Polymerized liposomes as stable oxygen-carriers

James A. Hayward[+*], Daniel M. Levine[+], Lawrence Neufeld[+], Sanford R. Simon[+], David S. Johnston[*] and Dennis Chapman[*]

[+]*Department of Biochemistry, State University of New York at Stony Brook, Stony Brook, New York 11794, USA* and [*]*Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine (University of London), Rowland Hill Street, London NW3 2PF, England*

Received 31 May 1985

We have produced a surrogate erythrocyte ('hemosomes') by encapsulating human hemoglobin in polymerized vesicles composed of diacetylenic phospholipids plus or minus cholesterol. Hemoglobin (in the presence or absence of allosteric effectors) was encapsulated by a freeze-thaw method in large, unilamellar vesicles composed of monomeric lipids. Entrapment was demonstrated by molecular-sieve chromatography. Brief irradiation with ultraviolet light produced polymeric hemosomes with polymerization kinetics and conversions similar to liposomes in the absence of protein. Photo-induced oxidation of the heme was eliminated or severly limited by a combination of prior ligation with CO and the maintenance of high intravesicular hemoglobin concentrations (5–10 mM internal hemoglobin). The inclusion of allosteric effectors within polymerized hemosomes facilitated near-quantitative conversion to the oxy-HbA form. Gas permeability of monomeric and polymeric hemosomes was demonstrated by spectroscopic methods. Reversible spectral shifts, corresponding to oxygenation-deoxygenation, were obtained after brief evacuation and exposure to oxygen or nitrogen. The gas permeability of polymerized hemosomes appears sufficient for the vesicles to act as oxygen carriers in vivo, a notion that is strengthened by their apparent hemocompatibility.

Phospholipid polymer *Liposome* *Hemoglobin* *Oxygen transport* *Blood substitute* *Hemosome*

1. INTRODUCTION

Efforts directed toward the development of oxygen-carriers as replacements for red blood cells have extended over several decades [1,2]. The characteristics required for red cell surrogates include chemical and physical stability, low immunogenicity, thromboresistance, appropriate oxygen affinity, adequate biological half-life and biological inertness (low pathogenic potential). Liposomes containing entrapped hemoglobin ('hemosomes') may fulfill these requirements [3–6]. However, their application may be limited, in part, by the instability of the bilayer capsule when it is comprised of conventional phospholipids.

We have recently described a new concept in biomaterials design, namely, the design of polymers which mimic the thromboresistant surfaces of blood cell membranes [7]. The simplest common feature among the blood-compatible cellular and model membranes is the high content of the electrically neutral phospholipids that contain the phosphorylcholine head group. We have synthesized phosphatidylcholines (PC) that contain diacetylenic groups in one or both of their acyl chains [8]. These phospholipid molecules form polymers upon irradiation with ultraviolet light; polymerization has been accomplished for diacetylenic phospholipids in the membranes of living microorganisms and in a series of biomembrane model systems (review [9]). ^{13}C-NMR and Raman spectroscopic studies demonstrated that conversion of the monomer corresponds to the formation of an alternating double-bonded and triple-bonded conjugated structure [10,11] (fig.1C).

Liposomes composed of polymeric phospholipids may offer specific advantages for hemosome

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

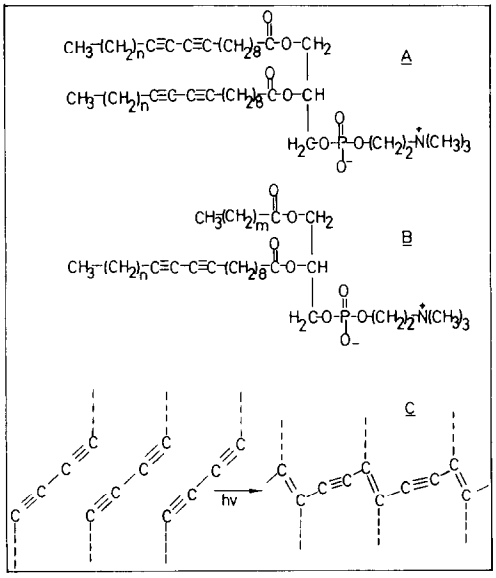

Fig.1. Structures of the monomeric, identical-chain (A) and mixed-chain (B) diacetylenic PCs, and the polyconjugated polymer (C) formed by irradiation. The number of methylene groups, m and n, may be varied.

applications because of their reduced permeability and rate of aggregation [12], their increased resistance to hydrodynamic shear and chemical disruption [13], and their apparent thromboresistance [7,14]. The incorporation of some polymeric phospholipids within the bilayers of liposomes dramatically reduces their permeability [14] and increases their resistance to the damage induced by sonication, organic solvents or detergents [13]. Polymerized vesicles of diacetylenic PC do not perturb platelet aggregation [14] nor blood coagulation in vitro [7].

2. MATERIALS AND METHODS

Monomers of diacetylenic phosphatidylcholines were synthesized as described previously [7,11]. Two classes of PC were synthesized (see fig.1): (a) identical-chain PC, containing diacetylenic groups in both acyl chains, and (b) mixed-chain PC, containing one diacetylenic chain and one fully saturated acyl chain. The number of carbons in the diacetylenic chain was 23 (C_{23}; $n = 9$) or 25 (C_{25}; $n = 11$) for the mixed-chain lipids or 20 (C_{20}; $n = 13$) for the identical-chain lipid. Mixed-chain lipids were prepared from egg yolk PCs (Sigma, London) containing palmitic acid ($n = 14$) and stearic acid ($n = 16$) in the ratios 70:30 (mol/mol) as revealed by gas-liquid chromatography. All other reagents were analytic or spectrophotometric grade. All lipids gave a single spot when analyzed by thin-layer chromatography [7]. The R_f values for the diacetylenic PCs were similar to those of a highly pure dipalmitoyl PC. Differential scanning calorimetry yielded a single, well defined endotherm for each lipid. The transition temperatures for the main gel-to-liquid-crystal transition have been reported [12].

Human adult hemoglobin (HbA) was isolated from outdated units of red blood cells by standard procedures [15]. Soluble HbA was dialyzed against phosphate buffer (0.1 M NaH_2PO_4, 0.01 M KCl, pH 7.4), concentrated in an Amicon ultrafiltration system using CO as the driving gas, and stored at 4°C. Stock HbA solutions were approx. 10 mM in heme as measured by the absorbance of the carbon-monoxy form (CO-HbA) at 540 nm.

Hemosomes were prepared by first dispersing a thin film of the dried lipid in phosphate buffer containing inositol hexaphosphate (IHP, 10 mM). IHP is an allosteric effector and lowers HbA affinity for gaseous ligands; the presence of IHP in polymeric hemosomes should increase O_2 delivery under conditions of low oxygen pressure (e.g. in peripheral tissue). Cholesterol was included in the mixed-chain hemosomes in order to minimize solute permeability of the monomeric hemosomes. The suspension (5–15 mg lipid/ml) was sonicated to clarity in a bath sonicator (Laboratory Supply Co., New York) under CO. CO-HbA was added to a final concentration of 5 mM. Following brief sonication, the dispersion was frozen rapidly in liquid N_2 and then thawed slowly to room temperature as described by Pick [16]; this method yields large, unilamellar vesicles.

Entrapped and free HbA were separated by molecular-sieve chromatography on cross-linked Sepharose 4B or 6B (Pharmacia); the hemosomes eluted in the void volume. Monomeric hemosomes were converted to the CO-HbA form and then polymerized by UV irradiation at 0°C in a quartz cuvette under CO for variable periods of time using a 254-nm light source (Spectronics C-81, Westbury, NY) at a dose of 360 $\mu W/cm^2$. Hemosomes used in gas-ligation experiments were polymerized for periods of 5 or 60 min. Conver-

to the oxy-HbA form was accomplished by alternate evacuation and gentle agitation in an oxygen atmosphere; hemosomes were deoxygenated in a similar fashion using nitrogen or by addition of an excess of sodium dithionite.

The kinetics of monomer conversion were followed by measuring the absorbance at 520 nm, the wavelength at which the polymer absorbance is the strongest. The hemosomes were deoxygenated by gentle agitation in a nitrogen or carbon monoxide atmosphere (before or after polymerization) or by addition of an excess of sodium dithionite (after polymerization).

The spectra of hemosomes were obtained on an Aminco DW-2 UV/Vis spectrophotometer in the dual-beam mode. Visible and ultraviolet spectra of monomers and polymers in solutions and dispersions were recorded on either a Unicam SP8-100 or a Cary 17 spectrophotometer. The pure polymer was isolated by chromatography of chloroform extracts on Sepharose-LH60 (Sigma).

3. RESULTS AND DISCUSSION

Encapsulation of HbA within the enclosed volume of unilamellar, diacetylenic PC vesicles can be demonstrated by the behavior of the dispersion in a column of molecular sieves. Non-irradiated vesicles elute in the void volume with approx. 2–3% of the total HbA, and are well resolved from the fractions of extravesicular HbA. Ultraviolet irradiation of the lipid-HbA dispersions before chromatographic purification resulted in a greater efficiency of encapsulation; 3–5% of the total HbA eluted with the light-scattering, early fractions. The increased entrapment may represent a decrease in HbA leakage during chromatographic isolation. However, the recovery of lipid from the column was poor for polymerized hemosomes. A large percentage of the phospholipid polymer remains tightly associated with the column matrix, evident by a red band at the top of the column, and has been observed by previous investigators [17]. Irradiated, large unilamellar vesicles are less strongly adsorbed than similarly treated multilamellar vesicles. Once isolated, the polymerized hemosomes remain essentially monodisperse when stored at room temperature for up to 8 h. On prolonged storage at 4°C some settling of the hemosomes occurs which can be eliminated by gentle agitation.

When unprotected, HbA in the ferrous form may be photo-oxidized to met-HbA by ultraviolet irradiation. Therefore, we examined the rates of CO-HbA oxidation for irradiated hemosomes and for unencapsulated CO-HbA (fig.2). The rate of met-HbA production, reflected by the absorbance at 405 nm, is much more rapid for free HbA than for hemosome-encapsulated HbA. No further photo-induced oxidation of HbA occurs in egg phosphatidylcholine after the first 5 min, even after prolonged (80-min) irradiation. Similarly, the concentration of CO-HbA, reflected by the absorbance at 419 nm, shows a much more rapid and extensive photo-induced decrease for free HbA than for hemosome-encapsulated HbA. These results demonstrate that HbA is protected against oxidative damage during irradiation by encapsulation at high HbA concentrations. It seems unlikely that this protection simply represents a decrease in light penetration (i.e., internal-filter effects) since the rates of polymerization for liposomes (HbA-free) and hemosomes were essentially identical (see below). Similar experiments (not shown) demonstrated the advantages of irradiating hemosomes in CO-form as opposed to the deoxy-form. Polymerizable hemosomes must be irradiated in the absence of oxygen, since even trace levels may inhibit the polymerization process. When irradiated

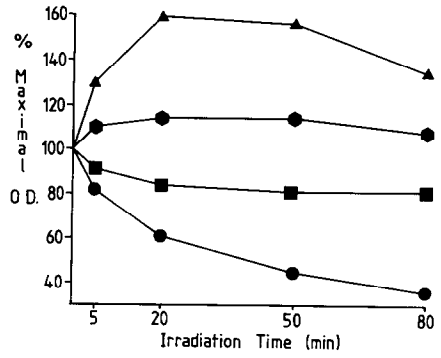

Fig.2. The effects of ultraviolet irradiation on the integrity of free HbA and HbA entrapped in egg phosphatidylcholine:cholesterol (9:1, mol:mol hemosomes: absorbance at 405 nm for free HbA (▲) and hemosomes (●); absorbance at 419 nm of free HbA (●) and hemosomes (■).

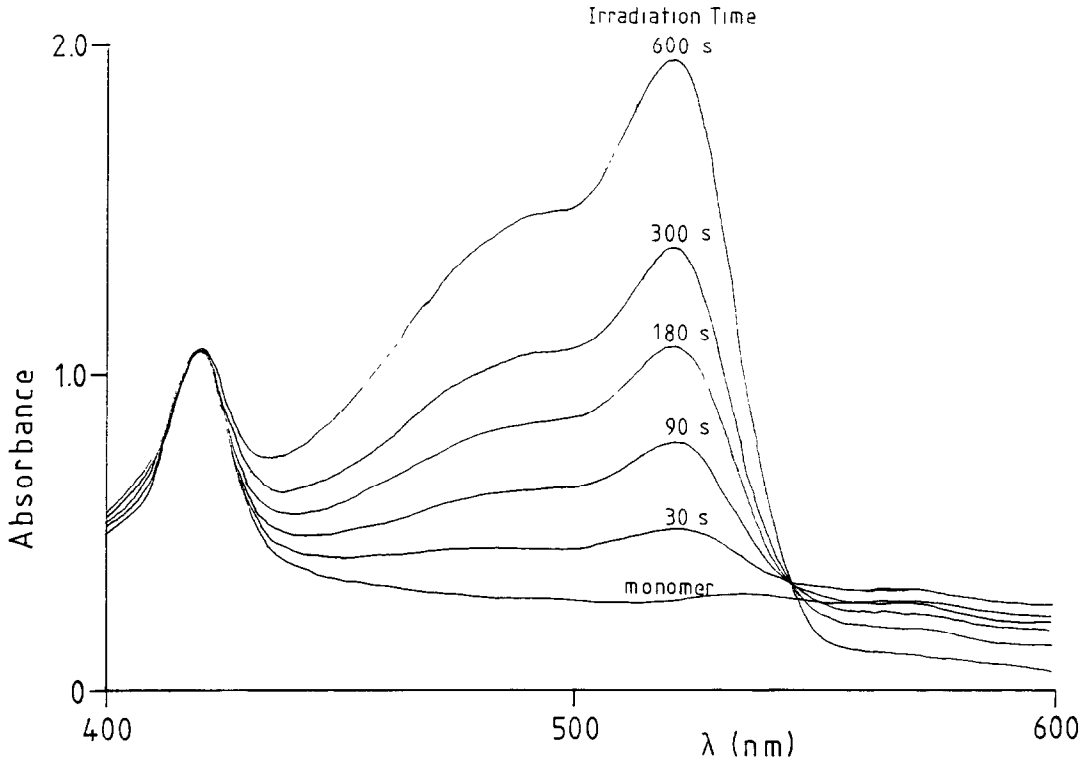

Fig.3. Visible spectra of diacetylenic hemosomes (C_{25}-mixed-chain phosphatidylcholine:cholesterol, 9:1) as a function of irradiation time.

under deoxy conditions, the spectra of hemosomes composed of egg phosphatidylcholine indicated a very rapid rise in the percentage of met-HbA which was accompanied by a decrease in the total absorbance. Prior conversion of the hemosomes to the CO-form eliminated these spectral changes and increased the rate of polymerization in diacetylenic hemosomes.

Monomeric hemosomes are characteristically pink; irradiation results in an orange hue which is sometimes difficult to discern in a concentrated solution of hemosomes. In fig.3 are presented visible spectra of diacetylenic hemosomes as a function of irradiation time. The spectrum of CO-HbA is essentially unaltered by polymerization. The generation of a negligible amount of met-HbA is apparent in the increased absorbance at 405 nm. Polymerization is manifest in the absorbances at 490 and 520 nm.

The kinetics of hemosome polymerization were compared with the kinetics of liposome polymerization to determine whether the presence of HbA affected the formation of phospholipid polymer (fig.4). The inclusion of cholesterol (0.1 mole fraction) in the bilayers of monomeric liposomes did not affect the rate or extent of polymerization. Addition of CO-HbA did not interfere with the extent of polymerization nor with the spectral characteristics of the polymer. A slight decrease in the rate of polymerization was noted upon addition of CO-HbA; however, this can easily be compensated for by using longer irradiation times without damage to HbA.

Extraction of polymeric liposomes into chloroform, followed by isolation of the high-molecular-mass polymer on a column of Sepharose-LH60, permits the determination of polymer weights and extinction coefficients. Based upon the absorbance of polymeric liposomes at 520 nm (fig.3), approx. 75% of the maximal color is developed after irradiation for 10 min. Analysis by gas-liquid chromatography indicates that greater than 70% of the monomeric phospholipids are converted to polymer during the same time

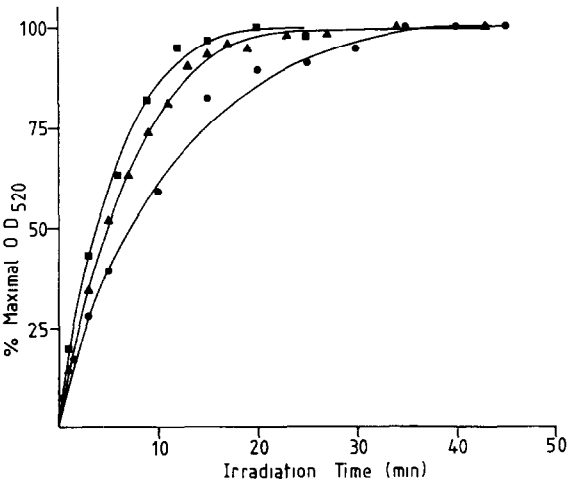

Fig.4. Kinetics of liposome (no added HbA) and hemosome polymerization expressed as percent of maximal absorbance at 520 nm: (▲) liposomes composed of pure diacetylenic (C_{25}-mixed-chain) phosphatidylcholine; (■) plus cholesterol (0.1 mol fraction); (●) hemosomes composed of the diacetylenic phospholipid plus cholesterol.

course. However, when the extracted samples were subjected to molecular sieving, only 25% of the total phospholipid weight elutes as a high-molecular-mass fraction while the remaining polymer elutes in the inclusion volume. We conclude from these results that the polymers have a broad distribution of molecular weights, only 25% of which is sufficiently large to elute in the void volume of the LH60 column. The spectral characteristics of mixed-chain polymers, in particular the presence of 2 absorbance maxima, are consistent with a wide distribution of polymer molecular masses. The polymers formed from the identical-chain lipid are more uniform in size (evident by narrow absorbance peaks), form smaller molecular-mass polymers (absorbance maximum at 460 nm) and are less intensely colored. The characterization of mixed-chain polymer fractions which differ in their relative molecular masses is in progress. The larger molecular masses of mixed-chain phospholipid polymers, and their enhanced reactivity in comparison with identical-chain monomers suggest that these phospholipids may be more appropriate for hemosome preparations.

The absorbance spectra of polymerized, diacetylenic hemosomes are shown in fig.5. Following polymerization, hemosomes in the CO-form (λ_{max} = 419 nm) were quantitatively converted to the oxy-form (λ_{max} = 415 nm) by evacuation and exposure to O_2. This initial change in ligation state was greatly facilitated by the presence of IHP. Our initial experiments were performed in Tris buffer; conditions under which complete conversion from CO-HbA to oxy-HbA was never at-

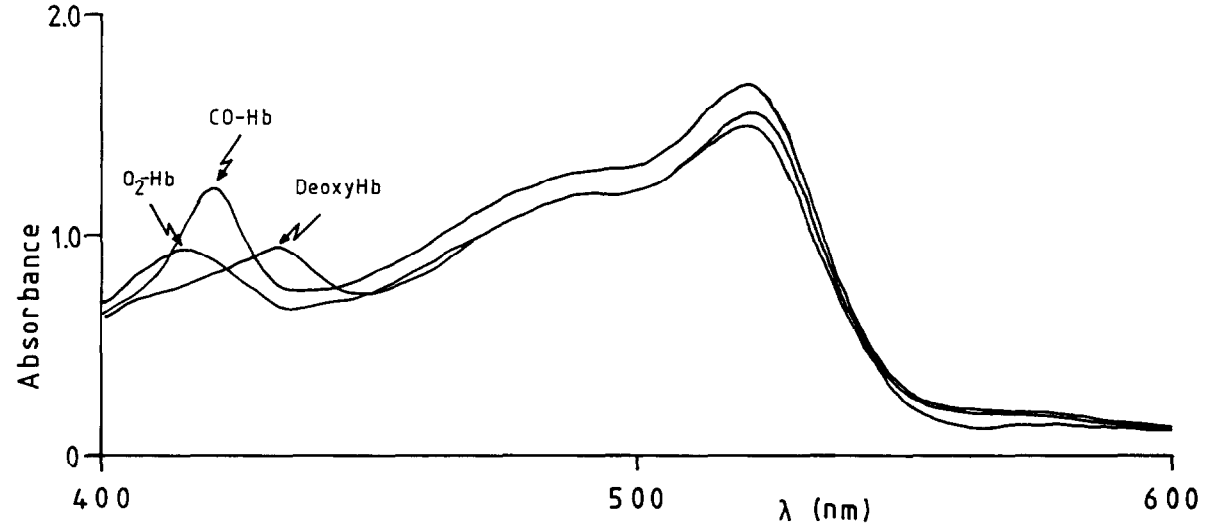

Fig.5. Visible spectra of polymerized, diacetylenic (C_{25}-mixed-chain phosphatidylcholine: cholesterol, 9:1) hemosomes. Reversible spectral changes accompany the exchange of gas across the polymerized bilayers.

tained. Both IHP and inorganic phosphate serve to lower the affinity of ligand-binding. IHP is a powerful allosteric effector and has the added advantage of low membrane permeability due to its polyanionic character. When the suspension is evacuated and then flushed with nitrogen, the Soret band of deoxy-Hb (λ_{max} = 430 nm) was obtained. These spectral changes were completely reversible. No difference in gas permeability could be detected between monomeric and polymeric hemosomes, nor between polymeric hemosomes formed from mixed-chain or identical-chain phospholipids. Moreover, polymeric hemosomes yielded the same gas-ligation behavior regardless of the duration of exposure to ultraviolet radiation for periods up to 60 min (the maximum duration tested).

In summary, these results demonstrate that: HbA is retained within the enclosed volume of polymeric hemosomes, the entrapped HbA is capable of reversibly binding dissolved gases, and polymeric lipids are gas permeant. Studies of gas-exchange rates and the preparation of oxygen-dissociation curves in the presence and absence of allosteric effectors are currently in progress. While it is envisaged that polymerized hemosomes are suitable for development as surrogate erythrocytes, the stability imparted by a polymeric, bilayer capsule may enable their eventual application as gas-carriers in other situations that necessitate a high degree of physical or chemical stability.

ACKNOWLEDGEMENTS

Financial support for this work was provided by Biocompatibles Ltd (J.A.H. and D.S.J.), a Postdoctoral Fellowship from the National Institutes of Health (5-T32-ES07088; D.M.L.), and by grants from the National Institutes of Health (HL-13527) (L.N. and S.R.S.) and the Leverhulme Trust (D.C.). The authors gratefully acknowledge the technical assistance provided by Fred Bunz.

REFERENCES

[1] Gruber, U.F. (1969) Blood Replacement, Springer, Berlin.
[2] Miller, I.F. (1978) CRC Crit. Rev. Bioeng. 3, 149–179.
[3] Djordjevich, L. and Miller, I.F. (1980) Exp. Hematol. 8, 584–592.
[4] Miller, I.F. (1981) Chem. Eng. Commun. 9, 363–370.
[5] Gaber, B.P., Yager, P., Sheridan, J.P. and Chang, E.L. (1983) FEBS Lett. 153, 285–288.
[6] Szebeni, J., Breuer, J.H., Szelenyi, J.G., Bathori, G., Lelkes, G. and Hollan, S.R. (1984) Biochim. Biophys. Acta 798, 60–67.
[7] Hayward, J.A. and Chapman, D. (1984) Biomaterials 5, 135–142.
[8] Johnston, D.S., Sanghera, S., Pons, M. and Chapman, D. (1980) Biochim. Biophys. Acta 602, 57–69.
[9] Hayward, J.A., Johnston, D.S. and Chapman, D. (1985) Ann. NY Acad. Sci. 695, 158–175.
[10] Pons, M., Johnston, D.S. and Chapman, D. (1982) J. Polym. Sci. Polym. Lett. Ed. 20, 513–520.
[11] Pons, M., Villaverde, C. and Chapman, D. (1983) Biochim. Biophys. Acta 730, 306–312.
[12] Johnston, D.S., McLean, L.R., Whittam, M.A., Clark, A.D. and Chapman, D. (1983) Biochemistry 22, 3194–3202.
[13] Juliano, R.L., Hsu, M.J., Peterson, D., Regen, S.L. and Singh, A. (1983) Exp. Cell Res. 146, 422–427.
[14] Juliano, R.L., Hsu, M.J., Regen, S.L. and Singh, M. (1984) Biochim. Biophys. Acta 770, 109–114.
[15] Allen, D.W., Schroder, W.A. and Balog, J. (1958) J. Am. Chem. Soc. 80, 1628–1636.
[16] Pick, U. (1981) Arch. Biochem. Biophys. 212, 186.
[17] Hupfer, B., Ringsdorf, H. and Schupp, H. (1983) Chem. Phys. Lipids 33, 355–374.