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Influence of the Headgroup of Azolium-Based Lipids on Their Biophysical Properties and Cytotoxicity

Andreas Rühling, Da Wang, Johannes B. Ernst, Stephanie Wulff, Roman Honeker, Christian Richter, Angélique Ferry, Hans-Joachim Galla* and Frank Glorius*

Abstract: A series of (un-)charged NHC derivatives bearing two pentadecyl-chains in the backbone was studied in detail to find cooperative effects between the membrane and the NHC derivative. The tendency to show lipid-like behavior is depending on the properties of the NHC derivative headgroup, which can be modified on demand. The surface activity was investigated by film balance measurements, Epi-fluorescence microscopy and differential scanning calorimetry. Additionally the cytotoxicity was evaluated against different cell lines such as eukaryotic tumor cell lines. These novel lipid-like NHC derivatives offer a broad spectrum for biological applications.

Over the last two decades N-heterocyclic carbenes (NHCs) have emerged as an exciting class of ligands in complex chemistry, as they are versatile in synthesis and broadly applicable in catalysis, metal surfaces and nanoparticle modifications.[1] In sharp contrast to this only little attention has been paid to the corresponding NHC salts, except for their use as ionic liquids and as transmembrane anion transporters.[2,3] Recently, we developed a new class of 4,5-dialkylimidazolium salts, which have a structural compliance with cationic lipids or surfactants. Their biological properties and influence on model membranes made of 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine (DPPC) were investigated in detail.[4] A strong dependence on the alkyl chain length was found, having a stronger membrane interaction the longer the alkyl chain becomes,[5] which was later on explained by molecular dynamic simulations.[6] Shorter alkyl chain derivatives create a vacancy in the hydrophobic part of the membrane, reducing the packing density of the lipids and therefore soften the structural integrity of the membrane, whereas a long alkyl chain derivative smoothly incorporates into the membrane and strengthens the integrity due to electrostatic interactions between the cationic imidazolium headgroup and the anionic phosphate of DPPC.

Driven by these findings it becomes interesting to investigate the influence of the headgroup on the membrane interactions and the biophysical properties, while the alkyl chain length remains to be two times a pentadecyl chain, displaying the previously most promising chain length.[5] However, the change in size of the headgroup was envisioned to have an impact on the membrane interactions, as bigger groups should disturb the dense packing of the DPPC molecules, leading to a fluidized membrane. Depending on the hydrophobicity / hydrophilicity one would assume to observe a difference in the interaction with DPPC. Another possible effect is the charge of the headgroup, because a permanent charge should be able to build a stronger hydrogen bonding network or have a larger amount of water molecules in the close coordination sphere of the headgroup.

To investigate possible headgroup effects a set of seven different NHC derivatives was synthesized, separated into two subgroups, charged (**A**, **1-5**) and uncharged NHC derivatives (**B**, **6-7**) (Figure 1, for synthesis see supporting information). Their biophysical properties and lipid-like behavior were studied and compared to the previously used 1,3-dimethyl-4,5-dipentadecylimidazolium iodide (**8**, C_{15}-IMe-HI).

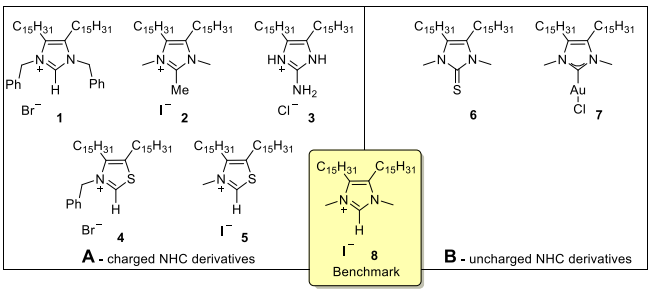

Figure 1. Subgroups of NHC derivatives, **A** – charged and **B** – uncharged and the benchmark compound C_{15}-IMe-HI.

The charged subgroup consists of an NHC salt with two sterically more demanding benzyl groups (**1**), an NHC salt in which the acidic C2-hydrogen was replaced by a non-acidic methyl group (**2**) to suppress hydrogen bonding via the C2-

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hydrogen, a highly hydrophilic guanidinium salt (**3**) and two thiazolium salts (**4,5**) which differ in the size of the nitrogen substituent and therefore overall size of the headgroup. In general thiazolium salts are considered to have the smallest NHC derived cationic headgroup. The uncharged subgroup contains a hydrophilic thiourea (**6**) and a hydrophobic Au-NHC-complex (**7**), which could later be used for catalysis or as an organometallic drug.

In order to understand if the molecules **1**-**7** behave like lipids, film balance measurements were conducted with a 10 mol% solution of **1**-**7** together with the lipid DPPC. The plasma membrane is mimicked by DPPC and if **1**-**7** show a lipid-like behavior the formation of a stable film would be observed (Figure 2). From the compounds of **A** only **1** and **4** show a weaker film stability in comparison to pure DPPC, which can be attributed to the large benzyl group as the nitrogen substituent pushing the DPPC molecules apart, an effect being more pronounced for **1** than **4**. The weakened film stability is displayed by the higher surface pressure at the same area per molecule in comparison to pure DPPC, indicating a collapsing film. For **2** and **5** the membrane is slightly more stable than for pure DPPC, but weaker than with **8** as benchmark.

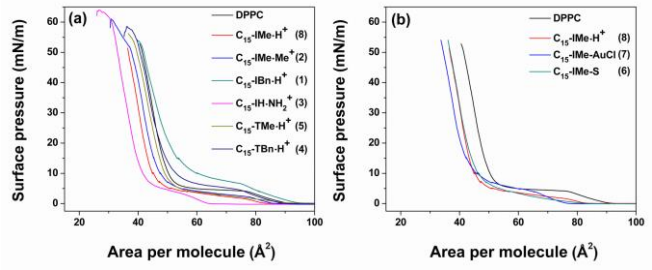

Figure 2. Surface pressure-area (π-A) isotherms of the monolayers of (a) pure DPPC and DPPC/X = 0.1 **1**-**5**,**8**; (b) pure DPPC and DPPC/*X* = 0.1 **6**-**7**,**8** at the air-water interface at 293 K. All measurements were performed on pure water (pH 5.6).

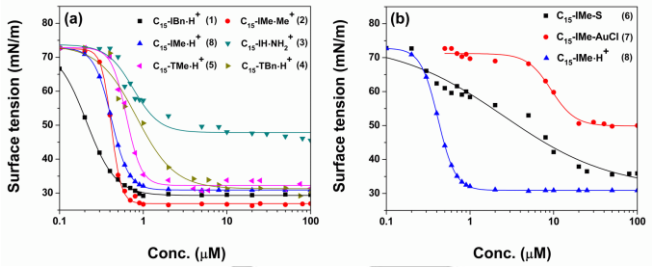

Figure 3. Surface tension-concentration (γ-C) isotherms for (a) **1**-**5**,**8** (b) **6**-**7**,**8** in pure water (pH 5.6) at 293 K.

It can be concluded that the increased hydrophilicity of imidazolium salts and the acidic C2-hydrogen are beneficial for hydrogen bonding and attractive electrostatic interactions with DPPC. Interestingly **3** forms even more stable films than **8** together with DPPC, although guanidium salts are used in high concentrations for protein denaturation. Presumably the higher hydrophilicity enhances the interaction with DPPC and therefore the film stability. For the uncharged subgroup **B** both **6** and **7** show an attractive interaction with DPPC, leading to a stronger film. The thiourea **6** is on par with **8** and **7** forms even stronger films. This might be explained by the partial dissociation of the chloride in the monolayer, leading to a cationic gold species, which strongly interacts with the anionic part of DPPC and is very hydrophilic in the headgroup area. In terms of the Gouy-Chapman layer model this dissociation can be interpreted that only the cationic part has an influence on the film stability. Additionally this model explains why no influence of the counter ion was observed for other NHC derivatives (see supporting information). The surface activity behavior is supported by measuring the critical micelle concentration (CMC) applying film balance measurements (Figure 3). A lower CMC equals an easier formation of a lipid-like assembly, which could be beneficial for an incorporation into a membrane. The lowest values were found for **1** (0.5 µM) and **2** (0.9 µM), both being lower than for benchmark **8**. The lower hydrophilicity of the headgroup of **1** and **2** seems to be key for a low CMC. The thiazolium salts **4** and **5** show a medium to low CMC (7.9 µM and 1.8 µM) being attributed to their slightly higher hydrophilicity compared to **1** and **2** because of the shrink in headgroup size. However, **3**, which showed a strong interaction with DPPC films, has a higher CMC, rendering it less suitable for a lipid-like behavior. This can be explained by the ability to coordinate more water molecules around the headgroup, resulting in a higher hydrophilicity. This explanation together with the chloride dissociation of **7** can be used for its high CMC (18.4 µM). Thiourea **6** shows a high CMC value of 17.7 µM, presumably due to the lack of charge.

Table 1. Surface properties for **1**-**7**,**8**

Compound	CMC / µM	γ_{min} / mN·m-1
1	0.5 (0.4~1.3)[a]	29.4 ± 0.2[a]
2	0.9 (0.7~1.0)	26.9 ± 0.2
3	4.1 (2.5~5)	47.8 ± 0.7
4	7.9 (5~10)	31.3 ± 0.9
5	1.8 (1~2)	32.2 ± 0.4
6	17.7 (5~25)	31.2 ± 3.2
7	18.4 (15~20)	49.9 ± 0.7
8	1.4 (1.0~5.0)[a]	31.0 ± 0.1[a]

[a] Data from Ref. [5].

For further investigations the focus was set on the two most and least promising compounds to get a better understanding for the requirements for a lipid-like behaving molecule, i.e. **1**-**3** and **7**. All four compounds show interactions with DPPC, but as a single component only **1** and **2** are promising for a lipid-like behavior. To visualize these circumstances and find out more about the lateral domain formation within the mixed DPPC/NHC derivative lipid monolayer Epi-fluorescence was measured for our four candidates as 10 mol% solution in DPPC together with 0.05 mol% BODIPY as fluorescent dye (Figure 4). Pure DPPC shows the formation of well-known kidney shaped domains with the tendency to grow in size at higher pressure. For **1** a significant fluidization effect is found, demonstrated by the disappearance of the lc domains at 4 and 5 mN/m. Domains are visible at 10 and 15 mN/m with an almost unchanged shape, but

reduced in domain size and number. This could be taken as an indication for **1** being squeezed out of the DPPC mixed monolayer into the aqueous phase (the total concentration of 10 mol% **1** is 0.021 µM, which is below the CMC of 0.5 µM). This is in agreement with the low CMC value of **1**. **2** and **7** both show a significant shrinking in domain size with **7** leading to even smaller domains in the pressure area from 4 to 7 mN/M and a change in shape to circular domains. The circular domains are uniformly distributed and at higher pressures self-quenching can be observed. In case of **3** the same trend is shown, but the domains become even smaller. The circular domains in combination with the stable, uniform distribution support the good miscibility of the compounds with DPPC, a requirement for stable film formation. Upon compression the disappearing domains represent a nearly homogenous phase, which can be attributed to the attractive lipid-like interactions between the two components.[7]

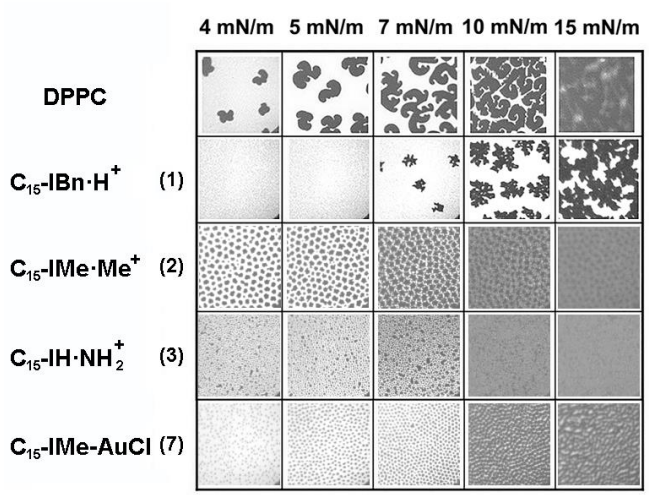

Figure 4. Epi-fluorescence images of the monolayers of pure DPPC and DPPC/*X* = 0.1 **1**-**3** and **7** on pure water (pH 5.6) at 293 K. All samples were mixed with *X* = 0.005 BODIPY-DC. The frame size of all images is 120×120 µm.

To gain a more detailed understanding of the interaction between the NHC derivatives and the membrane related DPPC system a deeper look was taken on the thermotropic phase properties of DPPC liposome by conducting differential scanning calorimetry (DSC) (Figure 5). If the NHC derivatives show a cooperative lipid-like behavior a rigidification of the mixed DPPC bilayer should be observed in DSC, displayed by a higher phase transition temperature, respectively an unattractive interaction would fluidize the bilayer and therefore the phase transition temperature decreases. Pure DPPC undergoes the main phase transition at 42 °C, going from the solid to the fluid phase.[8] The mixed vesicles of DPPC/**1** show a decrease in the phase transition temperature when the molar ratio of **1** is increased. Upon 5 mol% **1** the phase transition occurs between 40 and 42 °C. At higher concentrations of **1** the temperature drops continuously down to 33 °C at 30 mol%. Pure **1** shows no phase transitions in the range of 20 to 60 °C. This result is in agreement with the film balance measurements, showing a weaker DPPC film if **1** is added to DPPC. In contrast to this the phase transition temperature is increased with **2** and **3**, with **3** having a stronger influence. Like for **1** up to 5 mol% the change in temperature is diminishingly small, about 3 °C difference. Going up to 30 mol% in both cases the phase transition becomes broader and culminates around 51 °C for **2** and 62 °C for **3**, respectively. Interestingly pure **2** shows a phase transition below around 28 °C, which is similar to the previously reported double phase transition for **8** below 35 °C.[6]

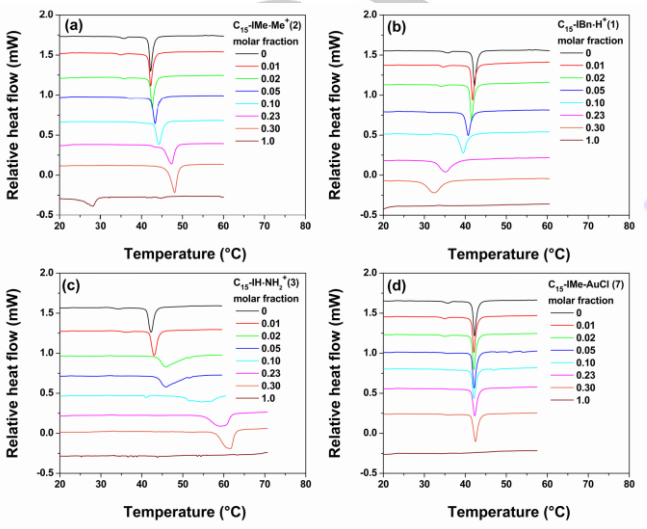

Figure 5. Differential scanning calorimetry (DSC) endotherms of large unilamellar vesicles (LUVs) of binary mixtures of DPPC with varying molar fractions of (a) **2**, (b) **1**; (c) **3** and (d) **7** in pure water.

This means that **2** and **3** both are capable of rigidifying the model membrane system as well as forming a uniform distribution within the membrane, which fits to the results obtained from film balance and the Epi-fluorescence measurements. Looking at all four compounds it becomes clear that an increase in size and hydrophobicity of the headgroup leads to a fluidization effect on the DPPC membrane (Figure 6). Vice versa decreasing the size and the hydrophobicity of the headgroup strengthen the stability of the membrane. For the gold complex the chain chain interaction obviously compensates the headgroup polarity effect thus just broadening the phase transition but not changing the phase transition temperature.

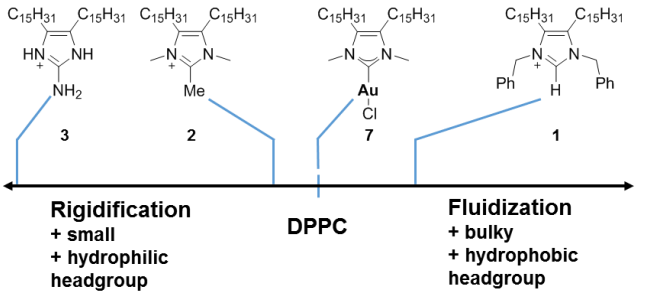

Figure 6. Illustration of the rigidification or fluidization potential of **1**-**3** and **7** of a DPPC membrane. The distances are based on the changes in the phase transition temperature of a 0.1 molar fraction of the component with DPPC. The phase transition temperature of pure DPPC was set to 0.

The above results attracted more interests toward the impact of the head group on the potential cellular effect of our NHC derivatives, with the aim to establish the relationship between their surface activity and their biological properties. Here the cellular toxicity of **1**-**3** and **8** was evaluated using a panel of cell lines including C6 glioma tumor cell line (Table 2). Because of the poor solubility in DMSO the Au-NHC complex **7** could not be investigated. A lower EC_{50} value corresponds to a higher potency to induce the cells death.

Table 2: Cytotoxicity comparison and the selectivity against C_6 glioma tumor cell line.

Compound	EC_{50} ± SD/μM		
	C6 glioma	*MDCK II*	*NIH3T3*
1	11.3 ± 2.0[a]	20.6 ± 4.2[a]	8.7 ± 0.7[a]
2	16.3 ± 0.6	23.6 ± 1.1	3.5 ± 0.9
3	67.2 ± 3.9	32.2 ± 3.3	12.2 ± 1.7
7	N/A	N/A	N/A
8	28.1 ± 0.7[a]	15.9 ± 0.5[a]	7.7 ± 0.2[a]
	$\frac{EC_{50}(MDCK\ II)}{EC_{50}(C6)}$		$\frac{EC_{50}(NIH3T3)}{EC_{50}(C6)}$
1	1.82		0.77
2	1.45		0.21
3	0.48		0.18
7	N/A		N/A
8	0.57		0.27

[a] Data from Ref. [5].

Note that the EC_{50} values of the tested NHC derivatives towards C6 glioma tumors decrease in the following order: **3** > **8** > **2** > **1**. This order is found to be negatively correlated with the spacing and hydrophobicity of their head group, implying that a more sterically disfavored and more hydrophobic imidazolium head group confer our NHC derivative with a higher anti-tumor activity. The possible reason is, that the more hydrophobic head group offers a relatively high partitioning into the interior of cell membranes, which is followed by strong membrane disturbance resulting from the space-consuming substituents on the imidazole ring. In particular, it has been reported that benzylated imidazolium-based compounds inhibit the cell proliferation,[9] causing G1-phase cell cycle arrest and apoptosis in tumor cells.[10] However, the same trend cannot be observed for the non-tumor cell lines.

Herein, a systematic investigation of the influence of the headgroup on NHC derivatives and their biological properties and cytotoxicity against epithelial and tumor cell lines was reported. The size and the hydrophilicity of the headgroup have a major impact on the cooperative lipid-like behavior. The more polar and hydrophilic the headgroup the better the incorporation into a model membrane, such as DPPC. If the headgroup is too bulky vacancies are created in the hydrophobic part of the membrane, which fluidizes and therefore weakens the membrane. Interestingly, even transition metal NHC complexes can be inserted into the DPPC membrane, without weakening, opening the pathway for a novel and simple class of organometallic drugs. For a specific set of two times a pentadecyl chain, the elaboration of the imidazolium core with sterically hindered and hydrophobic substituents considerably improves both the surface activity and anti-tumor activity. Thus, in conclusion we showed that the steric effect and the hydrophobicity of the head group of our NHC derivatives modulate both their surface properties and cellular effect.

Keywords: NHC salts • lipids • surface activity • cytotoxicity • antitumor reagents

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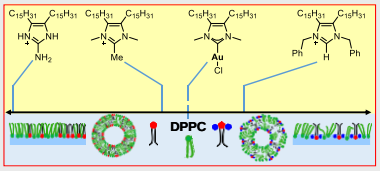

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