phase and the ratio of its ionic and neutral form concentrations in the organic phase is measured, while the hydrogen ion activity (i.e. pH) is measured in the aqueous phase.

Solvated H^+ ions reside mainly in the aqueous layer. Therefore the processes of protonation and deprotonation of a base B are combined by transfer of some of the ions (H^+ or BH^+) through the phase boundary. The exchange of protons can occur in the aqueous as well as the water-saturated organic layer. The pK_a^{ow} values are independent from the exact mechanism of proton transfer, because chemical equilibrium is independent form the path how the system arrives to equilibrium. Very importantly – if the organic and aqueous phases are at equilibrium, the solvated proton's thermodynamic activities are equal in both phases. This is true even if the equilibrium concentrations of H^+ differ by orders of magnitude.[56] The two phases remain electroneutral because of the migration of the counterions A^- into the organic phase in the same extent as the base B is protonated. The ions in the organic phase are mostly ion-paired. The overall

equilibrium can be written as follows ("O" and "W" are used to refer to the organic and aqueous phases, respectively):

$$(BH^+A^-)_O \quad \rightleftarrows \quad B_O + H^+_W + A^-_W \tag{1}$$

If the activity of counterion A^- and the ionic strength of the aqueous phase are constant and included in the standard state definition then the equilibrium constant K_a^{ow} can be expressed:

$$K_a^{ow} = \frac{a(B)_O \cdot a(H^+)_W}{a(BH^+A^-)_O} \qquad pK_a^{ow} = -\log K_a^{ow} \tag{2}$$

We assume here that $a(BH^+A^-)$ accounts for all of the BH^+ species in octanol, even if some of the them are present as free ions.

The nature and activity of the counterion A^- has significant influence on the pK_a^{ow} values. As demonstrated by equation (1) the higher the lipophilicity and activity of A^- the more the equilibrium shifts towards formation of BH^+A^-. This means that it is important that the identity and activity of A^- be defined when measuring pK_a^{ow} values. The anion A^- should be a sufficiently weak base to (1) avoid the distribution of its conjugate AH into the organic phase and (2) to ensure that $[BH^+A^-]$ is indeed an ion pair, not an associate of neutral forms linked by hydrogen bond (B···H-A).

2.2.2. Experimental determination of pK_a^{ow}

The pK_a^{ow} values were determined for the bases **1a-d** and **4a-d** (Scheme 1). As a reference compound, phosphazene 4-NO_2-$C_6H_4P_1$(pyrr)[11] was used. It has somewhat different structure and its experimental aqueous pK_a value is known.

Octanol solutions (1.5 ml) of each base were equilibrated with aqueous phases (1.5 ml). The aqueous phases had different pH values by shaking for 1 min. Thereafter the mixture was allowed to stand for 20 min until the formed emulsion had stratified. Confirmation experiments were carried out allowing equilibration for up to 200 min, no difference was found in results. With every compound the measurements were carried out at 4 different concentrations (ranging from 3 to 43 μmol L^{-1}). Three anions – chloride, bromide and *para*-toluenesulfonate – were used as counterions A^-, at ionic strength 0.1 mol L^{-1}. Detailed information of solution preparation and experimental conditions are presented in work II.

After agitation the phases were separated and their absorption spectra were recorded spectrophotometrically. For calculating the pK_a^{ow} values a wavelength, where the difference of molar absorptivities of the neutral and protonated forms was maximum, was used (Table 1). Spectrophotometry measures concentrations

and not activities. Therefore, as a first step "concentration-based" pK_{ac}^{ow} values (equation 3) were calculated.

$$K_{ac}^{ow} = \frac{[B]_O \cdot a(H^+)_W}{[BH^+]_O} \qquad pK_{ac}^{ow} = -\log K_{ac}^{ow} \tag{3}$$

The pK_{ac}^{ow} values were calculated by least squares fitting of the experimental absorbance changes with ones calculated by equation 4, varying the pK_{ac}^{ow} values and the absorbance values corresponding to the neutral and cationic species (B and BH^+).

$$A_{calc} = \frac{A_{BH+}}{1+10^{pK_{ac}^{ow}-pH}} + \frac{A_B \cdot 10^{pK_{ac}^{ow}-pH}}{1+10^{pK_{ac}^{ow}-pH}} \tag{4}$$

For every base B the pK_{ac}^{ow} values were determined at 4 different concentrations C_i and it was discovered that their concentration dependence is considerable. The obtained data enabled finding the pK_a^{ow} values as intercepts on the basis of equation 5 by extrapolating the $pK_{ac}^{ow}{}_i$ values to $C_i = 0$ mol L^{-1}:

$$pK_{ac\ i}^{ow} = pK_a^{ow} - A\sqrt{C_i} \tag{5}$$

According to the Debye-Hückel theory the slope A has to be the same for all bases in the same solvent. The value 40 was found for A for our conditions. The pK_a^{ow} values obtained from this data treatment are given in Table 1. The root mean square of residuals was 0.15 pK_a units.

2.3. Lipophilicity determination

As mentioned before the synthesized compounds/bases are highly lipophilic and their neutral forms are practically insoluble in water. Therefore, the direct measurement of the partition coefficient (P) values was impossible. Instead, the distribution coefficient (D) values were directly measured using an acidic aqueous phase. Ionization and ion-pair extraction were considered. The acidic aqueous phase provides sufficient solubility of the phosphazene bases due to protonation and ensures relatively constant pH of the aqueous phase that may otherwise be influenced by the ionization of the bases. The shake-flask method followed by chromatographic analysis (procedure based on ref 57) was used to measure the D values.

Two organic solvents were used, octanol and toluene. Octanol is certainly the most common solvent for lipophilicity studies and there are plenty of available data in the literature. Toluene is not so common solvent for these studies, however it allows to compare the log$P$ values of the bases in an aromatic and an aliphatic solvent. The HCl solution (0.005 mol L^{-1}, pH = 2.30 or 0.01 mol L^{-1},

pH = 2.0) was used as the acidic aqueous phase. The HCl solution and organic phase containing the dissolved base (6.6 – 125 μg) were transferred into vials to perform liquid-liquid extractions. Phases were mixed about 1.5 – 2 hours using an orbital shaker. Distribution ratios were measured by reverse phase (C18) HPLC with UV-Vis detection.

Distribution coefficients of bases were calculated from the ratios of the corresponding peak areas A taking into account the difference of injection volumes V (equation 6).

$$D = \frac{A_{org} \cdot V_w}{A_w \cdot V_{org}} \tag{6}$$

Considerable amounts of bases partitioned into the organic phase as ion pairs, due to the high lipophilicities of the phosphazene cations and abundance of Cl^- ions in solution. Therefore it was not possible to assume negligible concentrations of ions in the organic phase – a frequent assumption in such studies. The log$P$ values were calculated according to the following equation:

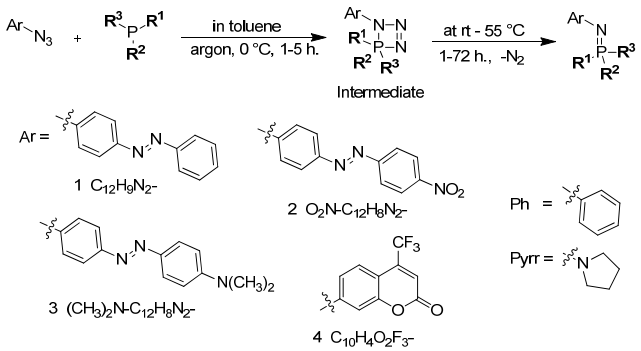
$$P = \frac{D\left(1 + \frac{[H^+]_{aq}}{K_a}\right)}{1 + \frac{[MH^+]_{org}}{[M]_{org}}} \tag{7}$$

The directly measured distribution ratios are denoted by D. The concentration ratios of cationic and neutral forms of the phosphazenes in the organic phase ($[MH^+]_{org}/[M]_{org}$) were estimated from the UV-Vis spectra.

The pK_a values were estimated from correlation analysis with MeCN pK_a values. The $[H^+]_{aq}$ values were measured in the aqueous phase using the glass electrode, after extraction. Each log$P$ value in Table 1 is an average of at least two measurements carried out on different days.

2.4. Synthesis

The Staudinger reaction was used to prepare phosphazenes **1a**–**4d** (Scheme 1), of which 15 have been synthesized for the first time, compound **2a** was previously reported by Katti et al.[58] For compounds **1a**, **1b**, **1d** and **2a** the Kirsanov reaction was also attempted, since the Kirsanov reaction was widely used in our group before[20], however it did not give the desired compounds. Detailed information about reaction conditions, NMR, HRMS and IR spectra are presented in work I (for compounds **1a**–**3d**) and III (for compounds **4a**–**4d**).

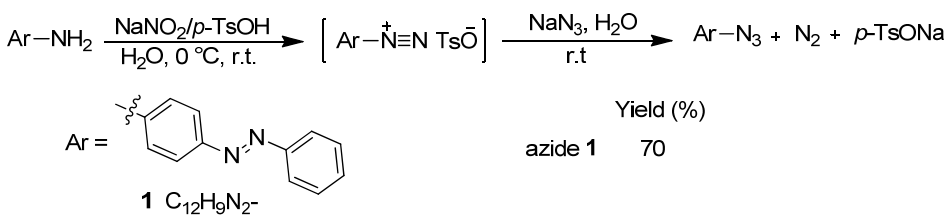


	Ar	R^1	R^2	R^3	yield (%)
1a	1	Ph	Ph	Ph	63
1b	1	Ph	Ph	Pyrr	23
1c	1	Ph	Pyrr	Pyrr	76
1d	1	Pyrr	Pyrr	Pyrr	57
2a	2	Ph	Ph	Ph	75
2b	2	Ph	Ph	Pyrr	49
2c	2	Ph	Pyrr	Pyrr	89
2d	2	Pyrr	Pyrr	Pyrr	69
3a	3	Ph	Ph	Ph	84
3b	3	Ph	Ph	Pyrr	86
3c	3	Ph	Pyrr	Pyrr	43
3d	3	Pyrr	Pyrr	Pyrr	11
4a	4	Ph	Ph	Ph	48
4b	4	Ph	Ph	Pyrr	73
4c	4	Ph	Pyrr	Pyrr	70
4d	4	Pyrr	Pyrr	Pyrr	27

Scheme 1: Synthesis of the iminophosphoranes **1a–4d** by the Staudinger reaction

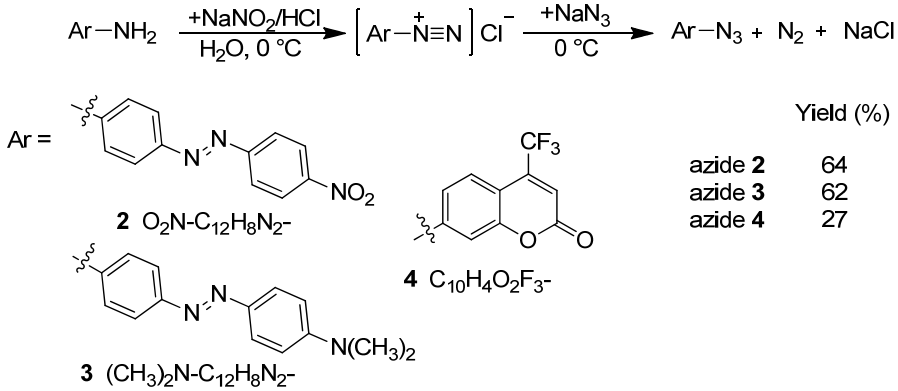
Generally reactions of azides with trisubstituted phosphanes in dry toluene proceeded at 0 °C to room temperature, with visible evolution of nitrogen and resulting in direct formation of the iminophosphorane. In the case of some phosphazenes containing tripyrrolidinophosphane **1d**, **2d**, **3d**, a higher temperature 50–60 °C was required. The Staudinger reaction proceeds via nucleophilic attack at the phosphane by the terminal nitrogen of the azide to afford an intermediate (linear phosphazide), that is usually not detectable (explored in work I), which then rapidly dissociates to products via a four-center transition state.[14]

Two methods were used for preparation of starting azides from commercially available corresponding amines. Azide **1**, 4-azidoazobenzene, was synthesized in high yield in a one-pot reaction (Scheme 2) according to the procedure described by Kutonova et al.[59–61]



Scheme 2: Preparation of 4-azidoazobenzene (azide **1**)

The preparation of azides **2** (4-azido-4′-nitroazobenzene), **3** (4-azido-4′-(*N*,*N*-dimethyl)aminoazobenzene) and **4** (7-azido-4-(trifluoromethyl)coumarin) according to the reaction in Scheme 2 was not successful, therefore the other common method to prepare azides was used (Scheme 3).[58]



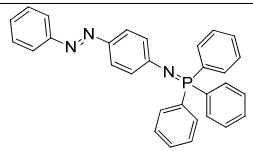
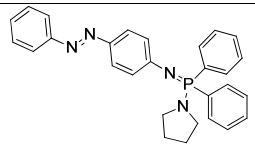
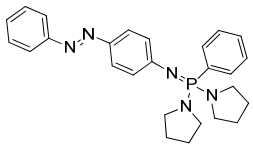
Scheme 3: Preparation of 4-azido-4′-nitroazobenzene (azide **2**), 4-azido-4′-(N,N-dimethyl)aminoazobenzene (azide **3**) and 7-azido-4-(trifluoromethyl)coumarin (azide **4**)

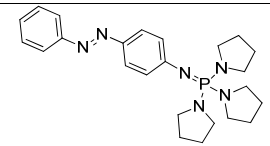
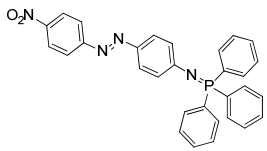
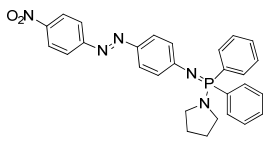
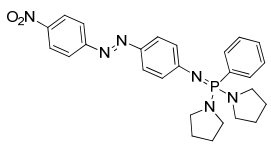
Pyrrolidino- and phenyl-substituents were used for the preparation of the phosphanes from Ph_nPCl_{3-n} (n=0,1,2,3) and pyrrolidine as described in refs 62,63.

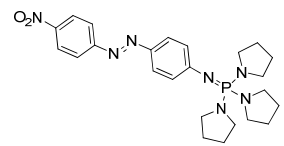
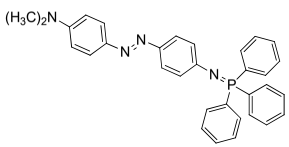
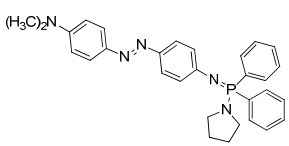
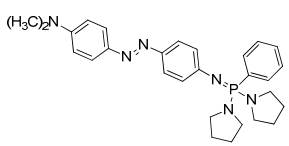
3. RESULTS AND DISCUSSION

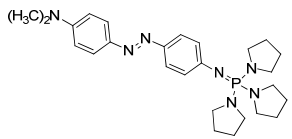
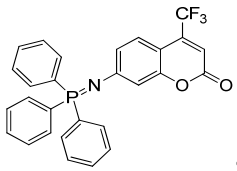
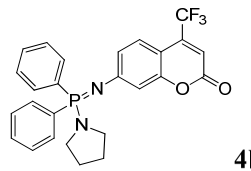
This section discusses the results of all experimental measurements presented in Table 1. 16 novel phosphazene bases were synthesized and characterized.

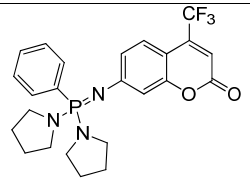
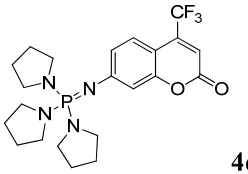
Table 1: Summary table of all results and structures of the synthesized compounds developed within this thesis

Compound	mp (°C)	λ_{abs} (nm) $BH^+/B/\Delta$	λ_{em} (nm) [a] $BH^+/B/\Delta$	Stokes shift (nm) $BH^+/B/\Delta$	Fluorescence lifetime (ns)	pK_a MeCN [b]	pK_a H_2O [c]	pK_a^{ow} [d]	log$P$ oct/w [e]	log$P$ tol/w [e]
 **1a**	167.6–168.4	336/410/74	-	-	-	15.6	7.7	4.3	7.2	8.4
 **1b**	135.9–136.8	340/411/71	-	-	-	16.8	8.4	5.4	7.1	8.0
 **1c**	115.6–117.2	342/415/73	-	-	-	18.4	9.2	6.7	7.7	7.1

Compound	mp (°C)	λ_{abs} (nm) $BH^+/B/\Delta$	λ_{em} (nm) [a] $BH^+/B/\Delta$	Stokes shift (nm) $BH^+/B/\Delta$	Fluorescence lifetime (ns)	pK_a MeCN [b]	pK_a H_2O [c]	pK_a^{ow} [d]	log*P* oct/w [e]	log*P* tol/w [e]
 **1d**	145.4–146.0	347/428/81	-	-	-	20.2	10.2	8.2	8.5	5.4
 **2a**	202.3–203.4	362/480/118	-	-	-	14.9	7.3	-	7.8	9.2
 **2b**	128.0–129.9	365/483/118	-	-	-	16.2	8.0	-	7.2	9.1
 **2c**	138.1–139.2	365/492/127	-	-	-	17.7	8.9	-	7.3	7.9

Compound	mp (°C)	λ_{abs} (nm) $BH^+/B/\Delta$	λ_{em} (nm) [a] $BH^+/B/\Delta$	Stokes shift (nm) $BH^+/B/\Delta$	Fluorescence lifetime (ns)	pK_a MeCN [b]	pK_a H_2O [c]	pK_a^{ow} [d]	log$P$ oct/w [e]	log$P$ tol/w [e]
 **2d**	146.1–146.8	371/512/141	-	-	-	19.5	9.8	-	7.9	6.1
 **3a**	227.5–228.5	539/423/116	-	-	-	16.2	8.0	-	7.5	8.0
 **3b**	179.5–180.9	543/423/120	-	-	-	17.4	8.7	-	7.8	7.3
 **3c**	134.6–135.8	548/423/125	-	-	-	18.9	9.5	-	8.6	6.3

Compound	mp (°C)	λ_{abs} (nm) $BH^+/B/\Delta$	λ_{em} (nm) [a] $BH^+/B/\Delta$	Stokes shift (nm) $BH^+/B/\Delta$	Fluorescence lifetime (ns)	pK_a MeCN [b]	pK_a H_2O [c]	pK_a^{ow} [d]	log$P$ oct/w [e]	log$P$ tol/w [e]
 **3d**	120.9–123.1	539/419/120	-	-	-	20.8	10.6	-	9.5[f]	5.3
 **4a**	165.6–166.4	330/388/58	397/486/89	67/98/31	2.4	13.9	6.8	1.8	6.5	7.3
 **4b**	127.0–127.6	328/393/65	402/497/95	74/104/30	3.2	15.2	7.5	3.2	6.2	7.2

Compound	mp (°C)	λ_{abs} (nm) $BH^+/B/\Delta$	λ_{em} (nm) [a] $BH^+/B/\Delta$	Stokes shift (nm) $BH^+/B/\Delta$	Fluorescence lifetime (ns)	pK_a MeCN [b]	pK_a H_2O [c]	pK_a^{ow} [d]	log*P* oct/w [e]	log*P* tol/w [e]
 **4c**	134.7–135.5	331/399/68	406/508/102	75/109/34	3.9	16.9	8.4	4.7	5.9	6.2
 **4d**	121.8–122.8	332/408/76	406/521/115	74/113/39	3.6	18.6	9.3	5.9	6.6	4.0

[a] Fluorescence emission properties are reported for spectra excited at the respective absorption maxima wavelengths for the neutral and cationic forms of the compounds. [b] Experimental pK_a values in acetonitrile from work I and III. [c] Estimated aqueous pK_a values. [d] Experimental pK_a^{ow} values from work II and III, counterion Cl^-. [e] Log*P* values estimated from directly measured log*D* values. [f] Value with high uncertainty, see the ESI of work I.

3.1. Spectral properties

Both the neutral and cationic forms of all the synthesized compounds have absorbance bands in the visible spectral range. In the case of unsubstituted **1a-d**, 4-nitro-substituted **2a-d** and coumarin based **4a-d** compounds, remarkable shifts of the absorbance bands toward the UV end of the absorption spectrum (blue shift) were observed upon protonation of the neutral compound. In the case of 4-dimethylamino-substituted **3a-d** compounds the absorption maxima displayed shifts to longer wavelengths (red shift) upon protonation.

Replacing the phenyl group that is attached to the phosphorus atom with pyrrolidino groups a red shift was observed with all the compound series. In addition, the distance between the absorption peak maxima of the neutral and cationic forms also increases when the phenyl groups are replaced by the pyrrolidino groups, which in fact is an anticipated and welcome characteristic.[13,64] Pronounced spectral differences in the visible region are characteristic of all synthesized phosphazene bases and their conjugate acids, therefore it is possible to use them as acid-base indicators.

Compounds **4a-d** are strongly fluorescent dyes. As mentioned before, many applications require relatively small lipophilic molecules that exhibit strong fluorescence and additional photophysical features. In the case of compounds **4a-d** the fluorescence emission peak maxima are located at wavelengths near ~500 nm (Table 1). Upon protonation of the neutral base we can see an interesting and useful feature, the emission maxima display substantial blue-shifts ($\Delta\lambda \approx 100$ nm). The fluorescence emission maxima are shifted to longer wavelengths by the sequential replacement of phenyl groups attached to the phosphorus atom by pyrrolidino groups. The replacement also increases the difference between the wavelengths of the emission maxima of the neutral and cationic forms. In general, the described shift in emission maxima is a highly welcome characteristic in the design of fluorescent indicator based sensors. Stokes shifts also increase (and are more apparent in the neutral compounds) with sequential replacement by pyrrolidino groups (Figure 1).

Besides the wavelength shifts of the emission bands, the emission intensities of the forms differ markedly and have different optimal excitation wavelengths. When the wavelength of the absorbance maximum of the neutral form is used for excitation, then a significant decrease in fluorescence intensity occurs upon protonation (Figure 2).

Fluorescence lifetime of the compounds is short, between 2.4 and 3.9 ns. Oxygen quenches fluorescence effectively and in general it is important to deoxygenate (purging with nitrogen or using an ultrasound bath) all used solvents.[53] Generally, when the fluorescence lifetime is below 5 ns the quenching by oxygen does not have a significant effect.[53,54]

All the absorbance and emission spectra are presented in supplementary information of works I and III.

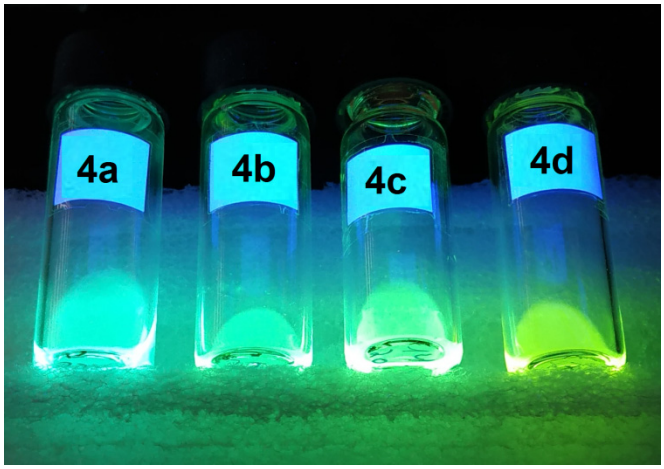


Figure 1: Compounds **4a-d** in MeCN under an UV lamp (365 nm)

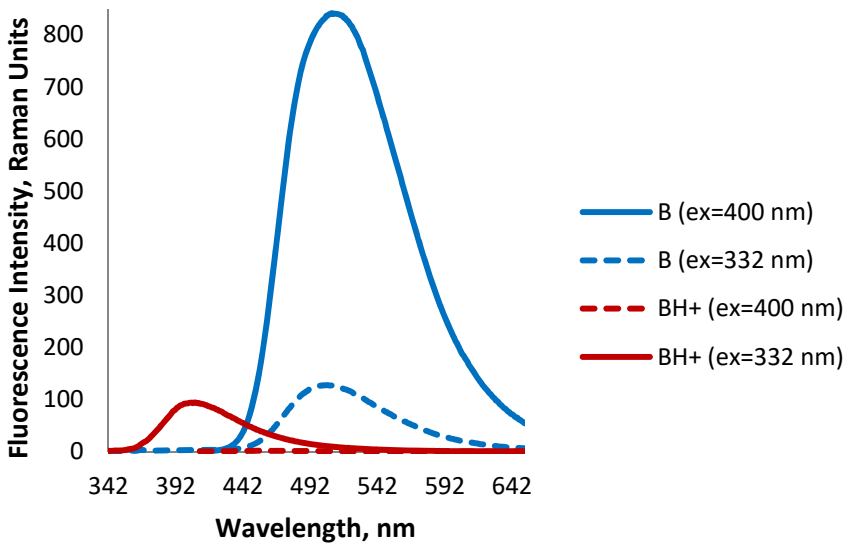


Figure 2: Fluorescence emission spectra of **4c** at excitation of 400 nm and 332 nm. Spectra displayed with solid lines represent spectra that were collected at their respective absorption maxima wavelengths for the neutral (B) and cationic (BH^+) forms of the compounds. All emission spectra have been IFE corrected.

3.2. Basicity

The pK_a values of the bases in MeCN range from 13.9 to 20.8 (Table 1). Based on the presented results it is evident that the basicity depends strongly on the number of pyrrolidino groups bound to the phosphorus atom. Adding one pyrrolidino group instead of an initial phenyl ring leads to an increase of basicity by 1.2 to 1.9 pK_a units. Comparing the bases **1a-3d** it is noticeable that the basicity is also dependent on the 4-substituents on the phenyl ring. Introducing the (–R) nitro group decreases the basicity of the compound by 0.6–0.7 pK_a units. Introducing the (+R) dimethylamino group increases the basicity by 0.5–0.6 pK_a units. Thus, the number of pyrrolidino groups have a greater impact on pK_a value.

The pK_a estimates in water range from 6.8 to 10.6 and follow the trend of pK_a values in MeCN.

The pK_a^{ow} values were obtained for compound series **1a-d** & **4a-d** and range from 1.8 to 8.2 pK_a^{ow} units (Table 1) using Cl^- as counterion. The pK_a^{ow} values follow the trend of pK_a values in MeCN and pK_a estimates in water. pK_a^{ow} values are 5.0 (**4a**) to 2.0 (**1d**) units lower than the pK_a estimates in water. The pK_a difference largely comes from different levels of stabilization of the cationic forms of the indicators in the organic phase, relative to the aqueous solution. The less stabilized is the cationic form, the larger is the pK_a difference. It is evident from the results that increasing the aromatic nature of the bases (by adding more phenyl rings) decreases the affinity of the protonated bases towards octanol.

As mentioned before it is important to define the nature and activity of the counterion A^-, since it affects the pK_a^{ow} value. Table 2 displays clearly that biphasic pK_a value is strongly dependent on the counterion A^-. The more lipophilic is A^- the higher is the value of pK_a^{ow} and the smaller is the difference between pK_a^{ow} and aqueous pK_a. Higher lipophilicity and activity of counterion A^- both promote formation of BH^+A^- and that is the reason why higher pK_a^{ow} value is obtained.

Table 2: Impact of the counterion on pKaow value

Compound	pK_a^{ow}	Counterion A^-
1a	4.3 [a]	Cl^-
1a	4.5 [b]	Br^-
1a	6.1 [b]	*p*-TsO^-

[a] Values obtained from a measurement series at 4 concentrations. [b] Tentative values obtained from a measurement series at one concentration.

3.3. Lipophilicity

The aim was to synthesize lipophilic bases and in addition their protonated forms should be also lipophilic. Table 1 shows the partition coefficients of the indicators in two solvent combinations: octanol/water and toluene/water. High lipophilicity of the synthesized compounds is clearly evident from the log*P* values. The number of pyrrolidino and phenyl fragments bound to the phosphorus atom influences the log*P* values markedly. The changes of lipophilicity within compound series depend on the number of pyrrolidino and phenyl groups in the molecules and differ between octanol and toluene.
In toluene, lipophilicity increases when the number of phenyl groups increases (and the number of pyrrolidino groups decreases) in the molecule and the span of log*P* values is 3.3 log units. In octanol/water system the trend is not as pronounced but the span of log*P* values are 2.0 log units.

Compounds with three pyrrolidino groups are the most lipophilic ones in octanol/water system. At the same time these compounds have the lowest lipophilicity in toluene/water system. The reason is the higher polarity of the phenyl ring compared to the alkyl moiety of the pyrrolidino group. Also, interaction of the nitrogen atom in pyrrolidino group with water molecules is sterically hindered by the neighboring substituents. Thus the phenyl groups turn out to be more polar, and favor partition into the aqueous phase in the case of the octanol/water system. If the organic phase is toluene, then there is quite some influence from the π-π interactions between the aromatic rings of the phosphazenes and the solvent, which is absent in the case of octanol.

Both forms, protonated and neutral, of the synthesized compounds are highly lipophilic and these compounds are suitable to use as indicators in low polar membranes.

SUMMARY

The aim of this thesis was to design and synthesize a family of novel lipophilic phosphazene-based indicators – active both via visible absorption and fluorescence – and to create an original method for quantifying acidity/basicity of lipophilic molecules in practically water-immiscible solvents (biphasic pK_a values). 16 novel phosphazene-bases have been synthesized using the Staudinger reaction and fully characterized. They are distinguished from other well-known indicator molecules by useful and unusual combination of properties, which are advantageous in the design of optical pH sensors. They have 0/+1 charge states and it was shown that both forms of these phosphazenes are highly lipophilic, thus lack of any localized charges in the respective cations. Therefore the synthesized compounds are well suited for acting as "silent spectators" in monitoring the acidity/basicity of different low-polarity systems.

pK_a values of the phosphazenes are in the basic range. To describe and quantify the basicity/acidity of lipophilic indicator molecules embedded in non-polar system that is at equilibrium with aqueous phase a novel approach biphasic p$K_{a,}$ is for the first time experimentally realized in this work. Importantly, the method is suitable to describe the acidity/basicity of indicator molecules in non-polar polymer matrix, since the pK_a value in the polymer matrix is different from the pK_a value in a solvent.

It was shown that these compounds show the desired spectral sensitivity to the pH of the environment. All synthesized compounds displayed absorbances in the visible spectral range and a shift of the absorbance band upon protonation of the neutral compound. Four of them have fluorescence emission maxima that also display substantial blue-shifts upon protonation of the neutral compound. In addition to the emission wavelength shifts, the absorbance extinction coefficients and maxima wavelengths also differ between the neutral and protonated forms of the compounds.

In conclusion, the synthesized compounds have all the desired and suitable properties to employ them in the design of optical pH sensor.

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SUMMARY IN ESTONIAN

Lipofiilsete fosfaseenidel baseeruvate indikaatormolekulide süntees ja eksperimentaalsed omadused

Käesoleva doktoritöö peamiseks eesmärgiks oli luua grupp uudseid fosfaseenidel baseeruvaid indikaatormolekule, mille vaba aluse ja protoneeritud vormi spektrid oleksid võimalikult erinevad ja asuksid nähtavas spektrialas või fluorestseeriksid. Lisaks töötati doktoritöö raames välja uudne meetod lipofiilsete molekulide happelisuse/aluselisuse kvantiseerimiseks kahefaasilistes süsteemides. Staudinger´i reaktsiooni kasutades valmistati 16 fosfaseenidel baseeruvat ühendit ning määrati nende omadused. Neid ühendeid eristab teistest teada-tuntud indikaatormolekulidest ebatavaline omaduste kombinatsioon, mis on kasulik optiliste pH-sensorite loomisel. Töös näidati, et sünteesitud ühendite neutraalsed ja katioonsed vormid on kõrgete lipofiilsustega ja katioonidel puudub selgelt väljendunud laengutsenter. Käesolevad struktuurid on sobilikud erinevate madala polaarsusega süsteemide happelisuse/aluselisuse monitoorimiseks, sest kirjeldatud omadustega ühendid käituvad sellistes süsteemides kui "vaiksed pealtvaatajad".

Doktoritöö raames määrati sünteesitud ühendite pK_a väärtused atsetonitriilis. Lipofiilsete molekulide happelisuse/aluselisuse kvantiseerimiseks süsteemides, kus vesilahus on tasakaalus veega mitteseguneva keskkonnaga/solvendiga, töötati välja uudne meetod – kahefaasiline pK_a – mida varem eksperimentaalselt rakendatud pole. Meetod sobib indikaatormolekuli happelisuse/aluselisuse kirjeldamiseks madala polaarsusega polümeermembraanis, sest indikaatormolekuli pK_a väärtus polümeermembraanis võib märgatavalt erineda pK_a väärtusest lahuses. Näidati, et sünteesitud ühendid omavad soovitud spektraalset tundlikust keskkonna pH muutuse suhtes. Kõik ühendid neelavad nähtavas spektrialas ja protoneerumisel toimub selge neeldumisspektri muutus. Neli ühendit omavad intensiivset maksimumi fluorestsentsispektris, mille asukoht ja intensiivsus muutuvad protoneerumisel.

Kokkuvõtteks võib väita, et sünteesitud ühendid on sobivate omadustega, et neid rakendada optiliste pH-sensorite loomisel.

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