THE INTERACTION OF PVP COMPLEXES OF GOSSYPOL AND ITS DERIVATIVES WITH AN ARTIFICIAL MEMBRANE LIPID MATRIX

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Abstract: In this paper, we present the results of a study on the membrane-active properties of gossypol, its derivatives and their polyvinylpyrrolidone complexes as assessed by differential scanning calorimetry and by the fluorescent probe method. The latter revealed the change in polarization of the incident radiation caused by the action of the polyphenol on the artificial membrane lipid matrix.

Key words: Gossypol and its derivatives, Lipid membranes, Differential scanning calorimetry, Membrane fluidity

INTRODUCTION

Gossypol is a binaphthyl dialdehyde polyphenol that is extracted from cotton seeds (Fig. 1A). It is widely known for its antifertility activity [1], but it has other properties, including antiviral, anti-inflammatory and antiphlogistic activities [2-5]. Chemical modifications of gossypol have been shown to produce various compounds with interesting and important pharmacological properties. Megosin,
batriden, gosolidon and other gossypol derivatives have been used in the treatment of various diseases [6-8].

Fig. 1. The molecular structures of gossypol in its aldehyde form (A), MGS-1 (B), megosin (C), and rometin, which is a complex of megosin with PVP (D).

Gossypol itself is toxic to humans and other animals, especially in high concentrations. It can cause diarrhoea, hypokalaemia, weakness, oedema, breathlessness, neuritis and paralysis [9]. Chemical modifications can decrease its toxicity. However, gossypol and its derivatives are water-insoluble, which inhibits their use in many medical applications. The biological availability of medical preparations depends to a great extent on their water solubility. One way of increasing the solubility of an insoluble or poorly soluble drug molecule is to associate it with a polymer that is cheap, water soluble, and chemically and biologically inert. Such polymers can transport the biologically active molecules in the body and deliver them to the cells, and can also control the durability of their action [10].

In our opinion, differential scanning calorimetry (DSC) is an effective method for distinguishing between these alternatives. As stated in the Materials and Methods section, one way in which the lipid multilamellar dispersions were prepared was by mixing the polyphenol or its PVP complex with lipids, then subjecting the dispersion to DSC. This enabled us to compare the effects of the investigated compounds on the lipid melting curves and to estimate the perturbation of the lipid bilayer structure.
In this study, we synthesized complexes between polyphenols and polyvinylpyrrolidone (PVP). PVP is chemically inert at physiological pH. Making complexes by enveloping gossypol or one of its derivatives in the PVP chain conferred water solubility on the polyphenols. PVP molecules have no intrinsic AOA, which enables us to propose the following alternative ways in which AOA may take effect in the preparations studied:

(a) the complex penetrates into the whole of the hydrophobic region of the lipid bilayer;

(b) the complex exerts AOA only on the surface of the lipid matrix without penetrating into the bilayer;

(c) the complex interacts with the polar part of the lipid matrix, then dissociates to release the hydrophobic polyphenol molecules, which penetrate into the hydrophobic region of the lipid, whereupon the AOA takes effect on lipid peroxidation.

The antioxidant properties of gossypol and its derivatives become prominent when they interact with either artificial lipid or biological membranes [11-13, 15]. Since they are water-insoluble, their antioxidant activity is presumably conferred onto the hydrophobic region of the membrane lipid matrix. However, it has been shown that water-soluble complexes of PVP with these same compounds also have antioxidant activity in membranes, which begs the questions: since they are parts of a water-soluble complex, how do these polyphenol molecules function as antioxidants in biological membranes, and at what depth in the lipid matrix do the antioxidant reactions take place?

MATERIALS AND METHODS

Chemicals

The lipids dimiristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) were from Sigma. Gossypol (2,2’-[1,1’,6,6’,7,7’-hexahydroxy-3,3’-dimethyl-5,5’-diazopropyl-8,8’-diformal]-dinaphthalene; Fig. 1A) and its derivatives megosin (bis-2,2’-[[(7,7’,8,8’-tetrahydro-1,1’,6,6’-tetrahydroxy-5,5’-diazopropyl-3,3’-dimethyl-7,7’-dioxo)-2,2’-binaphthyl]-8,8’-methyleniminoethane]-sodium salt; Fig. 1C) and MGS-1 (bisdimethylaminoethyliminogossypol; Fig. 1B), and their supramolecular complexes with N-polyvinylpyrrolidone (8000 MM), pogosin, rometin (Fig. 1D) and MGS-2, were synthesized in the Polyphenol Laboratory in the Institute of Bioorganic Chemistry of the Uzbekistan Academy of Sciences (Fig. 1). The complexes formed by incorporating gossypol and its derivatives into PVP polymers were stabilized by hydrogen bonds between the functional groups of the interacting molecules [27, 28]. The active polyphenol compounds constituted 9-10% of the complexes, i.e. the ratio of the number of PVP molecules to gossypol or derivative molecules in the complexes was approximately 10:1.
Preparation of multilamellar lipid dispersions
Multilamellar lipid dispersions were prepared from either DPPC or DMPC (300 μM) in 10 mM Tris-HCl buffer, pH 7.5, using the method of multilamellar dispersion preparation described in [14]. Samples for differential scanning calorimetry (DSC) were prepared in one of the following ways. In the first method, samples of the lipids in organic solvents were mixed with ethanolic solutions of gossypol, megosin or MGS1, or their PVP complexes. The solvent was removed by drying, then the buffer was added and the suspension was shaken for 5 min at 25-30°C (internal addition). In the second, a multilamellar lipid dispersion was prepared in buffer, then the water-soluble complex of the polyphenol preparation under investigation was added (external addition). Gossypol, its derivatives and their polyvinylpyrrolidone complexes were added in the same way.

Liposome preparation
Liposomes were prepared by sonication. PC:PE (98.5:1.5 by mass percent) was dissolved in 2:1 chloroform-methanol and rotary-evaporated under a vacuum in a round bottom flask. The dried lipid film was hydrated with 3 ml Tris HCl (10 mM, pH 7.4), then vortexed for 5 min. The sample was cooled on ice in a flask under an argon atmosphere, and then sonicated in a bath-type sonicator (Ultrasonic Disintegrator Type UD-11 Automatic “Techpan”, Poland) for 5x20 s with intervals of 30 s. After sonication, the liposome dispersion was centrifuged at 3000 g for 10 min. The lipid concentration in the initial suspension was 5 mM and the final concentration was 0.5 mM. The molar ratio of liposomes to fluorescent probe was 500:1.

Differential scanning calorimetry (DSC)
Thermodynamic phase transition curves of the multilamellar dispersions were obtained using a DASM-4 differential scanning microcalorimeter (Russia) at a recording speed of 1°C/min. The experimental curves were digitized and entered into a computer. The lipid melting enthalpies were determined from the areas under the peaks by integrating the experimental curves. Errors in the enthalpy calculations were less than 10%. Phase transition temperatures were defined by the corresponding peak maxima, and the precision of the measurements was 0.05°C.

Membrane fluidity measurements
The fluidity of the liposome bilayers was measured using a steady-state fluorescence polarization technique. The fluorescent probe used was 1,6-diphenyl-1,3,5-hexatriene (DPH), a non-polar molecule that inserts into the membrane with its long axis parallel to the phospholipid acyl chains. The measurements were made on a Perkin Elmer luminescence spectrofluorimeter (LS-50B) equipped for fluorescence polarization measurements. A 0.5 mM lipid suspension was stirred with 10 mM Tris HCl buffer containing 145 mM NaCl, pH 7.4. 1 μM of DPH solution (in tetrahydrofuran) was added to the cuvette and
the sample was stirred and incubated for 30 min in the dark at about 22°C. The temperature of the cuvette holder was controlled by a water thermostat (MLW-U). Polarization measurements were also taken at 22°C. The fluorescence anisotropy (r) of the probe was calculated by the fluorescence data manager program using the following equation:

\[ r = \frac{I_{VV} - G I_{VH}}{I_{VV} + 2 G I_{VH}}, \]

where \( I_{VV} \) and \( I_{VH} \) are respectively the vertical and horizontal fluorescence intensities with vertical polarization of the excitation light beam. The factor \( G = I_{HV}/I_{HH} \) (grating correction factor) corrected the polarizing effects of the monochromator. The excitation wavelength for DPH was 348 nm and the fluorescence emission was measured at 426 nm.

**RESULTS**

**DSC measurements**

The melting thermograms of the multilamellar DMPC dispersions show both a narrow peak with a maximum temperature, \( T_m \), the so-called main phase transition from the gel to the liquid-crystal state, of approximately 24.1°C, and a less strongly marked pre-transition peak at temperature \( T_{pt} \) (about 14°C; Fig. 2). The half-width of the main peak defines the cooperativity of the melting process, while the area under the peak measures the melting enthalpy, \( \Delta H_m \), the heat content of the melting process.

Tab. 1. The thermodynamic parameters of the polyphenols’ interactions with DMPC lipid bilayers.

|          | DMPC multilamellar dispersion (internal addition) | C_{pol}/C_{lip} | \( \Delta H_{tot}^a \) (J/mol) | \( \Delta H_{error}^a \) (J/mol) | \( \Delta H_{main}^b \) (J/mol) | \( \Delta H_{ht}^c \) (J/mol) | \( T_{main}^d \) (°C) | \( T_{ht}^e \) (°C) | \( W_{main}^f \) (°C) | \( W_{ht}^g \) (°C) |
|----------|--------------------------------------------------|----------------|-------------------------------|--------------------------------|-------------------------------|-------------------------------|------------------------|------------------------|------------------------|------------------------|
| Control  | 0                                                 |                | 26.51                         | 2.66                           |                                |                               |                        |                        |                        |                        |
| Gossypol |                                                  | 0.01           | 34.14                         | 0.95                           | 25.50                         | 7.72                         | 23.45                  | 26.80                  | 1.4                    | 1.5                    |
|          | Scan 4                                           | 0.02           | 31.31                         | 0.45                           | 17.11                         | 13.76                        | 23.55                  | 26.80                  | 1.7                    | 1.6                    |
|          | Scan 4                                           | 0.04           | 36.52                         | 0.449                          | 18.96                         | 17.113                       | 23.35                  | 26.65                  | 1.8                    | 1.7                    |
| MGS-1    | Scan 1                                           | 0.05           | 35.12                         | 1.96                           | 27.43                         | 4.72                         | 23.50                  | 26.20                  | 1.7                    | 3.0                    |
| Megosin  |                                                  | 0.02           | 28.74                         | 0.43                           | 18.60                         | 10.07                        | 24.20                  | 26.50                  | 0.7                    | 1.4                    |

Calorimetric parameters: \( ^a \Delta H_{tot} \), enthalpy of the total phase transition; \( ^b \Delta H_{main} \), enthalpy of the main peak phase transition; \( ^c \Delta H_{ht} \), enthalpy of the high temperature phase transition; \( ^d T_{main} \), phase transition temperature of the main peak; \( ^e T_{ht} \), phase transition temperature of the high temperature phase peak; \( ^f W_{main} \), half-width of the main transition peak; \( ^g W_{ht} \), half-width of the high temperature transition peak.
Fig. 2. The calorimetric curves for a DMPC multilamellar dispersion prepared in Tris-HCl (pH 7.5) in the absence (A, control) and in the presence of gossypol at a concentration ratio of gossypol to lipid of 0.01 (B), 0.02 (C), and 0.04 (D), and of megosin to lipid of -0.02 (F) and MGS-1 to lipid of -0.005 (G). The lipid concentration was 0.3 mM.

When gossypol was added to the multilamellar DMPC dispersion, the melting thermogram changed markedly. The character of the change depended on the gossypol-to-lipid concentration ratio ($C_{\text{pol}}/C_{\text{lip}}$). The results of several scans of such dispersions are shown in Fig. 2. First, the pre-transition peak disappears and a high-temperature shoulder appears on the melting curves. If the cooperativity of lipid melting obeys the Gauss law, the melting curve can be separated into a series of curves, the sum of which should produce the original. Fig. 2 shows such a decomposition, with the thin line corresponding to the
model curves and the bold line to the experimental data. Such decomposition of the initial thermogram into its components allows us to identify each lipid phase and estimate its individual thermogram parameters (the melting temperature, cooperativity and melting enthalpy). Repeat scans revealed a considerable increase in the areas under the high-temperature peaks, and there was further broadening and an additional shift of this peak to a higher temperature. The thermodynamic parameters for the melting of lipid samples containing pure polyphenols were obtained for each phase, as defined by the decomposition procedure (Tab. 1). The main phase transition peak shifted to a lower temperature (Tab. 1). These findings testify to a change caused by gossypol in the multilamellar lipid bilayers, in particular indicating that the structure became heterogeneous. Higher gossypol concentrations led to a more pronounced change in the thermogram (Fig. 2). Thus, the addition of gossypol makes the lipid bilayer heterogeneous, consisting of at least three types of lipid cluster. The same picture was observed for multilamellar DMPC dispersions when megosin and MGS-1 were added (Fig. 2). In the case of MGS-1, the extreme right of the high-temperature peak was less intense and pronounced than in the case of megosin.

As mentioned above, the main phase transition peak in the control sample is asymmetric, with a right high-temperature shoulder and a left low-temperature shoulder (Fig. 2). If the general experimental melting curve is decomposed using Gauss curves, two model lines are sufficient. The sum of the amplitudes of the right half of the first melting peak and the entire second peak yields the asymmetric high-temperature shoulder. Hence, for all other melting thermograms, the course of the main phase transition curve is considered to be defined by the sum of the first two Gauss peaks.

Gossypol is amphiphilic: its non-polar parts are naphthalene rings and isopropyl groups and its polar parts are carbonyl and hydroxyl groups [1]. The gossypol molecule can intercalate into the lipid matrix by its hydrophobic rings [20]. This is probably what we observed in the experiments we conducted.

These results show what type of change in the melting of the multilamellar dispersions can be caused by polyphenols interacting with the lipid bilayer. Thus, if water-soluble complexes can dissociate when added to the multilamellar phospholipid dispersions so that the polyphenol molecules can enter the bilayer, the results should be similar to those obtained with the pure polyphenols.

Our further investigations were devoted to studying the influence of water-soluble complexes of gossypol and its derivatives on the thermodynamic parameters of multilamellar phospholipid dispersions. We investigated the membrane-active properties of pogosin (the complex of gossypol with PVP), rometin (the complex of megosin with PVP) and MGS-2 (the complex of MGS-1 with PVP). We were interested in the mechanism of interaction of the water-soluble complexes with the lipid systems, and whether the complexes dissociate during this interaction or interact with the membrane without dissociating. Two ways of preparing the lipid dispersions were chosen. One involved a mixed
preparation of multilamellar dispersion containing polyphenol complexes with PVP (internal addition), and the other involved the addition of an aqueous PVP-polyphenol complex solution to a pure multilamellar lipid dispersion (external addition). Obviously, external addition should lead to a concentration of the membrane-active compounds on the outside bilayers of the lipid dispersions, whereas internal addition should lead to equal dispersion in all the bilayers. The concentration of PVP-polyphenol complexes was equimolar with that of the initial polyphenol concentration of the complex. Examples of the melting thermograms of multilamellar DMPC dispersions prepared by internal addition with pogosin or MGS-2 are shown in Figs 3 and 4. A comparison of the thermograms of samples comprising gossypol, megosin and MGS-1 with the water-soluble complexes reveals clear differences: pogosin and MGS-2 evoked less cooperativity in the lipid matrix melting process and increased the quantity of lipids in the high-temperature phases to a lesser extent than gossypol, megosin and MGS-1. However, repeat scanning of the same sample comprising either pogosin or MGS-2 showed further changes in the thermogram curves (Figs 3 and 4). Obviously, the systems studied are initially unstable, and some time is needed to reach equilibrium, which is observed after the fourth scan. This took approximately 24 h in our experiments. In general, one can see that thermodynamic parameters such as the half-widths and enthalpies of all the peaks change, as shown by decomposition of the experimental curve into different individual peaks.

Mainly, what has occurred is a change in the lipid from the low-temperature to the high-temperature phase intercalation of the polyphenol molecules into the lipid bilayer (Figs 3 and 4). Consequently, the ratios of areas under all the individual peaks are changed. All the parameters of each melting peak, such as the maximum melting temperature, enthalpy and half-width, are shown in Tab. 2.

Tab. 2. The thermodynamic parameters of the polyphenols’ interactions with DMPC lipid bilayers.

|          | DMPC multilamellar dispersion (internal addition) |          |          |          |          |          |          |          |          |          |          |          |
|----------|-------------------------------------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
|          | C_{pol}/C_{lip}                                  | ΔH_{tot} | ΔH_{tot} error | ΔH_{main} | ΔH_{ht} | T_{main} | T_{ht} | W_{main} | W_{ht} |          |          |          |          |
| Pogosin  | Scan 4                                          | 0.02     | 40.50    | 0.44     | 28.19    | 12.75    | 24.10    | 26.85    | 1.6      | 1.7      |          |          |          |
|          | Scan 4                                          | 0.04     | 28.47    | 1.80     | 16.11    | 11.24    | 23.65    | 26.90    | 2.6      | 1.4      |          |          |          |
| MGS-2    | Scan 4                                          | 0.008    | 30.40    | 0.37     | 21.14    | 8.89     | 24.25    | 26.95    | 1.0      | 1.4      |          |          |          |
|          | Scan 4                                          | 0.02     | 24.07    | 0.58     | 10.74    | 12.75    | 23.70    | 26.50    | 2.0      | 2.0      |          |          |          |

Calorimetric parameters: ^aΔH_{tot}, enthalpy of the total phase transition; ^bΔH_{main}, enthalpy of the main peak phase transition; ^cΔH_{ht}, enthalpy of the high temperature peak phase transition; ^dT_{main}, phase transition temperature of the main peak; ^eT_{ht}, phase transition temperature of the high temperature peak; ^fW_{main}, half-width of the main transition peak; ^gW_{ht}, half-width of the high temperature transition peak.
In Tab. 2, we give the values of the different thermodynamic parameters for the fourth scan, i.e. the point at when we considered that the membrane-active compounds had interacted fully into the lipid systems. It can be seen that when increasing amounts of the polyphenol complex were added to the multilamellar DMPC dispersion, the enthalpy rose in comparison with the control sample (pure lipid dispersion). The ratio of the major transition enthalpy $\Delta H_{\text{main}}$ (the sum under the first and second peaks) to the high-temperature phase transition enthalpy (the third peak) $\Delta H_{\text{ht}}$ is decreased, showing an increased proportion of the high-temperature phase in the general melting process.

From the data in Tab. 2, one can see that the temperature maximum of the major transition peak at lower concentration ratios of complexes to lipid is not much different from the typical control values. For the high-temperature phase, the melting temperature maximum lies in the range 26.5-26.85ºC, an interval of only 0.35ºC. The values of the half-widths of both the main and high-temperature phase transitions were much higher for lipid samples containing PVP-polyphenol complexes than for the control. This is evidence that the interaction of the complexes with the lipid system leads to a decrease in the cooperativity of the phase transition process that is typical of the pure lipid system.

Fig. 3. The calorimetric curves for a DMPC multilamellar dispersion in the presence of pogosin. The concentration ratio of basic polyphenol compound to lipid was 0.02 (A) or 0.04 (B).
As the polyphenol complexes produced changes in the multilamellar DMPC dispersions like those produced by the pure polyphenols, we must suppose that some of the PVP molecules surrounding a given polyphenol molecule must interact with the lipid bilayer, and that this is followed by dissociation of the complex. Any effect of pure PVP molecules on the thermodynamic properties of the lipid systems would be reflected in the form of the melting curve, and indeed, pure PVP molecules did influence the lipid bilayer (Fig. 5).

We considered that the second and third high-temperature peaks contributed to the changes in the melting curve, so these have to be subtracted from the general curve. The results of this subtraction for the fourth scans of pogosin and MGS-2 at different polyphenol:lipid concentration ratios are shown in Fig. 6. There is clear evidence of two phases with different thermodynamic parameters. The proportion of the high-temperature phase (the second phase) increased when more pogosin or MGS-2 was added to the lipid.
Fig. 5. The calorimetric curves for a DMPC multilamellar dispersion in the presence of PVP only. The concentration of PVP is the same as that used with the added polyphenol complexes.

Fig. 6. The calorimetric curves for a DMPC multilamellar dispersion after the subtraction of the PVP contribution from the curve for the general melting process in presence of pogosin at a concentration ratio of basic polyphenol compound to lipid of 0.008 (A) or 0.04 (B), or in the presence of MGS-2 at a ratio of -0.008 (C) or 0.02 (D).
Fig. 7. The calorimetric curves for a DMPC multilamellar dispersion in presence of polyphenol complexes at the given concentration ratios of basic polyphenol compound to lipid: A – pogosin at -0.02, B – MGS-2 at 0.02, and C – rometin at -0.02. The complexes were added externally.
Fig. 8. The calorimetric curves for a DPPC multilamellar dispersion prepared in Tris-HCl (pH 7.5) in the presence of gossypol or one of its derivatives at the given concentration ratios of basic polyphenol compound to lipid: A – gossypol at 0.008, B – pogosin at 0.01, and C – MGS-2 at 0.01. The complexes were added externally. The lipid concentration was 0.3 mM.
A high-temperature phase is also formed upon external addition of the polyphenol complexes (Fig. 7). The most complicated form of the melting curve was from the first scan when pogosin was added (C_{pol}/C_{lip} = 0.02). At that stage, not all of the membrane-active compound had interacted fully with the outer bilayer of the multilamellar dispersion. However, after several cycles of heating and cooling, the melting curves took the form shown in the upper part of Fig. 7. A similar picture was found when MGS-2 or rometin were added to the lipid samples. All the thermodynamic parameters for the fourth scans are given in Tab. 3.

Tab. 3. The thermodynamic parameters of the polyphenols’ interactions with DMPC lipid bilayers.

|          | DMPC multilamellar dispersion (external addition) |          |
|----------|--------------------------------------------------|----------|
|          | C_{pol}/C_{lip} | ΔH_{tot} | ΔH_{error} | ΔH_{main} | ΔH_{ht} | T_{main} | T_{ht} | W_{main} | W_{ht} |
| Pogosin  | 0.01              | 32.75    | 0.20       | 18.12     | 14.43   | 23.15    | 26.80  | 1.7      | 2.2    |
| MGS-2    | 0.01              | 20.75    | 0.39       | 11.58     | 9.56    | 23.55    | 25.80  | 1.7      | 2.0    |
|          | 0.02              | 25.61    | 1.28       | 10.40     | 13.92   | 23.55    | 25.50  | 1.5      | 2.6    |
| Rometin  | 0.02              | 37.59    | 0.64       | 28.94     | 9.30    | 24.40    | 26.60  | 1.3      | 1.8    |

Calorimetric parameters: *ΔH_{tot}, enthalpy of the total phase transition; \(^{2}\)ΔH_{main}, enthalpy of the main peak phase transition; \(^{3}\)ΔH_{ht}, enthalpy of the high temperature peak phase transition; \(^{4}\)T_{main}, phase transition temperature of the main peak; \(^{5}\)T_{ht}, phase transition temperature of the high temperature peak; \(^{6}\)W_{main}, half-width of the main transition peak; \(^{7}\)W_{ht}, half-width of the high temperature transition peak.

We also carried out experiments on multilamellar DPPC dispersions with external addition of pogosin and MGS-2 to examine the possibility that the lipid melting thermogram changed on repeat scans. As with the multilamellar DMPC samples, the melting thermograms showed the appearance of a high-temperature phase (Fig. 8), and the intensity of the high-temperature peak increased in subsequent scans. The result obtained with pure gossypol is shown for comparison. A high-temperature shoulder appeared, and its intensity increased after every scan. Thus, pogosin and MGS-2 caused changes in the form of the DPPC melting curve when they interacted with the lipid bilayer, and these changes were similar to those caused in the multilamellar DMPC dispersions.

**Fluorescence measurements**

The main purpose of the fluorescence measurements was to compare the actions of rometin and megosin on phospholipid liposomes consisting of phosphatidylcholine (PC, 98.5%) and phosphatidylethanolamine (PE, 1.5%). The results were of practical interest. Changes in the fluidity of the lipid bilayer upon the addition of the preparation were measured by the steady-state
fluorescence anisotropy of the probe DPH incorporated into the lipid matrix of the liposome. The concentration of rometin in the complex was equimolar with respect to that of megosin. When either megosin or rometin was added to the liposomes, there was an evident, dose-dependent increase in the anisotropy of DPH (Fig. 9). This indicates that both megosin and rometin decreased the fluidity of the liposome lipid matrix. This decrease was significantly pronounced at higher concentrations of the preparations studied. A comparison of the changes in anisotropy with increasing concentration revealed that the two complexes had similar effects. Pure PVP caused no change in the anisotropy (data not shown), so the changes in DPH anisotropy are attributable only to the interaction of megosin with the lipid molecules in the bilayer. It may be considered that the complex dissociates on interaction with the lipid bilayer, and then the megosin molecules interact directly with lipid. This result is highly consistent with the DSC data.

![Graph](image)

**DISCUSSION**

Our main interest was to study the interaction of PVP complexes of gossypol and its derivatives, pogosin, rometin and MGS-2, with multilamellar phospholipid dispersions, in order to elucidate the way in which these complexes exert membrane-active properties. On the basis of their biological effect [6, 23], it is reasonable to suppose that all these complexes dissociate into polyphenol and PVP molecules when they interact with the lipid. PVP is chemically inert in animals and has no biological activity [17]. To confirm this, we relied mainly on DSC. As lipid systems, multilamellar DMPC and DPPC dispersions were selected, and their thermodynamic properties were thoroughly studied [17, 18].
The most typical change in the form of the initial (control) thermogram when either a pure polyphenol or its corresponding PVP complex was added was the appearance of a high-temperature shoulder (Figs 3 and 4). For the DSC experiments, the polyphenol complex-lipid mixtures were prepared in two ways, termed internal and external addition. The external addition was required to show that the complexes dissociated only on direct interaction with the lipid bilayers. If that is so, then the values of the thermodynamic parameters and the forms of the thermogram of the melting-related changes differed only because different numbers of bilayers in the multilamellar system were available to the complexes.

The interaction of a pure polyphenol molecule with lipid bilayers and the dissociation of a polyphenol-PVP complex probably require a certain amount of time. We therefore supposed that during the first scan, the system comprising the DMPC lipid dispersion and either a pure polyphenol or a polyphenol PVP complex was not in thermodynamic equilibrium. Equilibrium was only reached after longer incubation. Therefore, we recorded the results of the fourth scan, which was done after eight or more hours, because of the repeated cooling and heating processes, after which the system can reasonably be supposed to have reached equilibrium. Measurements of the total enthalpy of melting after every scan revealed little change until the fourth scan (results not shown). We observed increasing amplitude of the high-temperature peak and a decrease in the base melting peak. This is clearly seen when the general experimental melting curve is decomposed into a number of Gauss curves (Figs 2, 3 and 4). If we consider that the sum of the two left peaks corresponds to the asymmetrical peak of the main phase transition, which is mainly particular to the pure multilamellar lipid system, the third high-temperature peak is probably the best indicator of interdigitation into the DMPC or DPPC bilayer [19, 20]. It is common knowledge that some molecules can be induced to interdigitate in a lipid bilayer phase [13]. The precise requirements for a relatively small molecule to interdigitate in this way are not known, but the following generalizations appear to be valid for many of the molecules tested. First, the guest molecule must displace water from a particular location in the interfacial region and reside at the membrane-water interface [20]. Second, its non-polar moiety must not extend too deeply into the acyl chain region [21]. It has been demonstrated that the ability to induce an interdigitated phase does not depend on the presence or absence of a charge on the molecule [22].

It is known that gossypol is amphiphilic, comprising non-polar naphthalene rings and isopropyl groups, and polar carbonyl and hydroxyl groups [1]. The gossypol molecule can partially intercalate into a lipid matrix via its hydrophobic rings [16]. This is probably what was observed in our DSC experiments. The details of the penetration of the gossypol molecule and its various derivatives into the hydrocarbon region of a lipid bilayer can be determined by interpreting ESR data [13, 24]. If gossypol penetrated entirely into the lipid hydrocarbon region, its complicated structure should lead to a marked perturbation of the packing of
the lipid molecules into the bilayer, excluding some of the lipid from cooperative melting. In our experiments, if only some of the gossypol molecules penetrated into the deep hydrophobic region at the first scan, the quantity penetrating should increase on subsequent scans, as seen in Figs 2, 3 and 4. The total enthalpy should then decrease and the main transition shift to either a lower or a higher temperature depending to a greater or lesser extent on the structure of the membrane-active molecule. In our case, the main transition temperature usually showed only a slight shift towards a lower temperature. Furthermore, the enthalpy values were more or less unchanged (within the 10% error) for all of the scans. Another confirmation that gossypol did not penetrate entirely into the hydrophobic region of the bilayer is that the gossypol molecule contains eight hydroxyl groups and is symmetrical, with a linear dimension of approximately 25Å [23], so it theoretically should be able to transport protons across the bilayer if the H⁺ concentration differs on the two sides of the membrane. Measuring the BLM using the voltage-clamp method showed that gossypol does not have such properties (unpublished data). Furthermore, we examined the influence of gossypol on the swelling of rat thymocytes and found that gossypol and some of its derivatives barely changed the thymocyte volume, so the membrane permeability to different ions was unchanged (unpublished data). Therefore, we have every reason to believe that gossypol fails to satisfy the conditions for molecules that penetrate completely into the lipid phase. The incorporation of gossypol and its derivatives can be facilitated by van der Waals interactions in both DMPC and DPPC lipid bilayers so that the gel state lipid is not destabilized to such an extent that an interdigitated phase is formed (Figs 2, 3, 4 and 8). We may suppose that when polyphenol molecules are located at the interfacial region of either DMPC or DPPC dispersions in the gel state, they anchor to the interface by virtue of their polar groups, with the non-polar parts of the molecules intercalating between the rigid acyl chains. Intercalation between hydrocarbon chains can induce the formation of voids between the chains in the bilayer interior. Since the energy needed to form holes in the hydrophobic region is extremely large, the chains must eliminate the voids [25, 26] either by bending cooperatively or by interdigitating [20]. Interdigitation is the more favourable event, demanding less energy than the cooperative tilt change.

Consequently, based on our results, the molecules of gossypol and its derivatives probably formed an interdigitated phase in the DMPC and DPPC lipid bilayers. The PVP molecules also changed the course of the thermogram and, correspondingly, some of the thermodynamic parameters of the melting process (Fig. 5). Pure PVP itself induces slight changes in the thermogram of melting of a DMPC dispersion (Fig. 5).

As mentioned above, in order for the PVP-polyphenol complex to dissociate, it must bond (for example via van der Waals interactions) to the polar part of the lipid bilayer, whereupon the dissociation process is initiated and continues for several hours. This is particularly evident in the DSC samples treated by external addition, where the result is presumably free of artefacts. Fig. 7 shows that the
complex indeed dissociates and gossypol molecules enter the lipid bilayer and interact to produce the appearance of an interdigitated phase. After the contribution of PVP is subtracted, the interdigitated phase is observed quite distinctly. The presence of either a multilamellar lipid dispersion or a liposome is the determining factor in the dissociation of the PVP-polyphenol complex, as confirmed by the fluorescence data (Fig. 9). The closely similar dose-response relationships for pure megosine and its PVP complex constitute evidence of the dissociation and direct interaction of megosin with the lipid bilayer. It is possible that this location of the molecules of gossypol and its derivatives enables the free radicals participating in lipid peroxidation to be trapped.

Gossypol, and especially its derivatives, are efficient medical preparations but have not found widespread use because they are water-insoluble; they have been deployed only in ointment or gel form. For other applications, it is very important that they be delivered to cell targets. For such purposes, complexes of the polyphenols with PVP or other chemically inert molecules (or molecules with biologically useful effects) that are also water soluble should be very efficient, provided the complexes dissociate during their interactions with biological membranes. The complex will then reach its proper target and the matrix, which served as the carrier, will either be removed from the organism or, if it has a useful biological effect, perform its own other function. It is thus crucial to study the molecular mechanisms by which different water-soluble complexes act on biological membranes using physical and chemical methods.

REFERENCES

1. Lui, G., Lyle, K.C. and Cao, J. Trial of gossypol as a male contraceptive. in: S.J. Segal, Editor. Gossypol: a potential contraceptive for men, Plenum Press, New York (1985) 9-16.
2. Montamat, E.E., Burgos, C., Gerez de Burgos, N.M., Roail, L.E. and Blanco, A. Inhibitory action of gossypol on enzymes and growth of Trypanosoma cruzi. Science 218 (1982) 288-289.
3. Ral dof, R.J., Deck, L.M, Royer, R.E., and Vander Jagt, D.L. Antiviral activities of gossypol and its derivatives against herpes simplex virus type II. Pharmacol. Res. Commun. 18 (1986) 1063-1073.
4. Benhaim, P., Mathes, S.J., Hunt, T.K., Scheuenstuhl, H. and Benz, C.C. Induction of neutrophil Mac-I integrin expression and superoxide production by the medicinal plant extract gossypol. Inflammation 18 (1994) 443-458.
5. Hou, D.X., Uto, T., Tong, X., Takeshita, T., Tanigawa, S., Imamura, I., Ose, T. and Furii, M. Involvement of reactive oxygen species-independent mitochondrial pathway in gossypol-induced apoptosis. Arch. Biochem. Biophys. 428 (2004) 179-187.
6. Baram, N.I., Ismailov, A.I., Ziyaev, H.Z., and Redzepov, K.Dz. Biologic activity of gossypol and its derivatives. Chem. Nat. Comp. 40 (2004) 199-205.
7. Royer, R.E., Mills, R.G., Deck, L.M., Mertz, G.J. and Jagt, D.L.V. Inhibition of human immunodeficiency virus type I replication by derivatives of gossypol. Pharmacol. Res. 24 (1991) 407-412.
8. Lin, T.S., Schinazi, R.F., Zhu, J., Birks, E., Carbone, R., Si, Y., Wu, K., Huang, L. and Prusoff, W.H. Anti-HIV-1 activity and cellular pharmacology of various analogs of gossypol. Biochem. Pharmacol. 46 (1993) 251-255.
9. Waites, G.M., Wang, C. and Griffin, P.D. Gossypol: reasons for its failure to be accepted as a safe, reversible male antifertility drug. Int. J. Androl. 21 (1998) 8-12.
10. Castelli, F., Pitarresi, G. and Giammona, K. Influence of different parameters on drug release from hydrogel systems to a biomembrane model. Evaluation by differential scanning calorimetry technique. Biomaterials 8 (2000) 821-833.
11. Dodou, K. Investigation on gossypol: Past and present development. Expert Opin. Investig. Drugs 14/11 (2005) 1419-1495.
12. Gordienko, N.V., Zamarayaeva, M.V., Gagelgans, A.I., Baram, N.I. and Ismailov, A.I. Membranotropic action of gossypol and its derivatives 1. Influence on functional characteristics of membranes of sarcoplasmic reticulum. Biol. Membranes 10/2 (1993) 462-469.
13. Gordienko, N.V., Zamarayaeva, M.V., Gagelgans, A.I., Salakhutdinov, B.A., Aripov, T.F. and Ismailov, A.I. Membranotropic action of gossypol and its derivatives 2. Study of structural reconstruction of membranes induced by gossypol and its derivatives. Biol. Membranes 10/2 (1993) 470-477.
14. Aripov, T.F., Rozenshtein, I.A., Salakhutdinov, B.A., Lev, A.A. and Gotlib, V.A. The influence of cytotoxins from Central Asian cobra venom and melittin from bee venom on thermodynamic properties of phospholipids bilayer. Gen. Physiol. Biophys. 6 (1987) 343-357.
15. Kovacic, P. Mechanism of drug and toxic actions of gossypol: Focus on reactive oxygen species and electron transfer. Curr. Med. Chem. 10 (2003) 2711-2718.
16. Tanphaichitr, N., Namking, M., Tupper, S., Hansen, C. and Wong, P. Gossypol effects on the structure and dynamics of phospholipid bilayers: A FT-IR study. Chem. Phys. Lipids 75 (1995) 119-125.
17. Ivkov, V.G. and Berestovsky, G.N. Dynamic structure of lipid bilayer, in: Ivkov, V.G. and Berestovsky, G.N. Publishing House Nauka, Moscow (1981) 293.
18. Wesołowska, O., Hendrich, A.B., Lania-Pietrzak, B., Wiśniewski, J., Molnar, J., Ocsovszki, I. and Michalak, K. Perturbation of the lipid phase of a membrane is not involved in the modulation of MRP1 transport activity by flavonoids. Cell. Mol. Biol. Lett. 14 (2009) 199-221.
19. McDaniel, R., McIntosh, T. and Simon, S.A. Nonelectrolyte substitution for water in phosphatidylcholine bilayers. Biochim. Biophys. Acta 731 (1983) 97-108.
20. McIntosh, T., Mc Daniel, R. and Simon, S.A. Induction of an interdigitated gel phase in fully hydrated phosphatidylcholine bilayers. *Biochim. Biophys. Acta* **731** (1983) 109-114.

21. Auger, M., Jarrell, H.C., Smith, I.C.P, Siminovitch, D.J., Mantsch, H.H.. and Wong, P.T.T. Effects of the local anesthetic tetracaine on the structural and dynamic properties of lipids in model membranes: a high-pressure Fourier transform infrared study. *Biochemistry* **27** (1988) 6086-6093.

22. Simon, S.A. and McIntosh, T.J. Interdigitated hydrocarbon chain packing causes the biphasic transition behavior in lipid/alcohol suspensions. *Biochim. Biophys. Acta Biomembr.* **773** (1984) 169-172.

23. Gdaniec, M. and Ibragimov, B. Gossypol, in: A. Mickiewicz, M. Nicol, D. David, T. Fumio, and R. Bishop (Eds) *Compr. Supramol. Chem.* **6** (1996) 117-145.

24. Ionov M., Gordiyenko N., Olchowik E., Baram N., Zijaev K., Salakhutdinov B., Bryszewska M. and Zamarava M. The immobilization of gossypol derivative on N-polyvinylpyrrolidone increases its water solubility and modifies membrane-active properties. *J. Med. Chem.* **52** (2009) 4119-4125

25. Israelachvili, I., Marcelja, S. and Horn, R.C. Physical principles of membrane Organization. *Quant. Rev. Biophys.* **13** (1980) 121-200.

26. Gutowicz, J. and Terlecki, G. The association of glycolytic enzymes with cellular and model membranes. *Cell. Mol. Biol. Lett.* **8** (2003) 667-680.

27. Ismailov, A.I., Baram, N.I., Biktimirov, L., Ziyaev, H.L, Zamarava, M.V., Gagelgans, A.I., Gordienko, N.V. and Uysupova, S.M. Patent 2123. *Rasmii Akhborotnoma* **4** (2002) 17-24.

27. Biktimirov, L., Ziyaev, Kh., Khodzhaniyazov, B., Ziyamov, D., Baram, N. and Ismailov, A. Complex derivatives of gossypol with N-polyvinylpyrrolidone. *Chem. Nat. Comp.* **32** (1996) 177-179.