Fullerene-based Biocomponents: New Concepts For Functionalising Membranes

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Abstract

Lipophilic hexakisadducts of fullerene C_{60} (**1**) form unprecedented rod-like nanoaggregates in phospholipid-membrane bilayers, resulting in modification of the micromechanic properties and stabilisation of the membrane.[1, 2, 3] Lipofullerenes with amphiphilic side chains (**2, 3**) enable additionally derivatisation and molecular recognition at the membrane surface. The amphiphilic spacer acts as a transmembrane anchor and provides the terminal functionality outside of the membrane.[4] New systems derived from parent compound **3** carry two functional groups each and can be easily modified due to the modular synthesis. Terminal functionalities to be investigated include D(+)-biotin (**3a**) and IDA (iminodiacetic acid) ligands (**3b**), as used in nickel-histidine tags. Modification of the lipophilic region, for instance with unsaturated addends (**3B**) is also possible. These addends should allow polymerisation inside the membrane and potentially lead to a tremendous increase of the membrane rigidity. Furthermore, mono- and bilayer-forming fullerene derivatives without the membrane-forming support of lecithins are investigated and exhibit interesting features.[5]
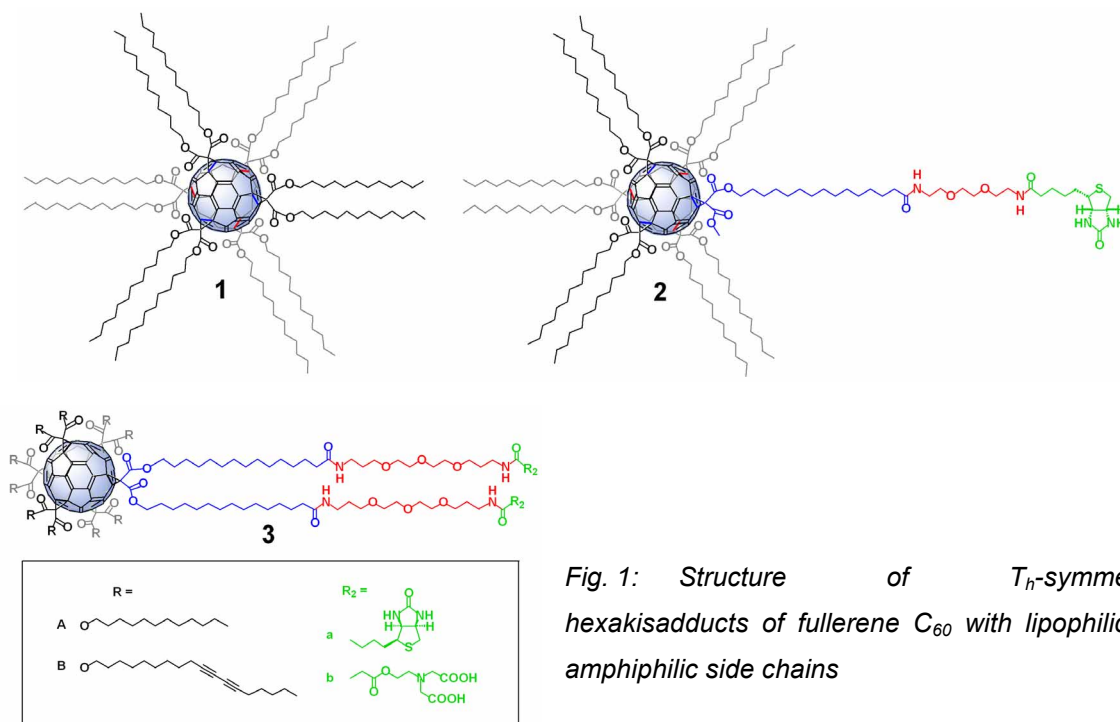
Fig. 1: Structure of T_h-symmetrical hexakisadducts of fullerene C_{60} with lipophilic and amphiphilic side chains

Introduction

The specific modification and functionalisation of artifical membranes enables their implementation as biomimetic systems for various applications in the field of molecular recognition and sensors. Our new approach to novel biocomposites is based on lipophilic fullerene derivatives, which show extraordinary aggregation phenomena in phospholipid bilayers. [6] Fundamental studies on bilayers and monolayers which consist of lipophilic or amphiphilic fullerene molecules, alone or mixed with artifical lecithins, establish a basic understanding of these systems.[a]

Investigation of fullerene derivatives in bilayers

Because of its T_h-symmetrically attached six pairs of long alkyl chains attached to fullerene C_{60}, lipofullerene **1** is soluble in lipid bilayers and shows interesting effects on the physical properties of these membranes.[1, 2, 3] In bilayers with the lecithin dipalmitoyl-*sn*-glycero-phospatidylcholine (DPPC) used as the membrane building unit, unprecedentedly high loadings of up to 25 mol% of lipofullerenes are achievable. The lipofullerene adopts self-assembled rod-like nanostructures in the bilayer, which stabilize it with respect to deformation in magnetic fields and modulate its micromechanic properties. Morphological studies of the multilamellar vesicles carried out with freeze fracture TEM show long range ordered superstructures in the gel phase, which are drastically reduced or even lost in the fluid phase of the bilayer system. Attempts to utilize these reproducible and promising aggregation phenomena are in progress.

The mixed [1:5]-hexakisadduct **2** was the first example of a lipofullerene with an amphiphilic spacer and consists of ten long alkyl chains within the five bisdodecyl malonate addends and a linker malonate carrying a (+)-biotin unit at the end of an amphiphilic spacer.[4] The malonates are attached to the fullerene core in an octahedral addition pattern, achieved by successive cyclopropanation sequences with spacer precursor malonate functionalized with an amphiphilic spacer and dodecyl malonate. The final step was the attachment of biotin to the precursor. **2** is proven to act as transmembrane anchor within a lipid membrane. The amphiphilic spacer intercalates the lipid layer and places the terminal functionality outside the membrane. As a model system for a biological receptor ligand interaction we have chosen the streptavidin (SA) / biotin system, using the (+)-biotin biofunctionality attached to the fullerene spacer moiety. Example **2**, as the first prototype of this new class of transmembrane anchors, was completely characterised by ^{1}H- and ^{13}C-NMR, IR- ,UV- and mass spectroscopy. The expected ability of its biotin unit to interact with streptavidin was proven by Reflectometric Interference spectroscopy RifS. The experiments provide evidence for strong and selective coupling to the protein.
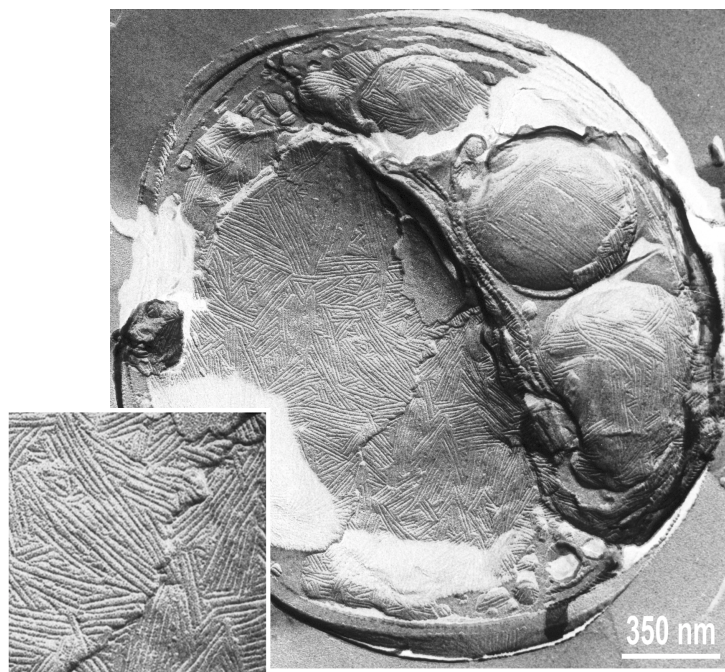
*Fig. 2:   Freeze fracture-TEM of DPPC-MLV containing 10 mol% **2***

Ongoing studies of DPPC bilayers containing fullerene derivatives reveal the following preliminary findings concerning **2**: electronmicrographs of binary multilamellar vesicles (MLV) show rod-like aggregation of **2** in the DPPC membrane similar to the aggregations of lipofullerene **1** (figure 2). Likewise, the length and coherence of these structures depend on the phase state. Investigations by deuterium NMR demonstrate the stabilisation of the MLVs by **2** and an additional increase of stability by coupling of avidin to the biotin anchors. The dynamics of the binary bilayers were studied by ^{2}H-NMR T_2-relaxation experiments. The observed decrease of the T_2 times of the MLVs can be explained by the perturbed diffusion of the DPPC molecules at the curved surface of the fullerene rods.[7]

Investigation of monolayers

The capability of **2** to form stable monolayers by itself was investigated in a series of Langmuir-Blodgett film experiments. In comparison to lipid monolayers, unexpected features in the pressure-area isotherm are revealed. For instance, the expansion isotherms show a large hysteresis, and a local maximum in the compression isotherm indicates slow rearrangment of the fullerene molecules. The distinct difference between the compression- and expansion isotherms appears to be due to dynamic and structural rearrangement processes.[8]

Intensive investigations of binary monolayers made up of DPPC lipid and amphifullerene **2** at the air/water interface were carried out to elucidate their phase behavior and to quantify how the specific binding of streptavidin to the biotin units depends on the different monolayer phase states.[8] The surface-sensitive methods of neutron reflection and infrared reflection-absorption spectroscopy, in combination with film balance techniques, allowed us to determine the monolayer thickness, density and molecular order, as well as the direct measurement of the amount of streptavidin coupled from the

subphase under different subphase conditions. As an exceptional feature of this binary system, it was shown that the specific protein binding behavior can be controlled by the lateral pressure π as a parameter. The effect of coupling streptavidin (SA) to the monolayer is depicted in figure 3.
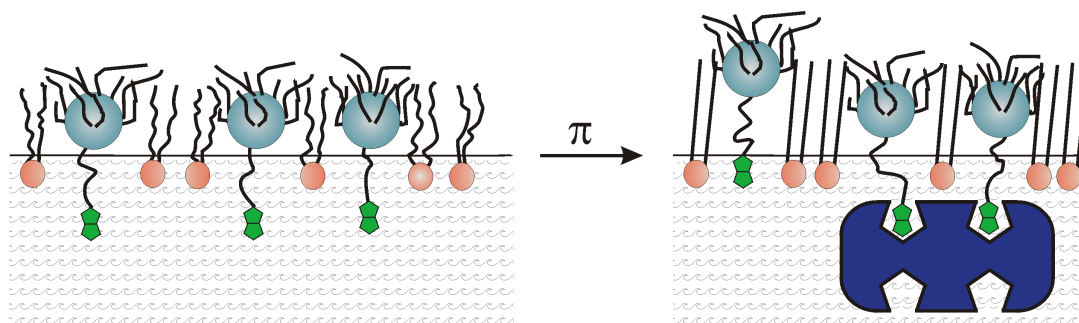
Fig. 3:   Model for the reversible squeeze-out of the fullerene molecules from the monolayer: at low pressure (LE phase) homogeneous distribution in the monolayer is observed (left), during compression the biotinated anchor is retracted into the lipid headgroup region, unless SA was preveously coupled to the monolayer in the LE phase, preventing the retraction (right).

Receptor (SA) binding takes place only at low pressure (LE phase) when the biotin anchor is sufficiently immersed into the aqueous subphase to enter the rather deep binding pocket of SA. High lateral pressure leads to partial demixing of the two components in three rather than in two dimensions. **2** is forced upwards during compression and may form an ordered superstructure on top of the LC-like ordered DPPC chains. The terminal biotin moiety is pulled into vicinity of the choline headgroups of DPPC and becomes inaccessible to SA binding. At low pressure, SA binding from the subphase is observed and remains bound as pressure increases, thus inhibiting the biotin anchor retraction. The neutron reflection and infrared reflection-absorption measurements in figure 4 demonstrate the pressure depending coupling of SA.[8]
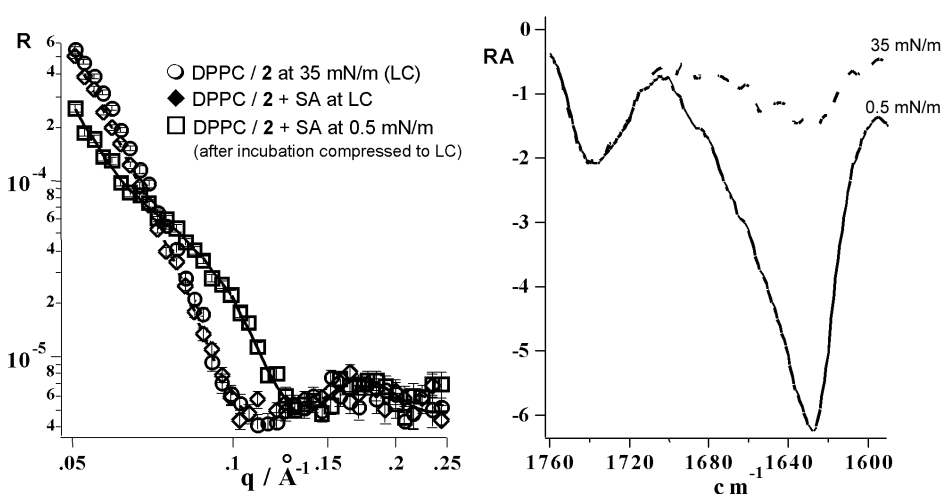
Fig. 4:   Variation of neutron reflection and infrared reflection-absorption at the membrane surface with pressure as evidence of SA binding

The aim of further investigations is to explore whether this rarely observed phenomenon of (at least partial) demixing of the two components in the third dimension can also be accomplished in bilayers. The related phenomenon of protein binding control through lateral pressure in such systems may well prove useful in biosensor applications.

Amphifullerenes with two functional groups for customised modification of membranes

Amphifullerene **3** contains two functional groups and represents a new generation of this promising approach for modulating membrane properties and surface functionalities. The modular synthesis facilitates the variation of the spacer proportions, the terminal biofunctionality and the lipophilic moieties. The combinations used in current syntheses and subsequent studies on membranes are depicted in figure 1. Besides (+)-biotin(**3a**), iminoacetic acid (IDA) ligands (**3b**) as well as polymerisable lipophilic units (**3B**) are under investigation at this time.
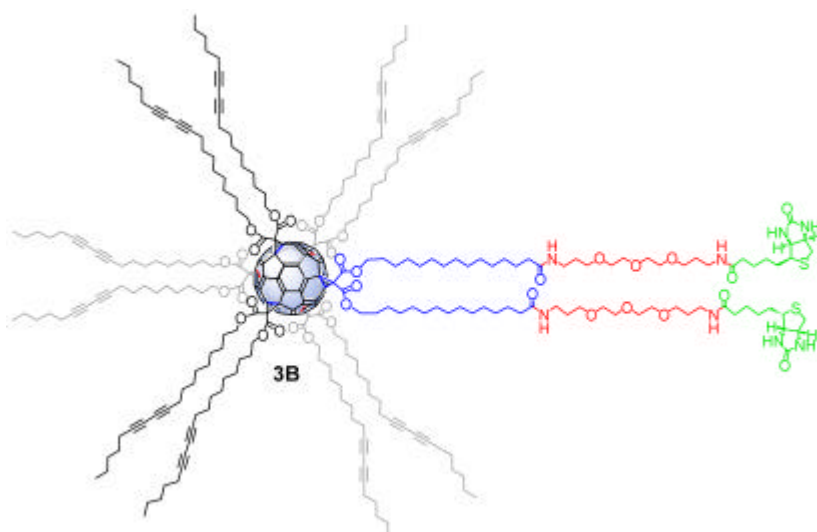

Fig. 5: *Bifunctional Amphifullerene **3B** with two terminal biotin anchors and polymerisable lipophilic side chains containing butadiyne units*

The butadiyne side chains allow 1,4-addition type polymerisation and are designed to fix the self-aggregated nanostructures in membrane bilayers covalently. The resulting poly-fullerene mesh should lead to an enormous stabilisation of the bilayer. Former attempts with lipofullerenes containing six pairs of polymerisable side chains (corresponding to **1B**) failed. Instead of polymerised fullerene rods inside the bilayer spherical polymer beads, which comprised only lipofullerenes, were obtained and the lipid vesicles where destroyed during irradiation with UV light to initiate the polymerisation.[9] The decelerated lateral diffusion of **3B** within the bilayer due to its transmembrane anchors should make the polymerisation process inside the intact membrane more likely. The expected very rigid membranes offer a variety of possible applications.

Artifical liposomes, mono- and bilayers consisting exclusively of fullerene components

A further extension of this new approach to functionalised biomimetics is based on membranes and liposomes consisting exclusively of fullerene derivatives without the membrane forming support of lipids. As mentioned above, amphifullerenes such as example **2** form stable monolayers in their own right and more amphiphilic derivatives like hexaadduct **4** (figure 6) form bilayers. This globular amphiphile is shown to form a wide variety of unilamellar vesicles of dimensions ranging between 50 and 400 nm and small cylindrical aggregates of about 5 to 200 nm.[10] The molecule carries five pairs of lipophilic alkyl chains and one pair of polyamide dendrons in octahedral positions. Due to the 18 carboxylic end groups of the dendrons, high water solubility and amphiphilicity of the molecule is obtained.
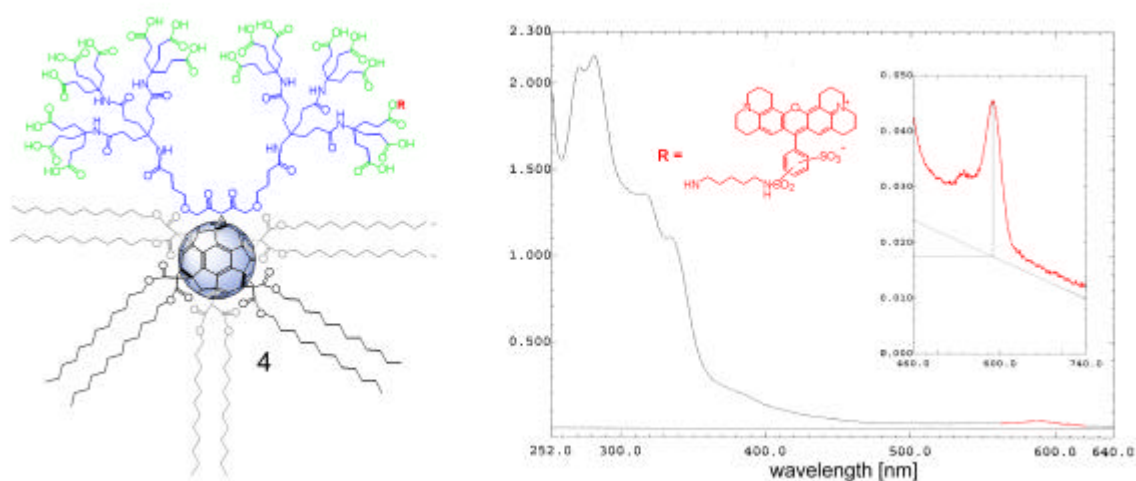

Fig. 6:   Structure of globular amphifullerene **4** (R = H) and UV/Vis spectrum of **4** partially labeled with the fluorescence marker Texas Red^Ò

Since several monolayer-forming fullerene hexaadducts are available, mixtures of differnt derivatives are within the scope of present investigations. The combination of different species each with specific properties and functional features, offers various possibilities for custom-made systems. First results of our examinations by means of fluorescence microscopy show interesting pressure-dependency of the mixing and demixing of monolayers made of **4** mixed with **2**.[11] Amphifullerene **4** was therefore partially labeled with the fluorescence marker Texas Red® (figure 6). The UV/vis spectrum shows the characteristic absorptions of fullerene hexaadducts at 271, 281, 316 and 335 nm as well as the absorbtion band of Texas Red at 587 nm. From the extinction coefficents of hexaadduct **4** and the dye the desired labeling rate of about 2% is established. Fluorescence microscopy elucidates under which conditions homogeneous monolayers or heterogeneous distribution of labeled **4** and **2** are obtained. Further experiments will show how the special characteristics of such binary composites could be applied as functionalised surface coatings or phase boundaries.

Conclusions and Outlook

Besides the above-mentioned features of these systems under current examination, other possibilities can be envisaged. For instance, electric conductivity within the bilayers by assembled fullerene derivatives could lead to new properties such as electrical signalling and controlling of integral membrane proteins. Therefore, new fullerene compounds are to be synthesized. Light-induced conductivity could be achieved with fullerene derivatives with only one or two malonate addends attached, but, unfortunately, monoadducts with unbranched alkyl side chains (like example **A** in the hexaadduct examples above) precipitate out of fullerene-lipid mixtures and do not assemble inside lipid membranes. Branched alkyl addends provide more possibilities for interactions with the lipophilic regions of the lipids and therefore may enable solubilisation of fullerene mono- or bisadducts in lipid membranes.

The fullerene-based biomimetics presented in this conference proceedings are only at the beginning of their exploration, but, already a variety of novel applications in customising the properties and functionalities of surfaces have been indicated. Several ongoing studies will investigate their fundamental features and show their possible potential for use in biosensors and related fields.

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