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Disruption of giant unilamellar vesicles mimicking cell membranes induced by the pesticides glyphosate and picloram

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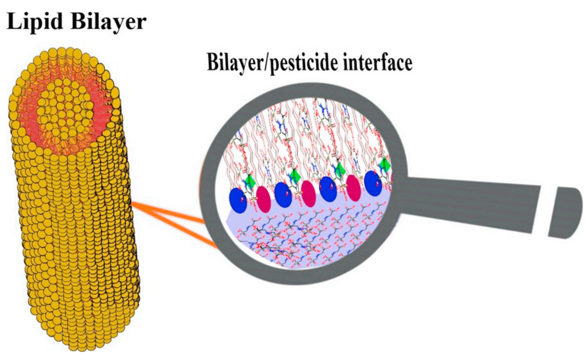
HIGHLIGHTS

- The disruptive effect of the two pesticides (glyphosate and picloram) on GUVs which is composed of the binary (DOPC/SM) and ternary system (DOPC/SM/Cholesterol) systems investigated with three experimental techniques.
- Stability test of GUVs (DOPC, DOPC/SM and DOPC/SM/Cholesterol) was performed in three aqueous medias (water, glucose and buffer).
- The important role of cholesterol on the overall phospholipid bilayers stability and permeability was investigated.
- The issue of the possible side interactions between the probes and the pesticides was addressed.
- Photobleaching of the laser power on the fluorescence probes was investigated, especially for higher incubation period.

GRAPHICAL ABSTRACT



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ABSTRACT

Giant unilamellar vesicles (GUVs) have been one of the most extensively investigated membrane model to study cell membrane-ligand interactions. In this study, we investigated the interaction between glyphosate and picloram with GUVs made with sphingomyelin (SM), cholesterol (CHOL), and dioleoyl-sn-glycerol-3-phosphocholine (DOPC) (DOPC/SM (1:1), DOPC/CHOL/SM (1:1:1)) in a physiological environment using confocal and phase contrast microscopy. At high pesticide concentrations (70 to 90 µM), we generally found the GUVs undergoing a physical such as contouring, elongation, and eventually lose their characteristic spherical shape. In addition, to determine the comparative effect of the pesticides, control experiments were performed using GUVs made with only DOPC and DOPC/SM 1:1. The results show that, at low concentration (0.5 µM), a significant effect was observed during a 30 min incubation time. These findings also suggest that cholesterol may play a

Abbreviation: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phophocholine; SM, egg sphingomyelin; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; Chol, cholesterol; l_o, liquid ordered; l_d, liquid disordered; T_m, phase transition temperature; $L\alpha$, liquid crystalline; Glyphosate, *N* - (phosphonomethyl) glycine; Rhodamine-PE, L-α-Phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl); NBD-DPPE, 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl).

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significant role in the permeability of the vesicle against the action of the pesticides, which have important biological implications on the lipid composition of the membrane.

1. Introduction

The term pesticide is used for a variety of chemical and biological products, including herbicides, insecticides, fungicides, nematicides and molluscicides. Pesticides are an integral part of agriculture throughout the world to control insect-borne diseases, preventing food loss and securing global food production. They have been used in small and large-scale agricultural farming to kill or inactivate living organisms such as rodents, insects, fungi and other so-called unwanted or undesirable plants. While most pesticides are designed to target a specific pest or pest group, non-target species may suffer from inadequate use of pesticides and their ensuing intermediates [1], as they are biocides with potential for poisoning organisms, affect the environment, food quality and human health. Particularly important is their handling by agriculture workers, since pesticides are sprayed with small knapsack sprayers (manually) or with aerial spray depending on the setting. Due to the lack of adequate training (guidelines) or government regulations handling highly toxic pesticides, small-scale farmers use these pesticides without protective clothing, thus leading to prolonged exposure to a highly concentrated formulation. This is especially relevant for a country such as Brazil, the third largest agrochemical consumer in the world [2], and as an exporter of several crops (coffee, soybean, maize, rice, cotton and wheat) [3]. Pesticides in Brazil are mostly sprayed with lever-operated knapsack sprayers due to their suitability for small-scale farms and in areas inaccessible to vehicles [4]. With these sprayers, workers may be exposed to a “cocktail” of hazardous agents through the skin (dermal), bare hands, eyes or oral with accidental ingestion.

The increasing demand for food has raised the use of pesticides. In Brazil, for instance, this use grew by > 160% from 2000 to 2012, with 381 kinds of pesticides approved for agriculture [5]. The most imported pesticides were picloram (3,5,6-trichloro-4-amino-2-pyridinecarboxylic acid) and glyphosate (*N*-(Phosphonomethyl)glycine), which accounted for 50% of the total amount imported [6]. Picloram (Pic) is a broad-spectrum herbicide, highly water-soluble, employed to kill unwanted broad-leaved plants on grass land pastures, in forestry, and in the garden. It is efficient in controlling weeds, but can it is also a potential groundwater contaminant and may cause damage to humans exposed to high concentrations. [1,7], Glyphosate (*N*-phosphonomethylglycine) is also a broad-spectrum pesticide which registered to use on over 100 species of weeds. Numerous studies have indicated that chronic exposure to glyphosate causes cancer, endocrine disruption, and reproductive-developmental toxicity among agricultural workers [2], particularly children working in risky labor situations [3].

Given the inevitable need to employ pesticides, it is urgent that new technologies are developed to increase efficiency in their delivery and reduce the amount sprayed, as in many proposed controlled release schemes [8]. Furthermore, new type of pesticides is needed which present fewer side effects on humans and other non-target organisms. In yet another avenue to minimize health risks, knowledge of the mode of action of pesticides and their physical properties is very important in order to establish stringent, efficient regulation for their use. Common to all of these possible initiatives and endeavors is the need to understand how pesticides interact with cell membranes, which are multi-component dynamic systems crucial to all cells for material translocation. Biomembranes are normally semipermeable, being permeable to water but selective to other solutes, and comprise a countless number of small, lateral lipid microdomains [9,10]. Lipid microdomains or lipid rafts are highly dynamic and form complicated lipid-protein network due to the strong protein-protein, protein-lipids or lipid-lipid interactions. These interactions are linked to cellular activities, including signaling communicating and exchange of information between extra and intracellular environment. These domains have different lipid and resident protein compositions, which depend on the organelles and types of cells, with phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (SM), and cholesterol being the principal contributors. Since the architecture and function of biomembranes can be influenced by environmental factors or diseases [11], understanding the effect of any foreign molecules on membrane integrity is relevant for human health. Membrane lipids represent the majority of ligand targets, which may be investigated either in natural or artificial membranes. Indeed, experimental work on such well-characterized membranes has shown that their structural integrity is affected by membrane-associated molecules (foreign molecules). In particular, the lipid phase determines the fluidity of the membrane, and thus regulates diffusion of membrane ligands. A model system such as giant unilamellar vesicles (GUVs) [12,13] is suitable to study biophysical and biochemical processes of the cell membrane and related functions due to their size and composition.

In this study, we employ GUVs as model membranes to verify the effects from the pesticides glyphosate and picloram. The composition of these artificial membranes was chosen so as to simulate the ocular membrane line with the ternary system containing 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC), egg sphingomyelin (SM) and cholesterol (CHOL) (DOPC/SM/CHOL). [14] Furthermore, in line with several studies on biomembranes, we investigated the importance of cholesterol in the stabilization and overall stability of phospholipid bilayers, since cholesterol affects the packing and mobility of lipids in different phases.

2. Materials and methods

2.1. Materials

All chemicals were of reagent or analytical grade except where stated otherwise. *N*-Phosphonomethyl glycine (MW 169.07), 3,5,6-trichloro-4-amino-2-pyridinecarboxylic acid (Picloram), glucose, sucrose, polyvinyl alcohol (PVA) (MW 145 K), and cholesterol (CHOL) were purchased from Sigma–Aldrich (Saint Quentin Fallavier, Brazil). The lipids DOPC, 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine (DPPC), egg sphingomyelin (SM), 1,2-diphytanoyl-*sn*-glycerol-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DPPE) and L-α-Phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (rhodamine-PE) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. Deionized water was taken from a Direct Q ultrapure water system (Direct-Q Water Purification System, Brazil). Phosphate buffer (pH 7.4, 50 mM, 50 mM NaCl) was prepared with doubly distilled water (18 MΩ cm) and the buffer solution refrigerated until used.

2.2. Sample preparation

2.2.1. GUVs Electroformation

GUVs were prepared using the electroformation technique [12]. Lipid mixtures in chloroform-methanol (7:3) used for GUV preparations are the following (in molar (weight) ratios): DOPC: CHOL:SM (1:1:1): with 1% Rh-PE and NBD-PE (1:1:1). For the electroformation process, a chamber is assembled by sealing two-indium tin oxide (ITO)-coated glass slides with a rectangular-shaped Teflon spacer (0.5 mm thick) in between. The swelling solution contains sucrose, DOPC/CHOL/SM and the two probes (Rhodamine PE and NBD-DPPE) were prepared from powder (1 mg/mL in methanol-chloroform (7:3)). Approximately 10 μL of this solution was spread on both ITO-coated slides and desiccated under vacuum overnight to remove residual solvent. Next, 5 mL of sucrose solution (0.2 M) were injected through the Teflon spacer into the chamber, and an AC voltage (10 Hz, 1 V amplitude) was applied for 1 h across the ITO slides using a function generator (minipa MFG-4202). GUVs were transferred to a 2 mL vial using a 5 mL syringe and were used immediately. A larger volume was used to avoid sample distortion. A digital multimeter was used to monitor the current and observe any fluctuation. Samples were imaged in 8-well chamber slides fitted with coverslip bottoms (ibidi GmnH, Martinsried, Germany). In this technique, a high yield of GUVs (90%) with size distribution (5 to 80 μm) was obtained within 1 h. For the experiments of the interaction between GUVs with the two pesticides, we apply the same procedure described above but without the two probes.

2.3. Microscopy

The GUVs images were acquired using a Nikon Eclipse C2si Spectral Confocal imaging system (Nikon Instruments Corporation, Japan) equipped with 20 and 40X plan-apochrome TIRF air objectives (NA = 0.75, working distance (W.D) 1.00 mm, and 0.95, W.D.) and 405, 488, and 561 nm lasers used as illumination/excitation of the sample and 515/30 nm detection filter. The phase contrast images were captured with 8-bit (412 × 272) and 14-bit (4908 × 3264 pixels) resolution of a Ds-Qi2 camera (Nikon, Japan). The collected data were processed using Nikon NIS-element software. Samples were imaged in 8-well chambers fitted with coverslip bottoms (ibidi GmnH, Martinsried, Germany). Each experiment was performed in triplicate to verify reproducibility and consistency before further analysis.

The statistical analysis of the size, diameters and shape distribution of the GUVs was performed using NIS-Elements AR software (Nikon, Japan). The counting and particles dimension of each vesicle was determined using "annotations and measurements" options in the software, and a histogram was constructed based on the structural and size alteration of the vesicles on the observed images. The collected images, each containing approximately 10–60 vesicles with different size distributions, were subjected to preprocessing procedures, e.g. smoothing or blurring.

3. Results and discussion

GUVs are useful systems to mimic cell membranes because of their size [15,16], ligand induced interaction with single vesicle can be directly observed from which biological relevant information may be obtained with optical microscopy, but one has to make sure that no artifacts hamper the interpretation of the data. The artifacts may arise from two main sources: interaction between the ligands and the fluorescence probes, and lack of stability of the GUVs. Here we test these two possibilities with confocal and phase contrast microscopic imaging. The confocal image in S1 shows the possible side interaction between the highly interactive functional group on the probe with the pesticides. Furthermore, when the GUVs with the probes were exposed for 30 min., the fluorescence intensity of the probes decreased, owing to a side effect from the laser power. Since these artifacts are also observed with other ligands (GUVS-antibiotics and other pesticides), we decided to conduct all the interaction experiments without the probes. With regard to stability, in a series of control experiments, we confirmed the stability of all GUVs investigated, as indicated in the images and discussion of Figs. S2 and S3 in the Supporting Information.

The main aim here is to investigate the interaction of glyphosate and picloram with GUVs made with the ternary equimolar mixture of DOPC, SM and cholesterol, i.e. DOPC/SM/CHOL-GUVs [17,18], which are the major lipid species in most plasma membranes. Fig. 1 shows the schematic picture of the ternary system, which consists of the vesicle core through which glyphosate and picloram interact with GUVs surfaces. Cholesterol is known to increase the rigidity and decrease the permeability of the bilayer with small molecules [19,20], thus protecting the host cell from membrane disruption by foreign chemicals.

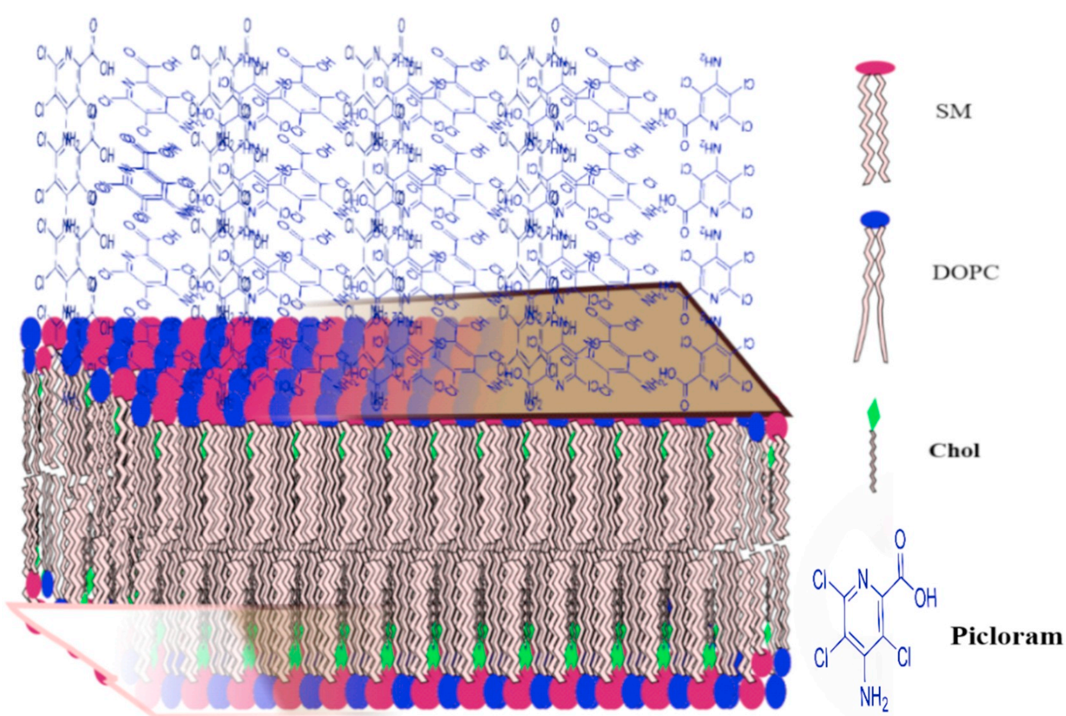

Fig. 1. Simplified schematic illustration of the interaction between DOPC/SM/CHOL 1:1:1 with picloram molecules. Colour scheme: red, SM, blue, DOPC, green, CHOL molecules in the bilayer. To simplify the cartoon, we choose picloram molecule as a representative. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

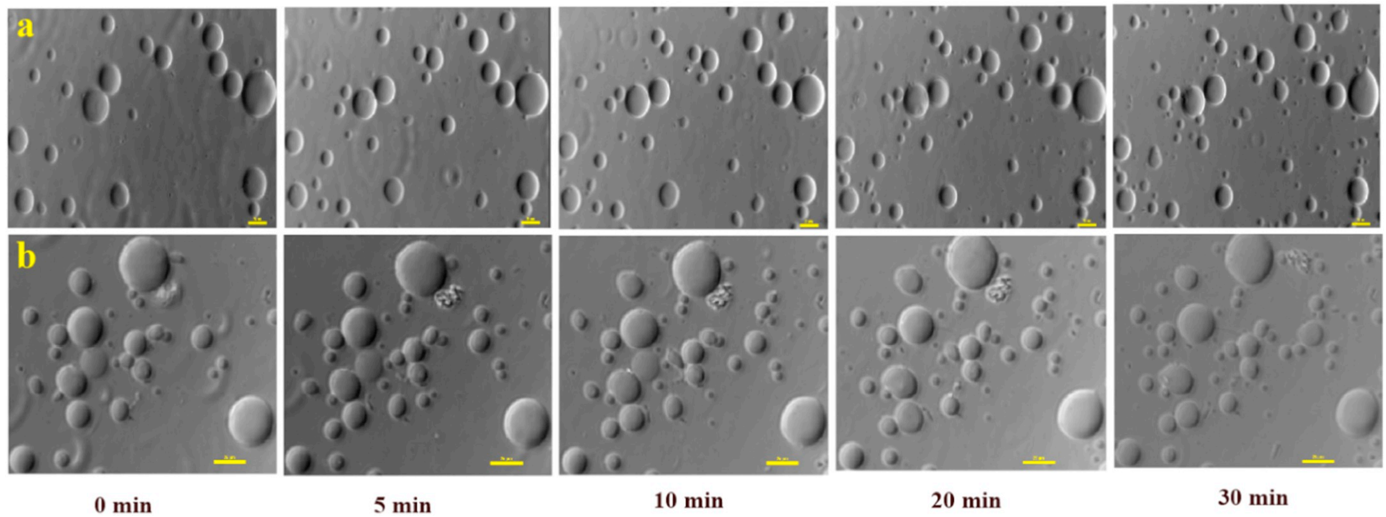

Fig. 2. Phase-contrast images of GUVs made with DOPC after incubation with a buffer solution of glyphosate. GUVs were created by electroformation in sucrose solution (a) 1 µL in buffer solution (200 µL, 0.5 µM) and (b) in 20 µL (70 µM) of glyphosate at room temperature. The numbers below each image show the elapsed time since the GUVs were exposed to glyphosate. The scale corresponds to 25 µm.

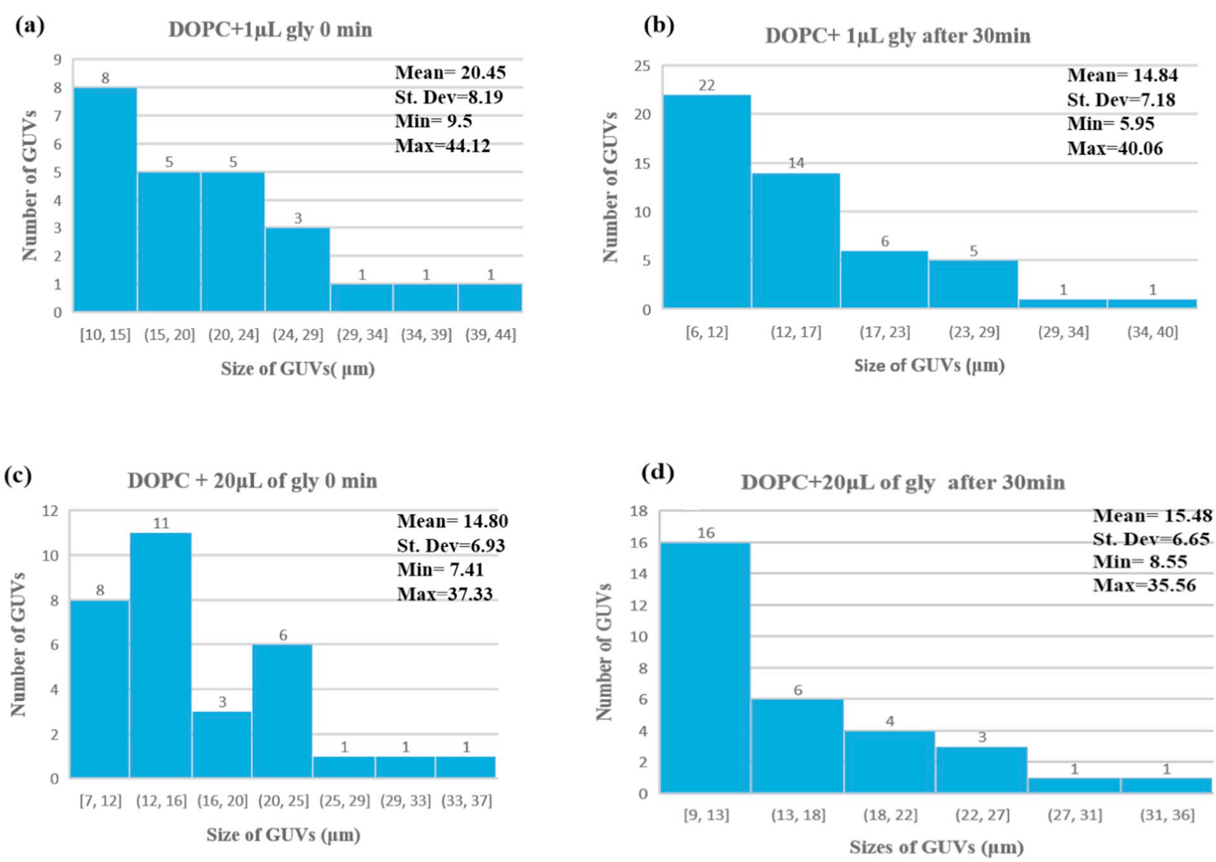

Fig. 3. Histograms showing the GUVs size distribution for (a) DOPC-GUVs incubated with 1 µL of glyphosate (gly) with incubation time 0 min; (b) after 30 min of incubation time; (c) 20 µL at 0 min; and (d) 20 µL at 30 min. The above histograms show continuous size and shape fluctuation as a function of incubation time and picloram concentration.

This has been attributed to the ability of cholesterol to reduce the mobility of lipids within the bilayer. In addition to causing a condensing effect on phospholipid packing, the incorporation of cholesterol in DOPC/SM/CHOL [21] induced an immiscible lipid phase with liquid disordered (L_d) and liquid-ordered (Lo) domains. Cholesterol preferentially affects the lipid packing of the Lo phase, which is enriched with SM, whereas it did not significantly alter the mobility and packing of lipids in the Ld phase containing mainly DOPC [22]. The existence of such domains is relevant for the phase behavior of lipid bilayers binding with ligands. Because we wish to understand the role of cholesterol, we also studied the interaction between glyphosate or picloram with GUVs made with DOPC and DOPC/SM.

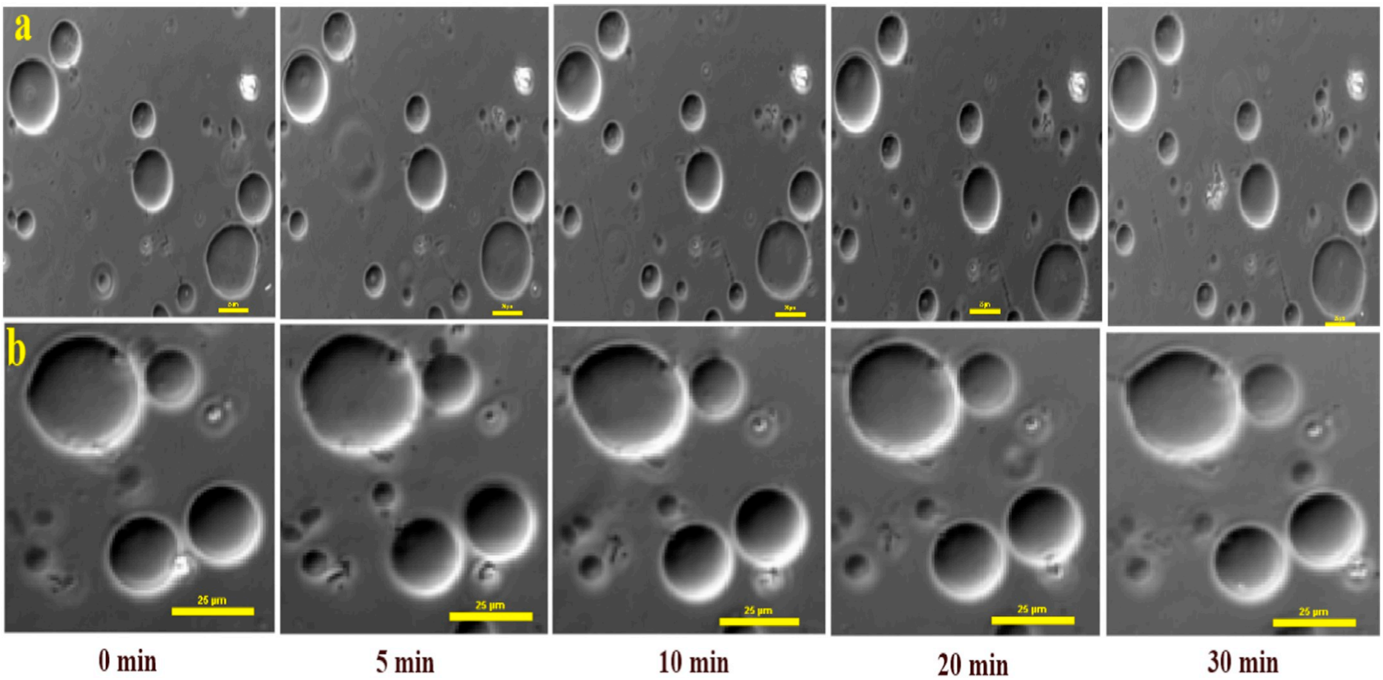

Fig. 4. Phase-contrast images of DOPC-GUVs incubated with (a) 1 µL, (b) 20 µL of picloram in buffer solution for varying incubation times, up to 30 min. The scale corresponds to 25 µm.

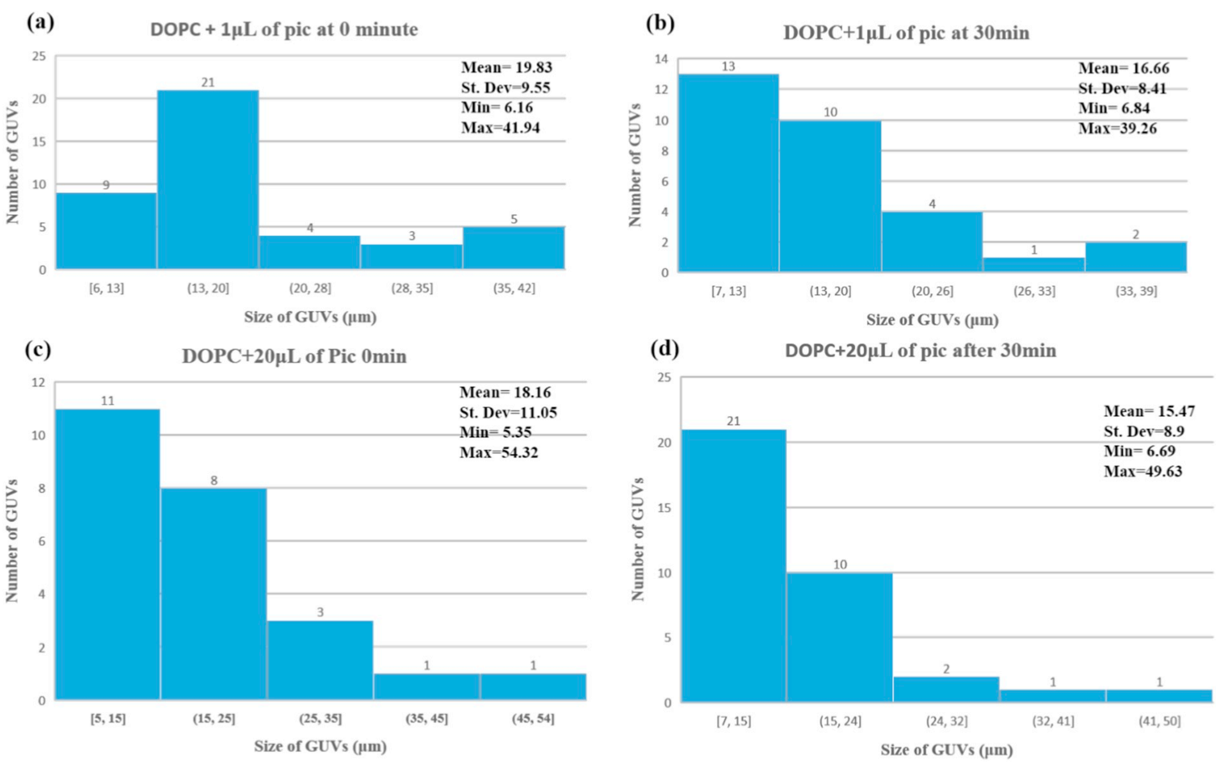

Fig. 5. Effect of different picloram (pic) concentrations and incubation periods on the integrity of DOPC-GUVs. The GUVs were incubated in PBS buffer solution at room temperature.

3.1. Interaction with DOPC GUVs

The synthesized solution of DOPC-GUVs contained a heterogeneous population of shapes ranging from 5 to 60 µm, most of which have a spherical shape which confirms the reproducible and stable preparation of GUVs. The DOPC-GUVs are in a liquid-crystalline phase at room temperature and are expected to show some degree of permeability and interaction with the pesticides [23].

We incubated various concentrations of glyphosate and picloram in buffer (10 mM PBS pH = 7.4) at room temperature and observed the mean diameter change of individual GUVs using phase-contrast microscopy. To ensure that binding equilibrium had been reached, sufficient time was allowed for all the reaction mixtures. After 30 min, 1 µL of glyphosate was mixed at room temperature with the working buffer

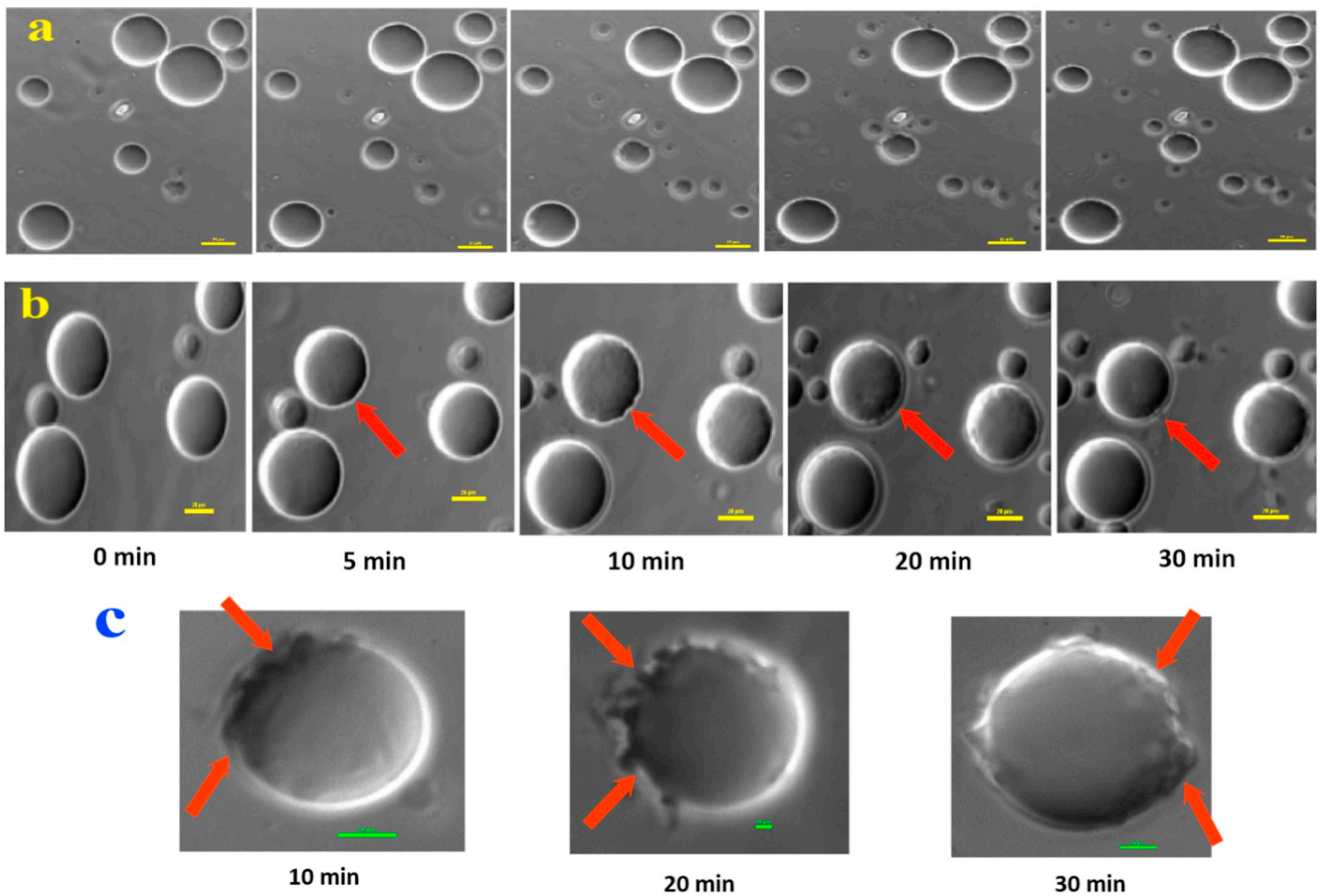

Fig. 6. The effect of glyphosate on DOPC/SM 1:1 M ratio: concentration and time study. (a) 1 μL, (b) 20 μL of glyphosate and (c) the disruptive effect of glyphosate on GUVs structural integration at 10, 20 and 30 min of the incubation period. The DOPC/SM-GUVs incubated for a maximum 30 min and the phase-contrast image collected at 0, 5, 10, 20, and 30 min of incubation time. The highlighted features (red arrows) indicate total alteration of the surface morphology of the vesicle's membrane after incubation of glyphosate for 30 min. The scale corresponds to 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

solution (200 μL), i.e. 0.5 μM glyphosate. At low glyphosate concentration (0–10 μL) the majority of GUVs show compositional and mechanical stability and retain their spherical shape, with the volume of the vesicles conserved during incubation. Hence, the pesticide concentration was too low to have a significant effect on the membrane/solution interface. In other words, at this concentration glyphosate does not disturb the head groups of the zwitterionic significantly, indicating that the pesticide does not penetrate into the GUVs. However, perturbation and/or alteration of GUVs was observed as the incubation time was increased to 30 min. According to the statistical evaluation, almost 38% of the GUVs (estimated by counting) was affected at the end of the incubation time, as illustrated in Fig. 2.

The GUVs with medium size are significantly impacted by increasing incubation time, having a diameter of 36–38 μm, as depicted in Fig. 3. This time-dependent structural reshuffling due to pesticide activity on GUVs as the incubation time increased suggests that hydration of the phospholipid head group at the interface loosens the packing due to the weakening of electrostatic interaction and/or accommodation of glyphosate molecules between adjacent phosphate groups.

At a glyphosate concentration of 70 μM, (i.e. much higher than in the experiments described above), within 1 s of incubation time, GUVs spontaneously transformed into a variety of shapes with effects including a decrease in size, protrusions, contouring, budding, rupturing and elongation. This may be inferred from a careful inspection of the images in Fig. 2. The decrease in GUVs size with increasing concentration as shown in Fig. 3 is difficult to understand. Our working hypothesis is that glyphosate with a slightly negative charge interacts with the zwitterionic bilayer, altering it and yielding different kinds of shapes and irregularities. This phenomenon may be due to increasing penetration of the glyphosate molecules caused by diffusion of the pesticide, followed by prolonged exposure of the vesicles to the pesticide. This leads to weakening of the hydrated bilayer interactions (e.g, water-water, liquid-water and lipid-lipid) at the bilayer interface. As the incubation time increased from 0 to 30 min, the rate of their deformation increased from 10 to 95%, indicating a multiplicity of biological actions exerted by glyphosate. It is likely that a strong interaction between the head group of the phospholipids with multiple charges of the glyphosate may also displace the loosely bound water or other species from the binding site.

Analogously to what is observed for glyphosate, picloram at low concentration and short incubation times (0.5 μM, 1 to 5 min) does not affect DOPC GUVs integrity. Between 5 and 20 min, < 5% of the GUVs are affected, and significant changes are observed for 30 min, as shown in Fig. 4. Also, for a high picloram concentration of 70 μM, the GUVs were altered within short incubation times.

The changes in GUVs size are more easily seen in the histograms of Fig. 5. At low picloram concentration (0.5 μM), the few large GUVs (35-42 μm) at the beginning (0 min) diminish in size with incubation time. For the picloram high concentration, Fig. 5d shows that up to 90% of the GUVs were affected.

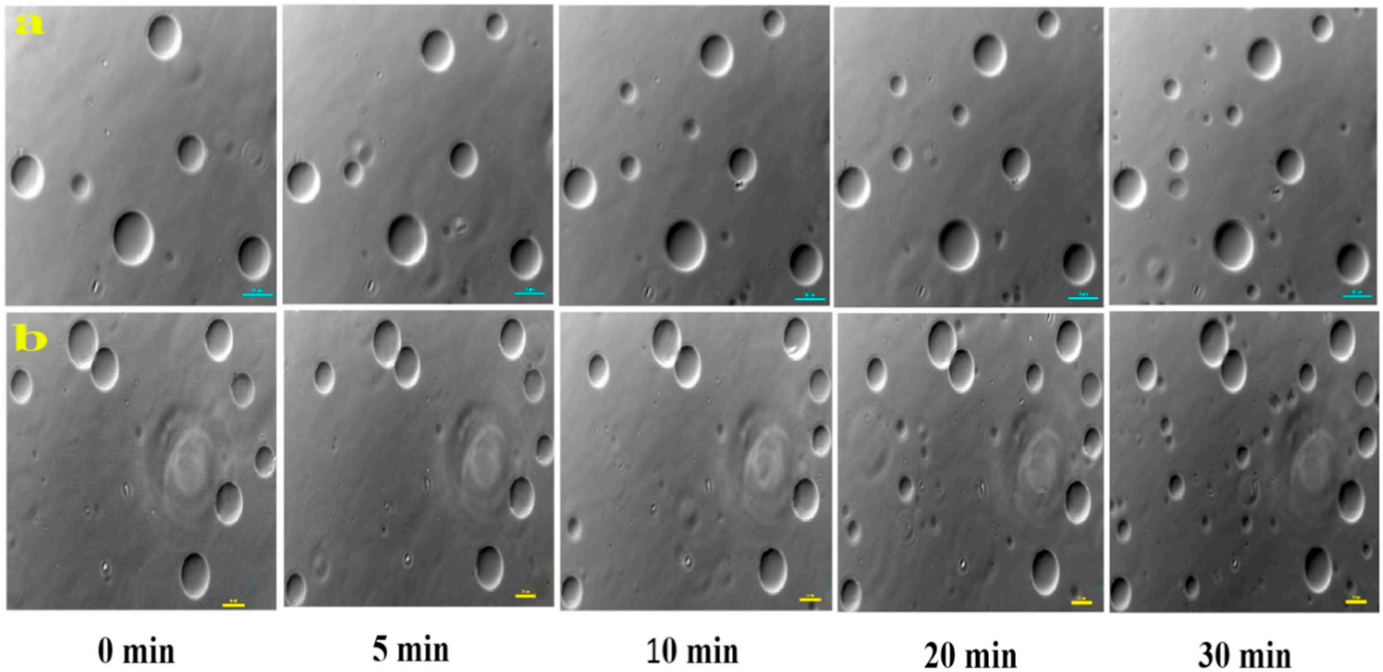

Fig. 7. Phase-contrast images of DOPC/SM 1:1 M ratio: (a) 1 μL and (b) 20 μL of picloram in buffer solution for various incubation times. The scale corresponds to 25 μm.

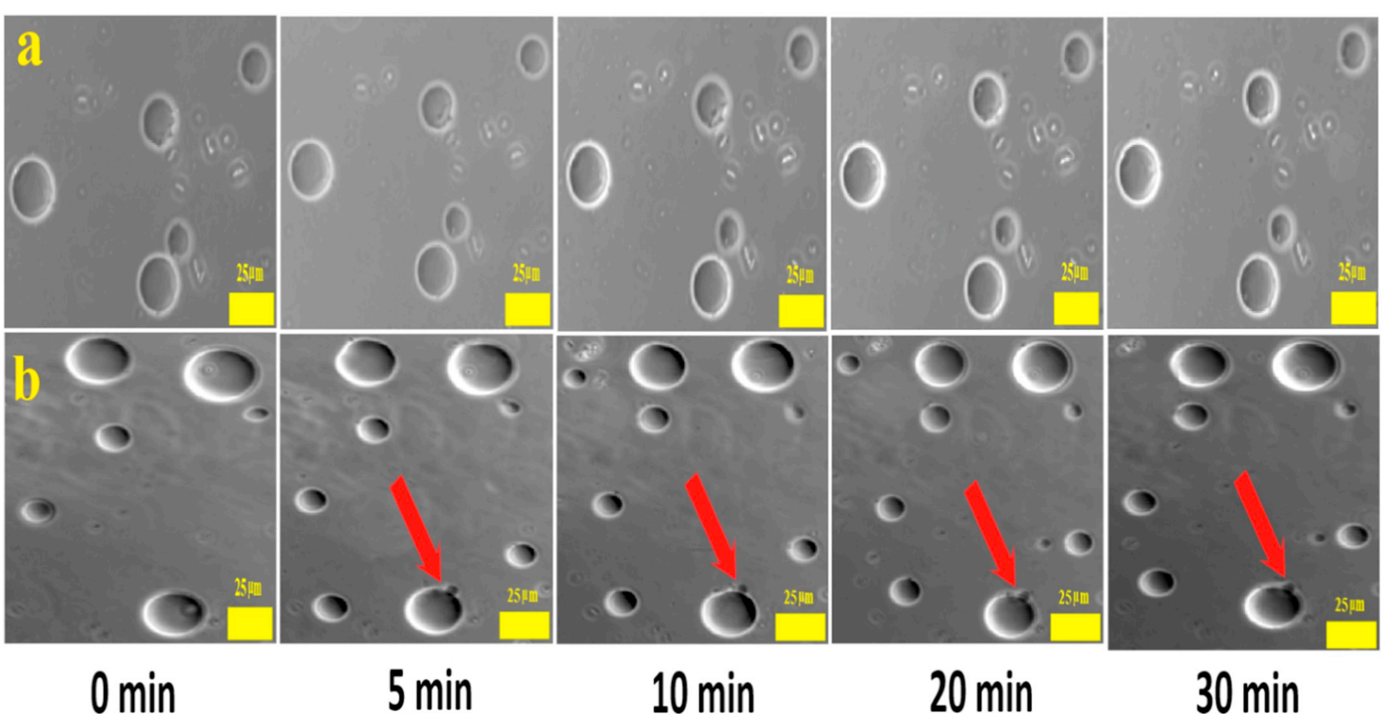

Fig. 8. Phase-contrast images of DOPC/SM/CHOL (1:1:1)-GUVs incubated with a solution of glyphosate: (a) 1 μL and (b) 20 μL glyphosate in a buffer solution at room temperature. Note that during the first 5 min of incubation there was no clear shape deformation for low glyphosate concentration.

3.2. Interaction with GUVs made with DOPC/SM/ 1:1 M ratio

Fig. 6 shows phase-contrast images of a highly heterogenous size distribution of DOPC/SM-GUVs incubated with glyphosate for up to 30 min in a buffer solution. At low concentrations, the vesicles edges were altered, particularly along the liquid disordered phase. As the incubation time increased (1 to 30 min), the number of GUVs affected increased and almost all the vesicles underwent some shape transformation, including GUVs breakdown (degradation) and shrinkage. For a high glyphosate concentration, Fig. 6b (S4) shows that the GUVs are affected from the beginning, with the destructive activity of glyphosate not being limited to a certain size of GUVs, in contrast with the effect at low concentration.

Fig. 7a shows that picloram at low concentration (0.5 μM) had little effect on the morphology of GUVS made with DOPC/SM, even after long incubation times (30 min), with only 20% of the GUVs being altered. In contrast, at 70 μM picloram, Fig. 7b indicates transformation into different shapes (oblate, prolate opening-up, and shrinkage), with

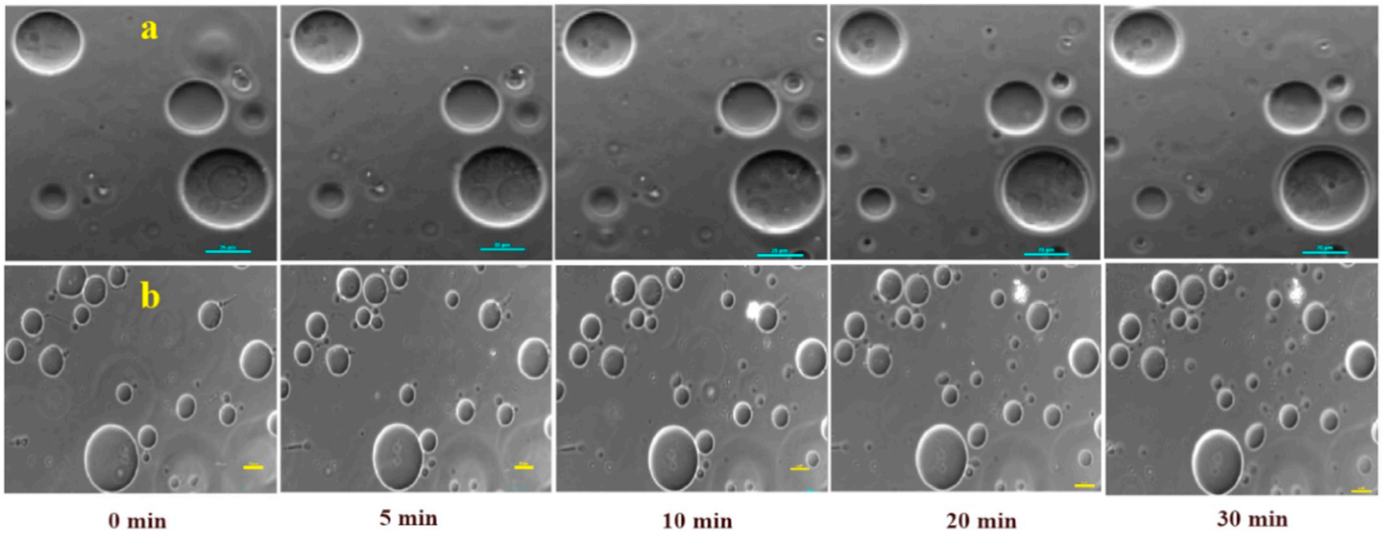

Fig. 9. Phase-contrast images for DOPC/SM/CHOL (1:1:1) GUVs exposed to picloram: (a) 1 μL and (b) 20 μL. GUVs were incubated for 30 min in buffer solution. The scale corresponds to 25 μm.

the majority of the GUVs being affected. Overall, one may conclude that picloram had smaller effects on DOPC/SM GUVs than glyphosate.

3.3. Interaction with GUVs made with DOPC/SM/CHOL (1:1:1)

We further investigated the effect of glyphosate on DOPC/SM/Chol-GUVs model using a similar experimental set-up as described above. At low glyphosate concentration (1uL, 0.5 μM), the majority of the GUVs show compositional and mechanical stability and retained their spherical shape and that the volume of the vesicles conserved during the incubation (5 min). The implication of this is as it often in the case of ligand-membrane interaction, the concentrations of the pesticide may be very low to have a significant effect on the GUVs stability (Fig. 8a). This also means that the DOPC/SM/Chol-GUVs accommodate all the stresses irrespective of the change of their environment. The robestness of such a composition underlying the synergistic effect of the three lipids, which promotes membrane integrity and stability when the membrane susceptible to such a low concentration of pollutants.

In contrast, for longer incubation times, the shape of some of the GUVs starts to change (shrinkage, disruption). At a high glyphosate concentration (70 μM), Fig. 8b shows some GUV disruption even within short incubation times with ca. 30% being affected. As the incubation time increased from 5 to 30 min, a pronounced shape transformation (prolate, bead-like) was observed for 30 to 80% of the vesicle populations (Fig. 8b). However, this effect is minor compared to that of DOPC/SM system, implies the important role of cholesterol in membrane'permeability.

For a low picloram concentration (0.5 μM), Fig. 9a shows intact GUVs up to 5 min., after which some alteration occurred with decreasing size of GUVs. It should be noted that the large GUVs with diameters of about 25–30 μm were practically unaffected up to 20 min of incubation. With 70 μM picloram, the GUVs had their shape altered as the incubation time increased to 30 min, and Fig. 9b indicates different shapes and loss of contrast due to the effects of picloram. A considerable number of GUVS (20–25%) had their shape changed within a few minutes of incubation. However, not all sizes of GUVs were affected and the overall organization of the bilayer remained intact.

4. Conclusions

We reported on the use of GUVs to mimic the cell membrane of ocular line with the ternary system DOPC/SM/CHOL in order to investigate the interaction with the pesticides glyphosate and picloram. Use of fluorescence microscopy was discarded since in control experiments, we found that the fluorescent probes interact with GUVs, even having their emission efficiency changed. Instead, we employed phase-.

contrast microscopy through which we could monitor the time-dependent effects induced by the pesticides on the GUVs. For the sake of comparison and to study the role of cholesterol, GUVs from DOPC and the binary DOPC/SM system were also employed. These three types of GUVs were highly stable in water, glucose, and buffer, with no change in size along the time scale of the incubation period (30 min).

At low concentrations, both the picloram and glyphosate are slow to react with the membrane surface in all of the three types of GUVs, only causing significant effects with longer incubation times (5 to 30 min). However, a significant disruption (increased permeability) of GUVs occurred at high concentrations of the pesticides, especially for glyphosate, right from the beginning of the exposure to the pesticides. Regardless of the concentration, disruption of the GUVs was consistently smaller for the DOPC/SM/CHOL system, thus pointing to a protecting role for cholesterol in the membrane model, as expected from the literature [24]. The results presented here do not allow one to correlate with the possible toxicity of pesticides in the work environment. Nevertheless, one may assume that effects from the pesticides on the model membranes are likely to mean that toxicity issues will arise. In this context, the disruptive effects in all GUVs for high concentrations (70 μM), are believed to correspond to harmful exposure of workers under different circumstances of glyphosate and picloram. This indicate that stringent regulation is required to protect workers during spraying of these pesticides.

Declaration of interests

None

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bpc.2019.106176.

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