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Fullerene derivatives in bilayer membranes: an overview

Martin Braun, Andreas Hirsch*

Institut für Organische Chemie, Henkestr. 42, D-91054 Erlangen, Germany

Abstract

Since lipophilic fullerene derivatives, so-called lipofullerenes, are found to be unprecedentedly soluble in lipid membrane bilayers, a new concept to modify the physical and mechanical properties as well as the functionalization of membranes by aggregation with fullerenes is accessible. Several model compounds (**1a/b, 2a/b/c/d, 3**) are realized and their behaviour in membrane bilayers was extensively investigated [M. Hetzer, S. Bayerl, X. Camps, O. Vostrowsky, A. Hirsch, T.M. Bayerl, Adv Mater 9 (1997) 913]. We present a brief overview of recent results to introduce this outstanding and promising approach to entirely new supramolecular structures. Alteration of the lipofullerene, for example by polymerizable butadiyn units (**2d, 4c–7c**) as well as hydrophilic moieties combined with biofunctional end groups as (+)-biotin (**3–7**) extends the possibilities to build tailor-made nanostructures with well- defined biocompatible functions.

Keywords: A. Fullerene; D. Biocompatibility

1. Introduction

Fullerenes are highly suitable cores for well defined and highly symmetrical macromolecules. Furthermore, the fullerene core enables the design of new macromolecular architectures targeted e.g. to modify their properties in self-assembled nanostructures. As components in lipid membranes fullerenes make composites with entirely new properties and structures approachable [1].

The intercalation of plain C_{60} or C_{70} molecules inside a lipid bilayer already should affect the whole membrane structure and should give exceptional material features as has been investigated in several studies [2–4]. For example, the extraordinary lubrication properties as well as the ability of fullerenes to act as photosensitizers or mediators for electron transport could be taken advantage of. However, the main limitation for these studies is the low solubility of plain fullerenes in bilayers [2–4]. To achieve much better solubility, lipophilic fullerene derivatives with long alkyl chains attached, so-called lipofullerenes, were synthesized. The chemical modification certainly alters the electronical, sterical and lubrication characteristics of the resulting derivative compared with the plain fullerene. Hence, membrane composites with different and unexpected physical and structural properties are formed, which could enlarge the wide range of already realized applications for bilayer membranes [5].

2. Membrane-soluble fullerene derivatives

2.1. Plain alkyl-lipofullerenes

To attain dissolution in bilayers, C_{60} was modified by attaching pairs of C_{12}- and C_{18}-alkyl chains respectively, corresponding to the chain length of lauric and stearic acid, to give lipofullerenes **1** and **2** (Fig. 1).

Monoaddition lipofullerenes **1a/b** contain one pair of alkyl chains and were synthesized by modified Bingel cyclopropanation [6] of C_{60} with the corresponding malonate promoted by CBr_4 and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). The T_h-symmetrical hexakisadducts **2a/b/c/d** with octahedral addition pattern are accessible by template activation of C_{60} with dimethylanthracene (DMA) [7] followed by complete sixfold cyclopropanation with the corresponding malonate and CBr_4/DBU [8,9]. These chemical modifications of C_{60} provide the basis for entirely new composite systems with synthetic lipids.

The procedure to achieve multilamellar vesicles (MLVs) consisting of the lipid dipalmitoyl-*sn*-glycero-3-phospha-

*Corresponding author. Fax: +49-9131-8526-864.

E-mail address: andreas.hirsch@organik.uni-erlangen.de (A. Hirsch).

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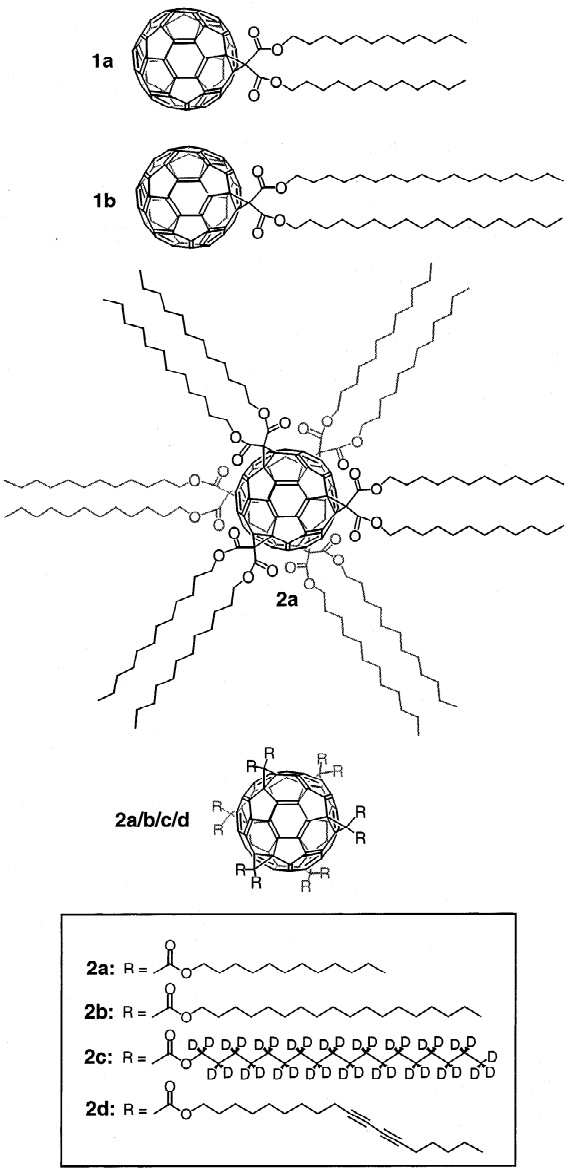


Fig. 1. Structure of monoadducts **1a**/**b** and T_h-symmetrical hexakisadducts **2a**/**b**/**c**/**d** representing lipofullerenes with different types of lipophilic side chains.

tidyl-choline (DPPC) and lipofullerene **2a**/**b**/**c**/**d** is depicted in Fig. 2. The intercalation of monoadduct **1** in between the leaflets of the bilayer turned out to be unsuccessful. Instead of homogeneous composite MLVs a heterogeneous mixture, with **1** precipitating, resulted under the same conditions. In case of the MLVs consisting of **2** and DPPC no macroscopic demixing or precipitation of lipofullerenes was observed up to 25 mol% lipofullerene content [8]. This remarkable proportion is supposed to be the highest known proportion of guest compound in lipid bilayers.

Freeze fracture TEM (transmission electron microscopy) was employed to study the morphological changes of the MLVs caused by the lipofullerenes **2**. In all cases predominantly large MLVs with diameters up to 5 μm and with the typical onion-like structure were observed. While plain MLVs show smooth fracture faces in the bilayer plane with so called 'ripple phases' at temperatures from 34 to 41°C, the inclusion of the lipofullerenes caused the formation of a rod-like surface structure in the entire temperature range between 25 and 70°C (Fig. 3). Although the lipofullerenes were expected to be uniformly distributed within the bilayer leaflets, they seem to self-assemble into rod-like structures of nanoscopic dimensions, which are sandwiched within the two monolayers of the bilayer. The increasing diameter (10–30 nm) of the rods due to increasing fullerene concentration also proves the suggestion, that the rods consist of lipofullerenes. Fig. 3 shows freeze fraction micrographs of MLVs of DPPC with 15 mol% **2a** and **2d**, respectively, under different bilayer conditions. In the fluid phase of the bilayer the rods appear rather disordered, but become stratified and long range ordered over distances of several micrometers in the gel phase. Here, the formation of superstructures reminiscent of bundles of individual rods (Fig. 3b) were also observed. With variation of the alkyl chain length and type (**2a**/**b**/**c**/**d**), no significant changes of these effects occured.

To examine the changes of the molecular order in the bilayer resulting from the intercalation of lipofullerenes by ^{2}H-NMR, several studies with lipofullerene **2b**, the analogous perdeuterated lipofullerene **2c** as well as with DPPC, selectively deuterated DPPC-d_8 and completely deuerated DPPC-d_{62}, were performed. It can be concluded that the presence of the lipofullerene does not cause significant alteration in the distribution of the methylene groups of the lipid chains from the DPPC, but that the methyl group reveals some significant effect. The interactions between lipofullerene and DPPC in the MLV's seem to take place mainly at the end groups of the molecules. For example the lipofullerenes are in contact between each other and the methyl groups of DPPC, while the rest of the DPPC molecules remains unaffected.

Extensive DSC (differential scanning calorimetry) measurements indicate that the phase transitions of DPPC and lipofullerenes are completely decoupled and that complete microscopic demixing predominates. Thus, the interaction between lipofullerenes and DPPC in the MLVs seems to be very small. The exceptional melting characteristics of plain **2** shown in the DSC, when heating causes the sample to undergo two major structural transitions (an exothermic transition and an endothermic melting transition), has also been investigated by ^{2}H-NMR and X-ray diffraction [10]. The resulting thermotropic phase behaviour can be described by a three-state model with an interdigitated state existing intermediately between the low-temperature, hard sphere fashion packing state and the fluid-like high-temperature state. The exothermic transition from the low-

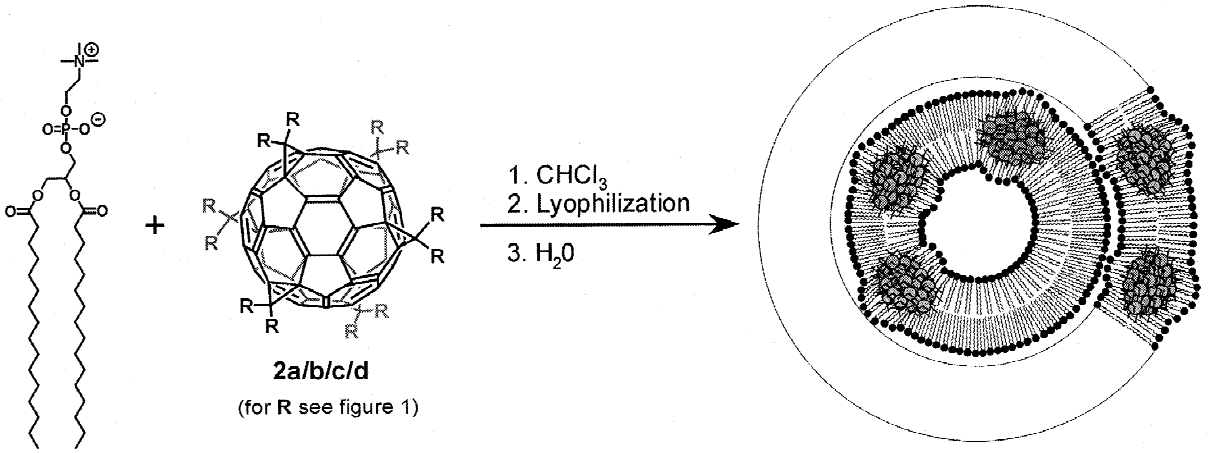


Fig. 2. Preparation of multilamellar vesicles (MLVs) with DPPC and lipofullerene **2a**/**b**/**c** or **d**.

temperature to this intermediate state is the effect of the denser packing due to the partial interdigitation of the alkyl chains belonging to neighbouring molecules.

The stabilization of the bilayer against deformation in a magnetic field by the lipofullerene rods is also remarkable. Unlike plain DPPC MLVs, which exhibit a microscopic magnetic field orientation due to the diamagnetic susceptibility anisotropy of the lipid [11,12] resulting in an elliptical MLV shape, the lipofullerenes seem to stiffen the fluid bilayer. This is comparable to the strut-like membrane cytoskeleton, despite the fact that the former seems to be located within the bilayer, while the cytoskeleton is attached at the membrane's outside.

2.2. Polymerizable lipofullerenes

Lipofullerene **2d** comprises octadecadiynyl chains to represent a hyperfunctional monomer for 1,4-addition type polymerization [13–17]. The intention of a polymerization of **2d** embedded in the membrane bilayer was to fix the rod-like self-aggregated structures covalently. Plain membranes consisting of lipids with butadiyne moieties are already well known to be polymerized resulting in networks of oligodiacetylenes [18]. The hyperfunctionality of **2d** enables an efficient formation of three-dimensional networks. An enormous stabilisation of the MLVs would arise due to the resulting polylipofullerene mesh. Furthermore, the removal of the lipids could lead to a three-dimensional framework of polymerized lipofullerenes.

The synthesis of **2d** was carried out in analogy to the synthesis of **2a**/**b**/**c** via template mediated cyclopropanation [8]. The membrane composite with **2d** and DPPC exhibits similar surface structures as the corresponding composites with the saturated lipofullerenes **2a**/**b**/**c** (Fig. 3c).

The polymerization of the aqueous suspension of DPPC-MLVs containing 15 mol% lipofullerene **2d** was initiated by irradiation with UV light (8 W, 265 nm, 12 h) at two different temperatures for the gel and fluid phase of the MLVs [19]. Precursor control experiments with plain DPPC under the same conditions investigated by DSC

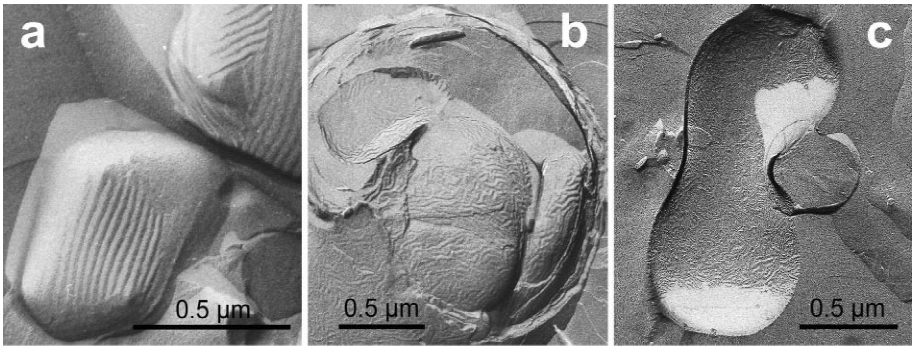


Fig. 3. Freeze fracture micrographs of MLVs of DPPC-d_{62} with 15 mol% of lipofullerene **2a** (a,b) and **2d** (c). The samples were quenched at either 25°C (a) or 60°C (b,c) [6].

showed no indication of thermal degradation or irradiation damage of the lipids.

In contrast to the control experiment, DSC as well as ^{2}H-NMR measurements revealed significant changes of the lipid molecular order and the dynamic bahaviour in the fluid phase samples (50°C) after the UV treatment. The deuterium NMR experiments also indicate that the lipid parts become exposed to a less flexible moiety within the bilayer, similar to the effect when plain DPPC bilayers become attached to a solid support.

Freeze fracture TEM of the fluid phase samples after irradiation shows almost no more rod-like structures (Fig. 3c). The number of lipid vesicles has decreased and perfectly spherical polymer beads with a diameter range from 100 nm up to several micrometers are observable (Fig. 4a). Under gel phase conditions no spheres were formed.

The smaller spheres (diameter $<$150 nm) are hollow and transparent, so the replica background below them was visible through the objects, whereas the larger beads are filled and thus impermeable to the electron beam. After irradiation it is possible to remove the lipids with organic solvents (Fig. 4b). The electron contrast of the remaining spherical structures reveals that they consist essentially of polylipofullerenes. Atomic force microscopy (AFM) was used to study the surface of the polylipofullerene beads (Fig. 4c). Single lipofullerenes stick out of the surface with an average distance of 16 nm to each other. After computational smoothing (second order base line correction) to compensate the surface curvature, an average coarseness of the polymer spheres of 0.9 nm and a maximum amplitude in height of 1.5 nm was determined (Fig. 4d). AFM also proved the filled polylipofullerenes to exhibit an unusually high strength.

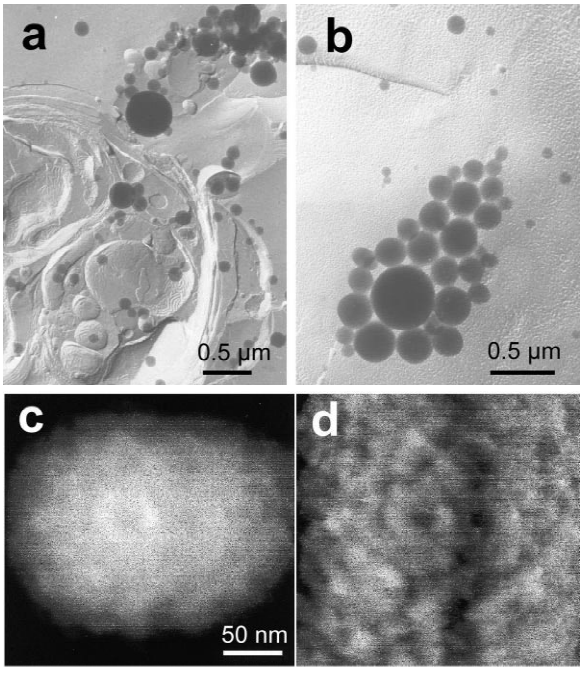


Fig. 4. TEM (a,b) and AFM (c,d) pictures of lipofullerene polymerspheres (a) after UV-irradiation of DPPC MLVs containing 15 mol% **2d**; (b) and (c) after extraction of DPPC; and (d) after computational smoothing of the surface [19].

The observed polymerisation of lipofullerenes is supported by the membrane and represents a special kind of emulsion polymerization where the monomer is polymerized in aqueous solution in presence of a detergent [20]. The formation of nanospheres is only observed from the fluid MLV-phase when the quasi two-dimensional laterale mobility of the monomers is maintained. Therefore the vesicles are suggested to hold an essential function as a template to enable isotropic diffusion-controlled polymerization.

2.3. Lipofullerenes with amphiphilic anchors ('amphifullerenes')

Lipofullerenes with at least one amphiphilic side chain represent a new class of transmembrane anchor systems. The biotinated lipofullerene **3** (Fig. 5) is a model compound, whereas the (+)-biotin unit is used to enable biocompatibility and molecular recognition.

Due to its lipophilic properties **3** is enclosed in DPPC bilayers while the amphiphilic side chain intercalates the lecithins. Hence the (+)-biotin unit as a biologically active linker is located at the membrane's outside and enables the addressing of membrane areas by labelling with streptavidin or avidin [21]. Fig. 6 shows a schematical representation and computer generated space filling model of the biotinated lipofullerene **3** embedded in a DPPC bilayer. The space filling model is based on MM+ force field calculations using the program package HyperChem [22].

The main advantages of this system compared with biotinated lecithins [23,24] are the stabilizing effects on the membrane vesicles and that the biotin-coupled protein is expected to be prevented from extracting the biotinated component out of the membrane.

Lipofullerene **3** represents a [1+5]-hexakisaddukt which was synthesized via subsequent reaction of C_{60} with the malonate carrying the amphiphilic spacer under modified Bingel conditions [6] to the corresponding monoadduct followed by the fivefold template mediated cyclopropanation with didodecyl malonates in presence of CBr_4/DBU and DMA [21,25]. In the final step of the synthesis (Scheme 1) (+)-biotin was attached to the deprotected precursor by Staab coupling [26,27].

Compound **3** was fully characterized by IR, UV/Vis, ^{1}H- and ^{13}C-NMR spectroscopy as well as by mass spectrometry. The UV/Vis spectrum shows the two typical absorptions for hexakisadducts of C_{60} with an octahedral

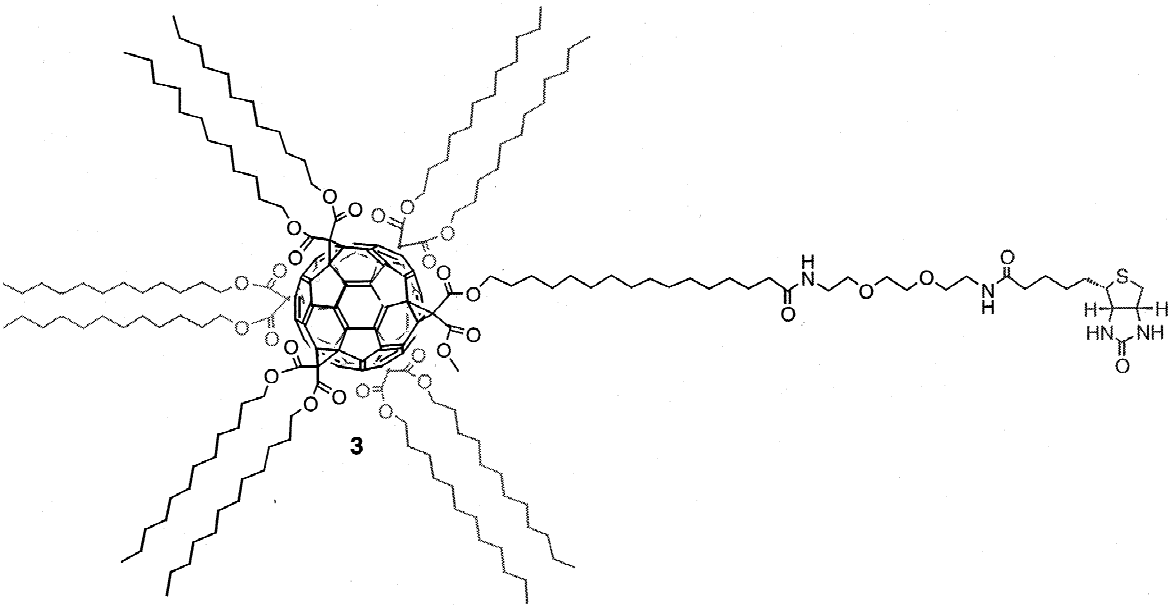


Fig. 5. Structure of the first realized lipofullerene with a biotinated transmembrane anchor.

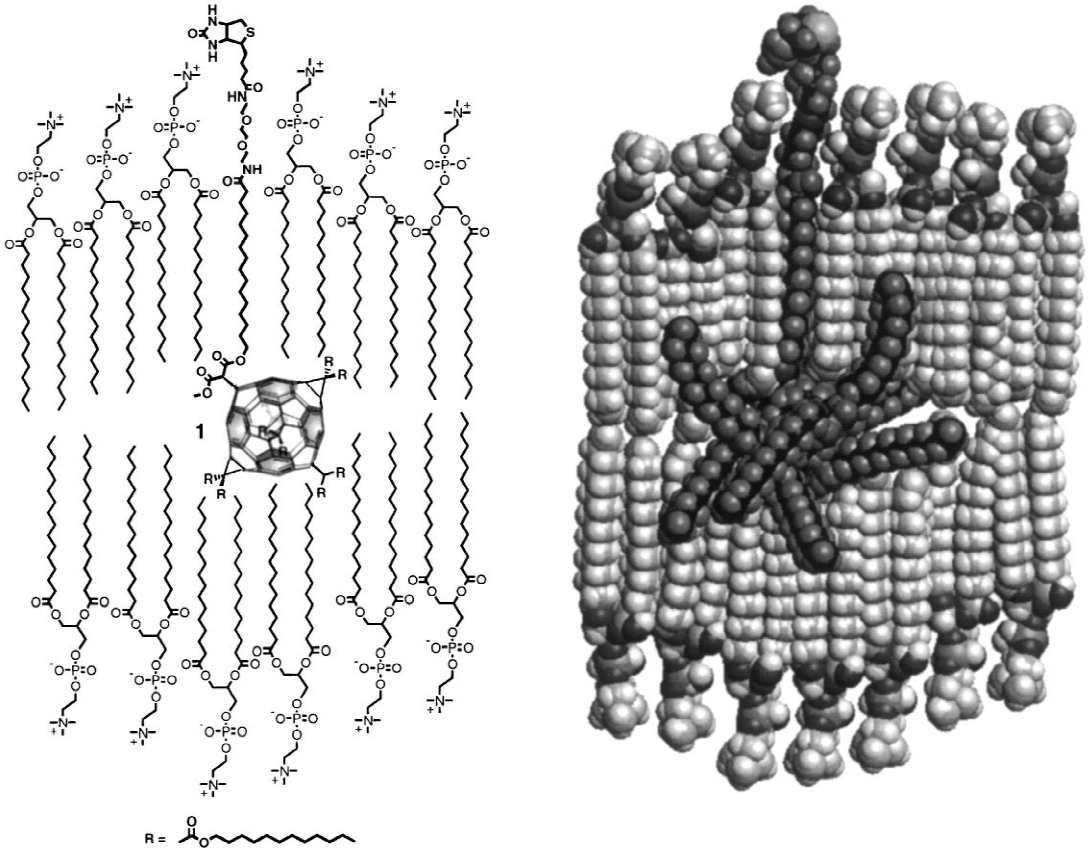
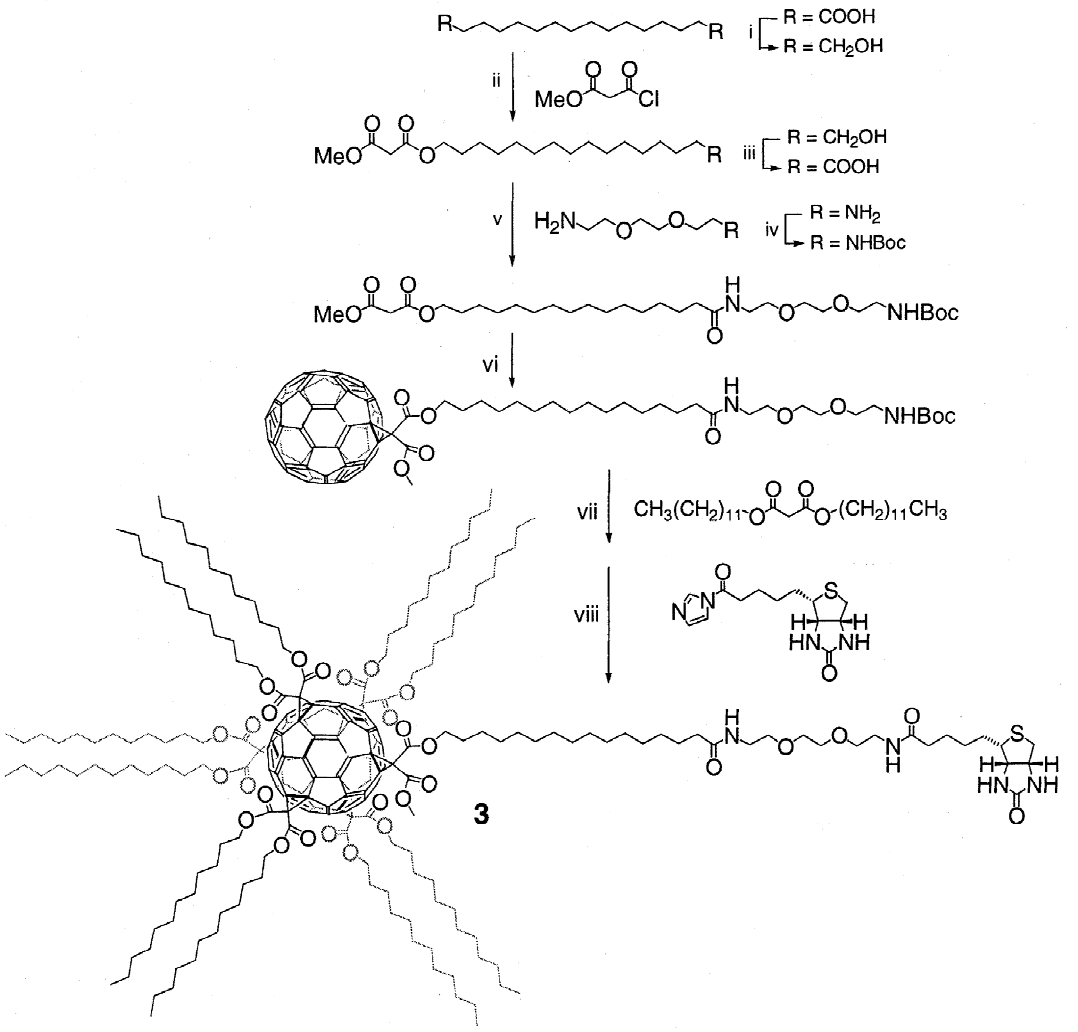


Fig. 6. DPPC bilayer enclosing the biotinated lipofullerene **3** depicted schematically (left) and as a space filling model (right).



Scheme 1. Stepwise synthesis of the biotinated lipofullerene **3**. (i) LAH, THF; (ii) pyridine, DCM; (iii) PDC, DMF; (iv) boc anhydride, dioxane; (v) CDI, THF; (vi) C_{60}, DMA, CBr_4, DBU, toluene; (vii) DMA, CBr_4, DBU, toluene; (viii) (a) TFA, DCM, (b) CDI, DMF, toluene [21].

addition pattern. The ^{13}C-NMR spectrum is also characteristic for such mixed hexakisadducts of C_{60} [25]. FAB mass spectroscopy results in the molecular ion peak and a quasimolecular ion peak at $m/z=3513$ and 3647 corresponding to $M+$ and $[M+Cs]^+$.

First experiments with RIfS (Reflectometric Interference Spectroscopy [28,29]), LB-film techniques and freeze fracture TEM provide evidence for the expected properties of **3** to couple selectively to the protein streptavidin, to build transmembrane systems with lecithins and to aggregate in between lipid membrane bilayers [21]. Compression experiments with the target molecule **3** spread as a monolayer at the air/water interface of a Langmuir trough also suggest a reversible phase behavior which is qualitatively similar to that of a lecithin monolayer. Lecithins mixed with **3** show different π/A-isotherms (surface pressure vs. area) than pure lecithin monolayers, which indicates the intercalation of the amphiphilic spacer.

Freeze fracture transmission micrographs of the membrane vesicles consisting of DPPC and **3** show the familiar rod-like nanostructures in the gel phase, whereas in the fluid phase the lipofullerenes are presumably more homogeneously distributed within the lipid bilayer. With regard to the decreased diffusion of lipofullerenes in between the bilayer because of the anchor, membrane morphologies different from the composites with plain

lipofullerenes result. We will report on these results in detail in an forthcoming publication.

3. Outlook

Lipophilic and amphiphilic fullerenes in combination with lecithin membranes allow for the formation of entirely new macromolecular systems. An enormous range of possibilities to modify the structure and properties of the membrane composites is accessible. For example studies with different chain lengths or numbers of chains as well as varying hydrophilic moieties should enable tailored membrane components. Therefore compounds **4–7** (Fig. 7) are within the scope of our next investigations.

Different amphiphilic and hydrophobic spacers consisting of oligoethylene or oligo-peptide moieties as well as varying numbers of spacers are means to influence the aggregation of the fullerenes. The diverging behaviour of similar [1+5]-, [2+4]- and [3+3]-hexaadducts could also be used to control particular features of the nanostructure's formation. The anchor system of course allows other biofunctionalities than biotin in order to adapt the membrane for various tasks. For example, chemically coupled systems via thiol-endgroups to gold surfaces are possible. The diffusion of the fullerenes with transmembrane anchors probably decreases with the number of anchors. Hence, the diffusion-reduced subphase in between the bilayer with polymerizable side chains (i.e. **4c**) could lead to a polymerization within the bilayer without destroying it. Drastically stabilized membranes would result and a wide field of new possibilities for example as macromolecular carrier or delivery systems would open up. In case of delivery systems it would be an additional challenge to open the container again and release the encapsulated molecules.

Acknowledgements

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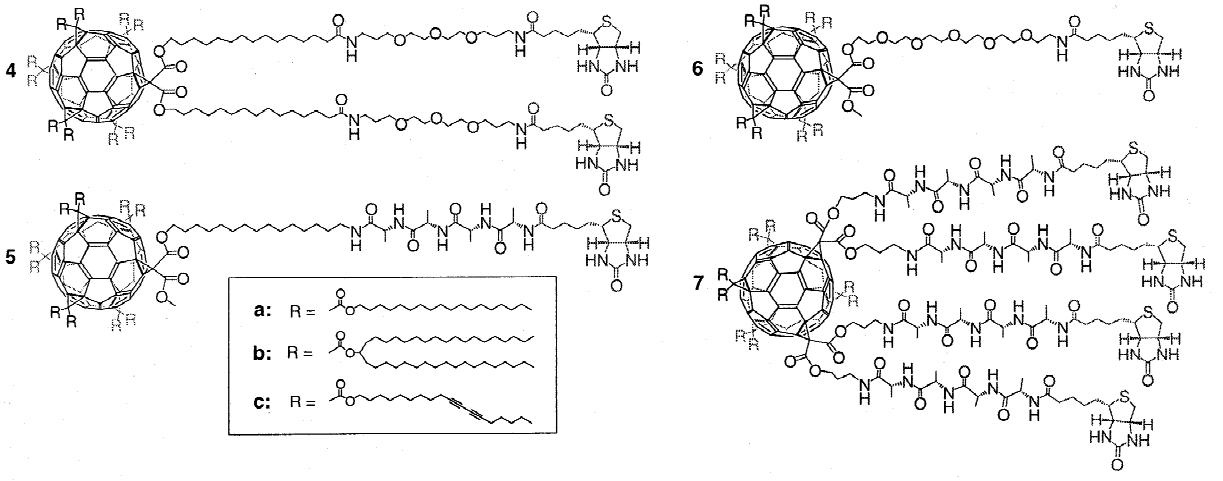

Fig. 7. Model compounds with amphiphilic and hydrophilic side chains to diversify the structure and properties of membrane composites.

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