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# SIMULATION OF GLYCYRRHIZIC ACID ASSOCIATES WITH CHOLESTEROL IN METHANOL

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There are experimental evidences that in the methanol solution of glycyrrhizic acid (GA) and cholesterol, the cholesterol molecules have two different types of the environment. One corresponds to free molecules and another corresponds to the molecules associated with GA. However, the nature of these associates remains unclear. The all-atom molecular dynamics simulation of GA solutions in methanol is performed. It is shown that, contrary to aqueous solutions, GA in methanol does not form small stable clusters, even in the presence of cholesterol. The arising associates do not have distinct structures and exist for no longer than dozens of nanoseconds. The concentrations of these clusters and their stability constants are estimated. It is necessary to assume the existence of larger-scale associates to explain the experimental data.

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**Keywords:** molecular dynamics simulation, aqueous solutions, glycyrrhizic acid, cholesterol, methanol, structure of associates.

## INTRODUCTION

The extract of licorice root, which contains glycyrrhizic acid (GA), has been well known in folk medicine and is used to treat and prevent different diseases [1, 2]. It was noted [3-10] that GA improved the solubility of some poorly soluble drug molecules, although the detailed mechanism has not been fully understood. A recent molecular dynamics simulation of GA dimers in water in the presence of a cholesterol molecule shows that, at low concentrations, GA molecules form stable dimers and in the presence of a hydrophobic molecule (cholesterol) stable associates arise, in which a cholesterol molecule is located near a dimer [11]. For methanol solutions, such studies have not been performed. However, the association of GA and cholesterol molecules in methanol was studied by NMR in [12] where it was found that there were two types of the local environment of cholesterol, which can be interpreted as free and GA-associated cholesterol molecules. The estimations showed that the stoichiometry of these GA/cholesterol associates was 2:1. However, the nature of these complexes remains unclear, and neither their size nor lifetime are known.

In this work, we examine the association of GA molecules with cholesterol in methanol using the all-atom molecular dynamics simulation, which allows us to obtain the data on the structure and properties of small associates of these molecules.

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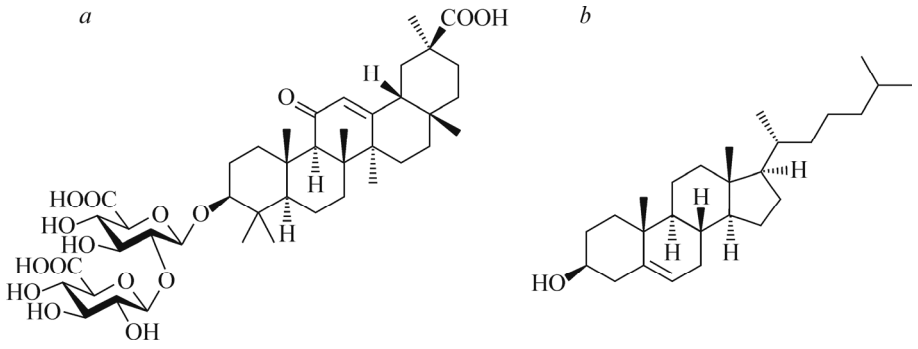


**Fig. 1.** Glycyrrhizic acid (*a*) and cholesterol (*b*) molecules.

## SIMULATION

Two GA isomers with different *trans*/*cis*-conformations in triterpene aglycone have been known [13-15]. These GA forms have somewhat different physical properties, in particular, the gel formation ability [15, 16]. In the literature, they are denoted as α- and β-forms, with mainly β-form occurring in nature. In addition, there are various conformations due to the type of glycosidic bonds, one of which links together the sugar ends and the other attaches them to the terpene skeleton (Fig. 1*a*). There can be two different configurations of these bonds, which are also called α- and β-bonds. In natural GA, both glycosidic bonds have the β-configuration [14, 15]. Sometimes, the researchers neglect this structural feature of GA in the simulation and deal with α-bonds, although only β-bonds occur in nature. In this study, we used both α- and β-glycosidic bonds. In both instances, the terpene skeleton was in the natural β-form.

In the case of α-bonds, we used the GA parametrization from our previous work [11]. We failed to find the β-bond parametrization in the literature and hence carried out our own using GAFF (for aglycone) [17] and GLYCAM (for glucuronic acids in the disaccharide moiety) force fields [18]. We took the parametrization from the Amber field for cholesterol [19] and that from the TraPPE field for methanol [20].

The molecular dynamics simulation was performed using the Gromacs software [21]. The simulation step was 2 fs; the interaction cutoff radius was 1 nm; the electrostatic interaction was calculated using the Particle Mesh Ewald method; the stretching vibrations of the bonds involving hydrogen atoms were constrained by the LINCS algorithm. The temperature in the system was maintained with two separate v-rescale thermostats: one for the dissolved molecules and another for the solvent. The pressure in the model box with periodic boundary conditions was maintained with a Parrinello–Rahman barostat; it was 1 bar for all models. The model configurations were visualized with the VMD software [22]. The molecules used are shown in Fig. 1.

## GA DIMERS IN METHANOL

In [11], to determine the association of GA molecules in the molecular dynamics simulation it was proposed to calculate the Lennard–Jones interaction energy $E_{LJ}$ between the atoms of the molecules not linked by covalent bonds. The Gromacs software provides the calculation of the interaction energy between a given group of atoms. In this case, these are the atoms of GA molecules beyond 1-4 interactions [23]. This energy does not have a special physical meaning, and we use it solely as a suitable measure to establish the approach of the molecules. (Note that $E_{LJ}$ slightly differs from the real Lennard–Jones interaction energy between different GA molecules, since in our case it also takes into account the Lennard–Jones interaction between the remote (in terms of bonds) atoms of the same molecule, which can approach each other when a molecule bends. However, it does not affect the identification of an associate).

When the molecules approach each other, the $E_{LJ}$ value experiences an abrupt jump. Fig. 2 shows the behavior of the $E_{LJ}$ energy for a model where two GA molecules are dissolved in a model cell with methanol. It is evident that the

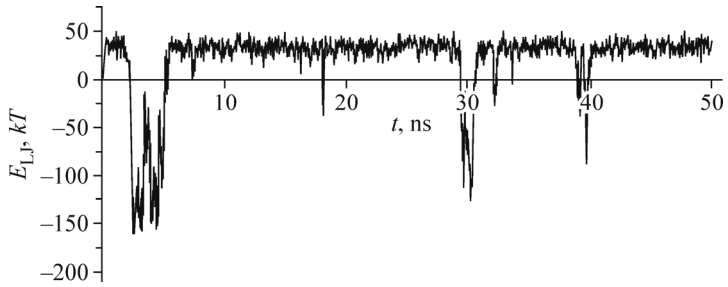


**Fig. 2.** Time behavior of the Lennard–Jones interaction energy $E_{LJ}$ between two GA molecules in the methanol solution in the molecular dynamics simulation. The outliers in the curve indicate that at a given point of time the molecules are in contact.

encountered GA molecules move apart soon after that. Note that different behavior is observed in water: the encountered GA molecules do not move apart until the end of the simulation (see [11] and Fig. 2 therein). Moreover, in methanol, the $E_{LJ}$ interaction for a pair of GA molecules does not exceed 160 $kT$, while in water it was about 250 $kT$. Thus, the simulation shows that GA molecules do not form stable pairs in methanol as they do in water. It should be emphasized that in these calculations we used GA with both α- and β-glycosidic bonds, and in both cases, the GA molecules exhibited no tendency to association.

## ANALYSIS OF SMALL ASSOCIATES IN SOLUTION

For a more detailed study of small associates of GA and cholesterol in methanol we carried out the molecular dynamics simulation of the solution containing eight GA molecules and two cholesterol molecules in methanol at 300 K and 285 K. The box size (8.7 nm) and the number of methanol molecules (9637) were chosen so that the GA concentration was 20.4 mmol/l, which is nearly two times greater than that in the experimental study [12]. For the specified temperatures, we obtained and examined the equilibrium molecular dynamics trajectories with lengths of 200 ns and 500 ns respectively. A typical configuration of these models is shown in Fig. 3.

To study the associates arising between GA and cholesterol molecules we built the *chronograms* of contacts once proposed by G. G. Malenkov [24]. Fig. 4 shows the chronogram for a temperature of 285 K. Solid lines (intervals with

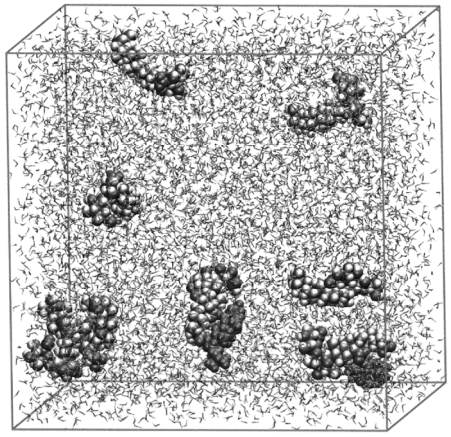

**Fig. 3.** Configuration of the molecular dynamics model of the solution containing eight GA molecules and two cholesterol molecules in methanol. The associates of two and three solute molecules are seen. The GA and cholesterol molecules are shown by their

van der Waals spheres; methanol molecules are shown by their skeletons.

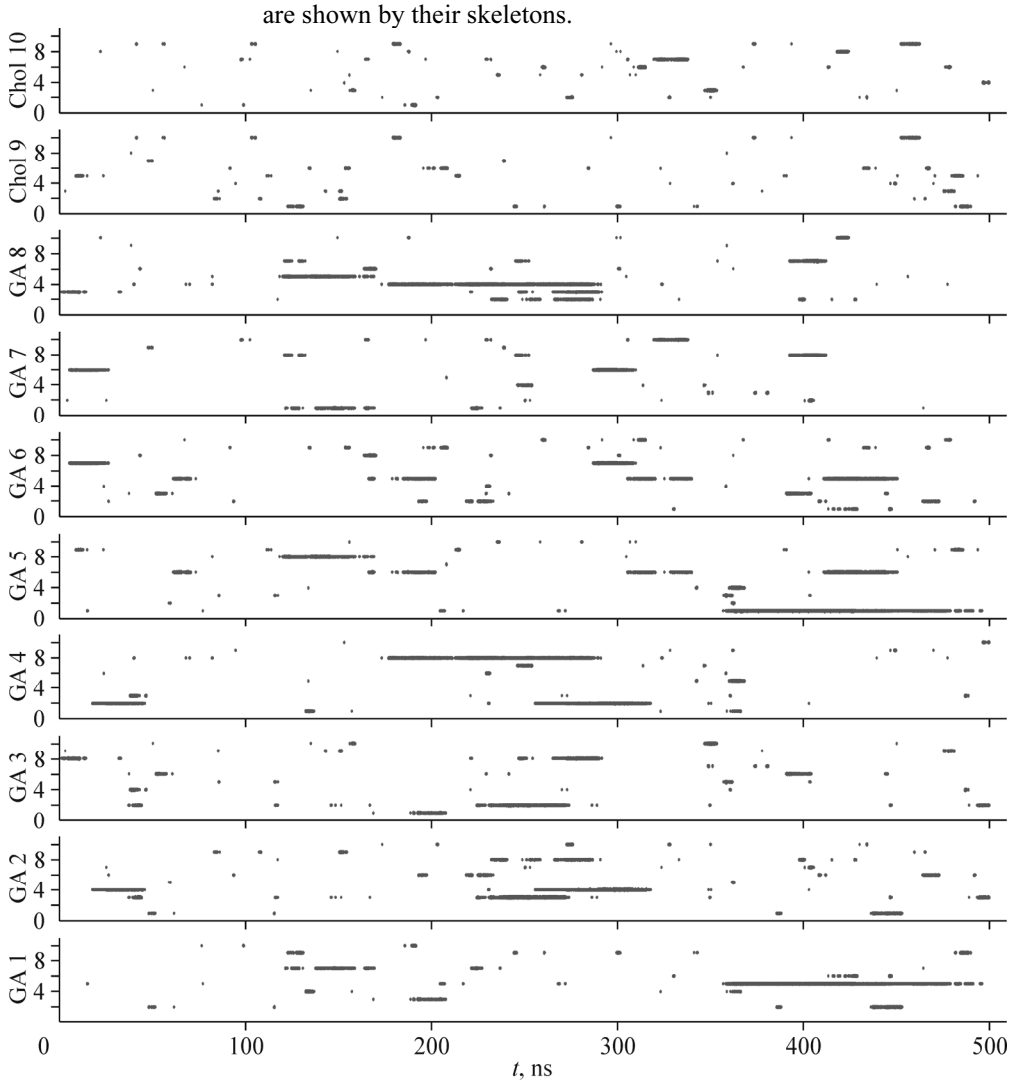


**Fig. 4.** Chronogram of contacts between the solute molecules of the methanol solution of GA and cholesterol at 285 K. Two upper bands refer to cholesterol molecules, the other eight refer to GA. The horizontal line segments show the contact time between the respective pair of molecules (see the text).

different length) denote the time periods during which a given pair of molecules was in contact. We regard as a contact a situation when the distance between any atoms of a pair of molecules was less than a specified value (in this case, we used a value of 0.35 nm corresponding to a distance for the hydrogen bond detection). The chronogram consists of ten bands (according to the number of molecules used, which were numbered from 1 to 10). Two upper (10 and 9) bands refer to cholesterol molecules and the others refer to GA. Each band contains ten tracks also numbered from 1 to 10. Thus, if at a certain time interval of our molecular dynamics trajectory an $i$ molecule contacts with a $j$ molecule, then a line corresponding to the given time period is depicted on the $j$-th track of the $i$-th band. Hence, the chronogram depicts the whole history of contacts between the molecules. (Note that the chronogram is symmetric to the replacement of $i$ by $j$.)

It is well seen from Fig. 4 that there are no stable associates of our molecules in this system. However, we can find the pairs living up to 100 ns, for example, the GA molecules with numbers 8 and 4, and also 1 and 5. Interestingly, cholesterol molecules have only short contacts both with each other and GA. The chronograms for other temperatures demonstrate a similar picture. For 300 K, the contact duration slightly decreased (the most long-lived pair existed for nearly 60 ns).

The information about the contacts between the molecules determines the connectivity graph, from which, using the standard algorithms for determining the connected components on the graphs [25], it is easy to find all clusters of our

**TABLE 1.** Concentrations of Associates with Different Compositions (mmol/l) in the Solution of Eight GA Molecules and Two Cholesterol Molecules in Methanol at 300 K (upper values) and 285 K (bottom values)

| Associate* | Cholesterol | | | Associate* | Cholesterol | | |
|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | | 0 | 1 | 2 |
| 0 GA | – | – | 0.05±0.02 | 3 GA | 0.53±0,20 | 0.09±0.06 | 0 |
| | – | – | 0.11±0.04 | | 0.41±0.08 | 0.05±0.03 | 0 |
| 1 GA | – | 0.57±0.14 | 0.01±0.003 | 4 GA | 0.03±0.02 | 0.01±0.004 | 0 |
| | – | 0.56±0.06 | 0.00±0.002 | | 0.29±0.19 | 0.04±0.02 | 0.00±0.001 |
| 2 GA | 1.99±0.23 | 0.16±0.08 | 0.01±0.008 | 5 GA | 0 | 0 | 0 |
| | 2.21±0.18 | 0.19±0.06 | 0 | | 0.06±0.05 | 0.00±0.003 | 0 |

* The monomer concentration was: $[GA_1] = 13.11$ mM and 12.04 mM for 300 K and 285 K, cholesterol $[Ch_1] = 4.06$ mM and 4.04 mM respectively. The total concentration of acid [GA] = 20.4 mM, cholesterol [Ch] = 5.1 mM. The errors were estimated from the spread in values when the whole MD trajectory was divided into five equal-time intervals.

molecules. By calculating the clusters for different time of the molecular dynamics trajectory we can estimate the average number of given-size associates in our model, and hence, calculate their concentration. Table 1 gives the concentrations of the associates for two different temperatures. In both cases, only small associates are observed. No greater than tetramers, which can contain cholesterol, are statistically distinguishable (see the shaded cells).

It can be seen that associates of two and three GA molecules without cholesterol are most probable. Therewith, cholesterol prefers to contact with one GA molecule and, much less, with two or three of them.

Knowing the concentrations of different associates, we can write down their stability constants. Since in our simulation we use a limited number of molecules of the dissolved components, the obtained associates cannot correspond to a real solution with the same concentration where the number of dissolved molecules is assumed to be unlimited. Even so, we estimated the stability constants for the simplest associates using complexes of two and three molecules

$$\text{(I) } GA + GA \leftrightarrow GA_2, \quad \text{(II) } GA + Ch \leftrightarrow GA\ Ch,$$

$$\text{(III) } GA_2 + GA \leftrightarrow GA_3, \quad \text{(IV) } GA\ Ch + GA \leftrightarrow GA_2\ Ch, \quad \text{(V) } GA_2 + Ch \leftrightarrow GA_2\ Ch.$$

Recall that the reaction constant $a + b \leftrightarrow ab$ is calculated as $k = [ab] / ([a]\,[b])$. Using this definition and Table 1 data, we obtain the following values of the stability constants for the associates: (I) 11.6, (II) 10.7, (III) 20.3, (IV) 21.4, (V) 19.8 at 300 K and (I) 15.2, (II) 11.5, (III) 15.4, (IV) 28.2, (V) 21.3 for 285 K. The constants are written in standard units (mol/l). Although the found constants can indicate only the order of magnitude, we can safely say that the present associates appear as a result of the dynamic equilibrium in the solution and cannot explain the existence of two different types of the environment of a cholesterol molecule observed in the NMR experiment.

## DISSOLUTION OF A GA CRYSTAL FRAGMENT

The experimentally observed existence of GA associates with cholesterol in methanol can be related with the presence of larger associates in a real solution. However, these are not macroscopic micelles, because, as the experimenters note, the solution remains transparent. The fact that in water GA molecules can form dimers with a specific structure (as shown in [11]) gives grounds to expect that in methanol there can also be stable associates with a specific structure, but larger than dimers. As such an aggregate we considered a fragment of the crystal structure of GA [14]. The GA crystal is loose and can be obtained only when the stabilizing additives (propionic acid) are introduced, because it contains wide channels available for guest molecules.

We built a crystal fragment from eight GA molecules stabilized by four cholesterol molecules, which were partially located in the channels between GA (Fig. 5*a*). This aggregate was placed in methanol at a temperature of 300 K. At first, we

performed a preliminarily relaxation of the environment for 100 ps. Then, we ran the standard simulation. Approximately in 10 ns, the cluster began to break down rapidly and in 40 ns there was no trace of it (Fig. 5*b*). Thus, the chosen aggregate

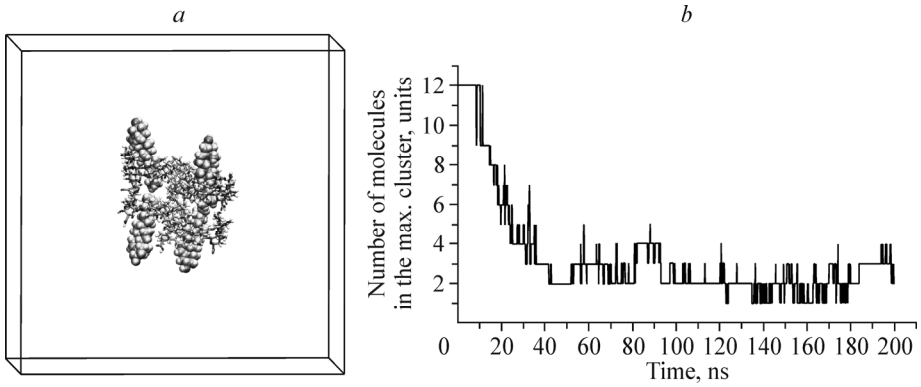


**Fig. 5.** Model box with the aggregate of eight GA molecules and four cholesterol molecules built based on the crystal structure of GA. The surrounding 9649 methanol molecules are not shown (*a*). The decay kinetics of this aggregate in methanol: the time dependence of the number of molecules in the largest cluster (*b*).

turned out to be a bad candidate for the role of stable nanoassociate of GA and cholesterol. However, the calculation does not close the question of the possible existence of a stable aggregate with a specific structure in methanol. It would be interesting to try other techniques for obtaining these clusters. For example, dealing with a solution with high GA and cholesterol concentrations it is possible to get a quite dense "self-organizing" aggregate, which can also be long-lived at low concentrations. However, this is the subject of a separate study.

## ASSOCIATION IN A WATER–METHANOL MIXTURE

We also examined the association of our molecules in a mixed methanol–water solution with 0.7/0.3, 0.5/0.5, and 0.3/0.7 molar fractions. For water, the TIP4P/2005 model was used. The concentration and the number of GA and cholesterol molecules were maintained the same as with pure methanol. For the model containing the greatest amount of water we used 11557 water molecules and 4800 methanol molecules; the box size was ~8.7 nm. The temperature of the solutions was 300 K; the trajectory length was 200 ns. We aimed to find out at what amounts of water a stable associate of GA and cholesterol molecules begins to form.

It appeared that as long as the amount of water was less than that of methanol, the molecules did not tend to associate. Qualitatively, they behave as in pure methanol: small associates arise, which exist for some time, then diverge, and the new ones form. However, when the amount of water increases, the situation changes fundamentally.

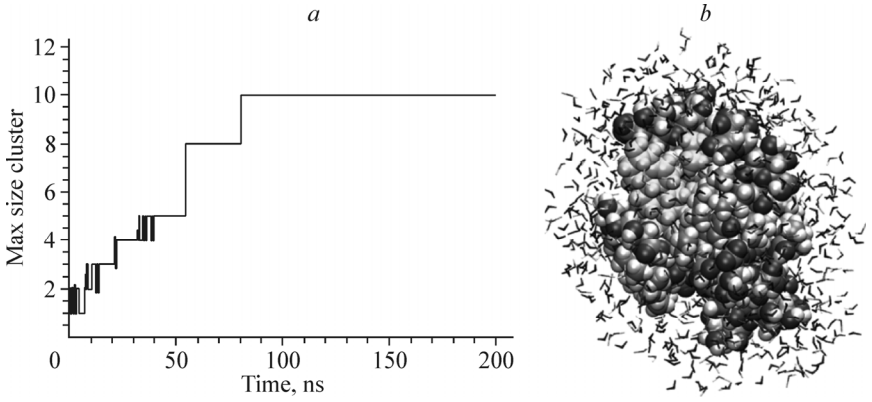


**Fig. 6.** Time change in the number of GA and cholesterol molecules in the maximum size associate in the 0.7/0.3 water–methanol solution (*a*). The

associate formed in 100 ns. The surrounding solvent molecules (water and methanol) are shown by their skeletons (*b*).

From Fig. 6*a* it can be seen that the molecules gradually stick together and already after 90 ns they all gather in a single associate, which is retained until the end of the simulation. The molecules swing and can even change their positions in the associate. The cholesterol molecules are partially located in the associate, however, sometimes they come out to its surface.

It can be assumed that a large cluster can also form at a smaller water fraction, however, a longer simulation can be needed to verify it. On the other hand, we think it is impossible to obtain stable nanoassociates in pure methanol using the classical simulation. This would require "macroscopic" simulation time.

## CONCLUSIONS

We applied the all-atom molecular dynamics simulation to show that, contrary to aqueous solutions [11, 26], small associates of both GA and GA with cholesterol are unstable in methanol. Using a chronogram of molecular contacts, the dynamics of the formation and dissociation of the associates is studied. The average concentration of different associates (dimers, trimers, and tetramers) is calculated and their stability constants are estimated.

Thus, the experimental evidence [12] for the existence of two states of cholesterol in the solution with GA in methanol (free and in an associate) can only be explained by the presence of larger formations of GA molecules containing cholesterol. However, the simulation of these associates requires special approaches and computational efforts.

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