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Structural Origin of ELOA Toxicity – Implication for HAMLET-Type Protein Complexes with Oleic Acid

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1. Introduction

Self-assembled proteinaceous complexes with oleic acid (OA) acquire distinct properties that are not characteristic of the native protein. Most notably, the newly obtained features include the ability to specifically kill tumor cells while sparing the healthy, normally functioning ones, as it is the case with human or bovine α -lactalbumin made lethal to tumor cells (HAMLET or BAMLET) [1,2] or to indiscriminately induce cell death in all tested cell lines, as it is the case with equine lysozyme (EL) complex with oleic acid (ELOA) [3,4]. While extensive information has been accumulated on the structural, functional and therapeutic properties of protein complexes with OA, many questions remain still unanswered, such as what is the structural origin of their toxicity, what are the specific targets at the cell surface and/or the cellular interior, what are the mechanisms of cellular uptake?

In this chapter, we summarize our current understanding of the structure and function of HAMLET-type protein complexes with oleic acid, using ELOA as an example.

2. Origin of HAMLET - Human α -lactalbumin made lethal for tumor cells

Complexes of human α -lactalbumin with OA were discovered by Catharina Svanborg and co-workers about two decades ago [5]. Initially, Håkansson et al. [5,6] and Svensson et al. [7] discovered that a multimeric human α -lactalbumin derivative isolated from the casein fraction of milk was a potent Ca^{2+} -elevating and apoptosis-inducing agent with a broad, yet selective cytotoxic activity. It was found that the apoptosis-inducing fraction of α -lactalbumin contained oligomers of α -lactalbumin that have undergone a conformational change towards a molten globule-like state [7]. Oligomerization appeared to have conserved

α -lactalbumin in a state with molten globule-like properties under physiological conditions. Multimeric α -lactalbumin was shown to bind to the cell surface, enter the cytoplasm and accumulate in cell nuclei [7]. Multimeric α -lactalbumin was also shown to increase the rate of respiration in isolated mitochondria by exerting an uncoupling effect, which was abolished completely by bovine serum albumin. Multimeric α -lactalbumin accumulated in the nuclei of sensitive cells rather than in the cytoplasm, vesicular fraction, or ER-Golgi complex [6]. Nuclear uptake was shown to occur rapidly in cells that are susceptible to an apoptosis-inducing effect, but not in nuclei of resistant cells. Nuclear uptake was shown to proceed through the nuclear pore complex and was critical for the induction of DNA fragmentation. Ca^{2+} was required for induction of DNA fragmentation by multimeric α -lactalbumin but nuclear uptake of multimeric α -lactalbumin is independent of Ca^{2+} .

Similar cytotoxic activity was observed by in vitro produced HAMLET complexes, in which human α -lactalbumin was converted into the apoptosis-inducing tumoricidal folding variant by binding OA [8-10]. The formation of HAMLET was carried out in chromatography ion exchange columns preconditioned with fatty acids. It was also identified that HAMLET formation is governed by stereo-specific lipid-protein interactions and that only unsaturated C16-C20 fatty acids in cis conformation, but not other fatty acids could induce HAMLET [11]. Among such complexes, only HAMLET complex with OA and cis vaccenic acid complexes were shown to kill tumor cells efficiently, while the C16 or C20 cis fatty acid complexes with α -lactalbumin showed low or intermediate activity [11].

HAMLET's remarkable tumor-selective cytotoxicity correlated with the conformational change of the protein that has taken place upon complex formation, i.e. conversion to molten globule-like state. However, α -lactalbumin in a molten globule state without OA does not possess such activity per se, indicating that the presence of both components is required. As partially unfolded α -lactalbumin can revert easily to its native state upon Ca^{2+} binding in natural cell culture media or within cells, the D87A Ca^{2+} -binding site mutant of α -lactalbumin was produced [12], which was lacking Ca^{2+} -binding property and remained partially unfolded at physiological conditions. Such mutant formed a tumoricidal HAMLET-like complex with OA, but the partially unfolded protein alone did not kill tumor cells. Another non-native α -lactalbumin variant with all amino acids building disulfide bridges substituted by Ala residues also did not exhibit cytotoxic activity in the absence of OA, while its HAMLET-like form displayed strong tumoricidal activity against lymphoma and carcinoma cell lines [13]. Together, these experiments consistently confirmed that both molten globule like protein conformation and specific fatty acids are required for the tumoricidal activity of the investigated complexes.

It has been suggested that naturally occurring HAMLET may have a protective function. In the stomach of nursing children low pH can induce the release of Ca^{2+} from the high-affinity Ca^{2+} -binding site of α -lactalbumin and activate lipases hydrolyzing free fatty acids from milk triglycerides, thereby providing naturally occurring conditions that favor the formation of α -lactalbumin lethal to tumors [14]. This could be important for lowering the incidence of cancer in breast-fed children by purging tumor cells from the gut of the neonate.

3. Equine lysozyme (EL) as a structural homologue of α -lactalbumin

The protein component of ELOA is equine lysozyme (EL), a protein that is abundant in mare milk and kumys (a fermented beverage produced from mare milk that is widely used in Middle Asia). EL belongs to an important calcium-binding sub-family within the extended family of lysozymes, *i.e.* in contrast to common c-type lysozyme EL possesses high affinity calcium binding site, resembling with this regards α -lactalbumins. Lysozymes and α -lactalbumins are characterized by not more than 35-40% in sequence homology, but share remarkably similar tertiary folds. EL serves as an evolutionary bridge between lysozymes and α -lactalbumins, combining the structural and folding properties of both. These are rather small molecules of about 14.6 kDa, consisting of two sub-domains – α -helical and β -sheet rich domains separated by a deep cleft. Lysozyme active site is located in this cleft (absent in α -lactalbumins). Calcium is coordinated by a loop positioned at the bottom of the cleft and important for the structural integrity of the protein, yet the physiological function of calcium binding to EL and other calcium-binding lysozymes is still unclear. The calcium-binding usually increases the protein stability against denaturing treatments, however in the case of EL, the significantly lower stability and cooperatively was observed compared to non-calcium-binding lysozymes even in its holo-form, while in the apo-form its thermodynamic stability is closer of α -lactalbumins than to c-type lysozymes [15,16]. EL forms a wide range of partially folded states under equilibrium conditions similar to these of α -lactalbumins [16,3,17,18]. However, EL molten globule is much more structured compared to the “classical” molten globules of α -lactalbumins, possessing an extended native-like hydrophobic core stabilised by interactions between three major α -helices (A, B and D-helices) in the α -domain [17,18]. Like c-type lysozymes, during refolding kinetics EL forms an ensemble of well-defined transient kinetic intermediates, possessing very persistent structures [19]. Importantly, the rapidly formed kinetic intermediate of EL (2.5 ms refolding time) is characterised by the same extended core structure as its equilibrium molten globule analogues populated under acidic conditions, indicating that the hydrophobic collapse into molten globule-like state is an essential step in protein folding. Given its distinct structural properties, EL may be used as an invaluable research object in revealing the general mechanism and role of intermediate states in protein folding.

4. Controlled ELOA production using ion-exchange chromatography

Similar to HAMLET, ELOA was produced at the solid-liquid interface in an ion-exchange chromatography column preconditioned with OA (Figure 1).

ELOA was eluted as a strong peak by using a 0-1.5 M NaCl gradient. In the absence of OA, free EL was eluted as a narrow peak at a low NaCl concentration [4]. EL was subjected to column chromatography without decalcification as it has been performed with human α -lactalbumin during original HAMLET production, indicating a difference in the generic properties of EL and α -lactalbumin. ELOA complex remains stable in its lyophilized form suitable for long storage as well as it can be kept in solution for up to a week. It is also important to note, that co-incubation of a 50 fold excess of OA mixed with EL in solution at

room temperature did not lead to ELOA formation as was evident from the lack of characteristic ELOA conformational transitions monitored by near-UV CD [4]. Thus, the application of a solid-liquid interface facilitating protein self-assembly and protein-OA interactions proved to be an efficient approach in production of both ELOA and HAMLET complexes. By comparison, the complex of hen egg white lysozyme with oleic acid was also produced under the same conditions, but it was very low populated, unstable and OA can be easily depleted from its structure. Hen egg white lysozyme is much more stable than EL and it is evident that the hydrophobic interface in the column chromatography is not sufficient to cause its partial unfolding and interactions with OA molecules.

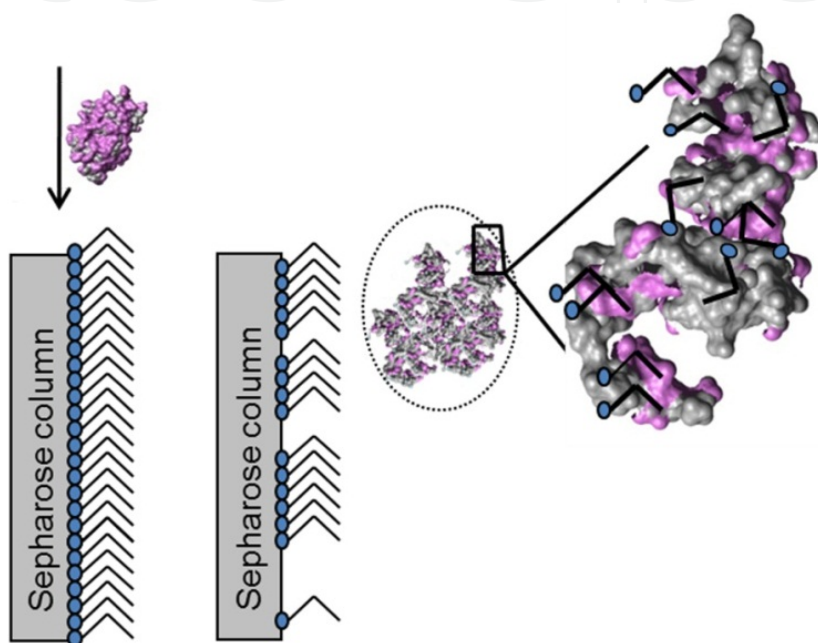


Figure 1. Schematic presentation of ELOA formation at the solid-liquid interface in a Sepharose chromatography column. The positively charged Sepharose matrix is preloaded with oleic acid (the hydrophilic carboxyl group is denoted by a blue circle and the aliphatic chain by a gray line with a “kink” at the position of the double bond). When folded, EL molecules (shown in space-filling representation, with exposed hydrophilic residues in purple and buried hydrophobic residues in grey) are added to the column some hydrophobic residues become exposed and interact with oleic acid molecules forming ELOA.

Indeed, hydrophobic and charged surfaces often facilitate the self-assembly processes by recruiting proteins and modifying their interactions [20]. Within the ion-exchange matrix bound OA molecules constitute an extended surface, facilitating both charged and hydrophobic interactions with EL molecules, while in solution OA, like many other small aliphatic molecules, would be present as a micelle. In addition, the solid-liquid interface may induce EL partial unfolding and expose its hydrophobic surfaces buried in the native state; this can also be critical for ELOA complex formation. It is worth noting, that hydrophobic interactions within the column chromatography may effectively model the interactions, which can take place at the hydrophobic and charged surfaces in biological systems. For example, the interactions with cell lipid membranes may be able to induce protein-ligand complexation otherwise not occurring in solution.

5. EL conformation in ELOA

Similar to human α -lactalbumin in the HAMLET complex, EL in ELOA acquires a partially folded state as evident from spectroscopic and NMR measurements [4]. The ELOA near-UV CD spectrum at room temperature shows the presence of less structure than in the native holo-state and even in the EL molten globule at 57 °C, i.e. the characteristic CD peaks are largely overlapped and the magnitude of the ellipticity is diminished at all wavelengths. ELOA spectra in the far-UV CD region recorded at both 25 ° and 57 °C exhibit the same shape as the EL molten globule spectrum at 57 °C, which together with the near UV CD data demonstrate disordering of the tertiary interactions, but preservation of the secondary structure.

Consistently with molten globule conformation, the 1D ^1H NMR spectrum of ELOA at pH 9.0 exhibit very broad aromatic and aliphatic resonances, indicative of conformational mobility in a millisecond time scale, and a complete absence of resolved methyl peaks in the up-field region of 2.5 - 0.5 ppm [4,21]. This is in contrast to the NMR spectrum of native EL characterized by well-dispersed resonances in both the aromatic and aliphatic regions. Examination of the 1D ^1H NMR spectrum of ELOA showed up-field shifts of the resonance of bound OA compared with the resonances of free OA. This unequivocally demonstrates that OA molecules are integrated in ELOA. Specifically, OAs interact directly with EL aromatic residues as manifested in the presence of cross-peaks between the protons of aromatic residues and OA observed in the ^1H NOESY spectrum of ELOA. Due to the poor chemical shift dispersion of the ELOA spectrum, it is impossible to assign the positive NOE cross-peaks to specific aromatic residues, nonetheless this is an absolutely clear indication that EL aromatic residues are directly involved in OA binding.

Similar to typical molten globule states, ELOA binds the hydrophobic dye ANS, which is commonly used to examine the partially folded protein conformations. Interaction with ELOA resulted in ca. 10-fold increase in dye fluorescence compared with free ANS in solution. A shorter wavelength shift of the spectrum maximum of ANS (from 515 to 495 nm) indicates that ANS in its bound form is involved into a more hydrophobic environment. These results demonstrate that the ELOA complex is characterized by some exposed hydrophobic surfaces, which attract hydrophobic ANS molecules.

The surface dynamics and exposure of aromatic residues of ELOA were also probed by photochemically induced dynamic nuclear polarization (photo-CIDNP) spectroscopy [21]. CIDNP method evaluates the surface structure of proteins and complexes by means of a laser induced photochemical reaction, which takes place only if the aromatic side-chains of histidine, tyrosine and tryptophan residues are accessible to a photosensitizer [22]. The ELOA CIDNP spectra were compared with those of holo EL and EL molten globule.

The CIDNP spectra of the native EL at several pH 4.5, 6.9 and 9.0 are well-resolved and were assigned by comparison with NMR chemical shifts [17,18,22]. ELOA and EL molten display less resolved CIDNP spectra, consistent with their millisecond conformational fluctuations, although it is still straight forward to distinguish tyrosine and tryptophan/histidine residues based on their emissive (negative) and absorptive (positive) polarizations, respectively. In

the EL molten globule state the characteristic emissive peak corresponding to the Tyr ϵ protons is the dominant feature, while the other peaks observed for the native state are not present, indicating that these residues are not surface-accessible. The ELOA spectrum contains the same emissive Tyr peak (≈ 6.75 ppm) as seen for the molten globule state. In addition, at ca. 7.7 ppm narrow absorptive photo-CIDNP signals assigned to either His 114 or Trp 63 or potentially both are present in ELOA spectrum, but not in the spectrum of EL molten globule. Both of these residues occur close to the EL inter-domain cleft, indicating limited conformational mobility in this region compared to the rest of the protein and hence that this region is affected by the presence of OA and may be an OA-binding site. Occupation of this cleft by OA may induce further large-scale changes in the relative positions of EL α - and β -domains, possibly lowering the affinity of the calcium-binding site. Hence, although there are clear similarities with EL molten globule, ELOA is characterized by some more structured regions arising from OA binding. These structural changes may be also related to ELOA functional activity as exposed hydrophobic residues in these regions may promote the ELOA interactions with the hydrophobic environment in lipid bilayers and cell membranes.

The thermal unfolding transition of ELOA, monitored by far-UV CD ellipticity at 222 nm, was manifested in an overall decrease of the CD signal and occurred over a very broad range of temperatures from 30 °C and up to 80 °C. In EL alone, dissolved at both pH 9.0 and pH 7.0, two unfolding transitions were observed over the same temperature range, however, these transitions were not distinguished in ELOA. This indicates that the conformational changes in ELOA and EL may have different structural origin. It is interesting to note, that HAMLET is less stable towards thermal denaturation than holo human α -lactalbumin, while exhibiting the same stability towards urea denaturation [23]. This demonstrates that OA may produce some destabilizing effect on proteinaceous compounds in both ELOA and HAMLET, but to different degree and with different manifestation in their thermal unfolding transitions.

6. ELOA stoichiometry and comparison with EL amyloid oligomers

The question which is still debated concerns how many protein and OA molecules can be involved in the HAMLET-type complex formation and which conditions can affect this process. Firstly, in the case of ELOA the analysis of 1D ^1H NMR spectrum enabled us to determine the amount of bound OA per protein molecule by comparing the peak areas of the bound OA, reflecting the contribution of 2 olefinic protons, with the peaks corresponding to EL aromatic proton resonances [4]. This value can vary from 4 to 48 OA molecules per EL molecule, depending on the specific chromatographic conditions during ELOA formation. In general, increasing saturation of the column with OA resulted in the formation of ELOA with higher OA content. Secondly, the number of EL molecules in ELOA was determined by pulsed-field gradient NMR diffusion measurements and estimated to be 4–9 in most cases [4]. Thus, both number of OA and protein molecules can vary significantly within the ELOA complexes and the largest ELOA lies at the upper scale among the HAMLET-type complexes.

At the same time, the size of ELOA complexes tends to decrease upon dilution to micromolar concentration range. Their dimensions were characterized by AFM using the volume measurements of the round-shaped particles naturally attached to the mica surface, under the assumption that they acquire a shape of spherical cup due to their interactions with mica [24,22]. At the concentrations used in this study ($< 1.5 \mu\text{M}$) ELOA was predominantly present in the form of low molecular weight complexes - monomers to pentamers, while some larger aggregates were observed in a lower quantity. This finding was corroborated by fluorescence correlation spectroscopy (FCS). By comparing the average residence time of fluorescently labeled ELOA (*ca.* 120 μs) to the residence time of the reference fluorescent dye (*ca.* 35 μs), FCS indicated that ELOA is predominantly present as a low molecular weight complex (20-30 kDa) in diluted solutions [22].

It is interesting to draw comparison between ELOA and EL amyloid oligomers since they display some common properties. Amyloid oligomers attracted particular attention among protein self-assembled complexes due to their critical involvement in several amyloid and conformational diseases [25-27]. Oligomerisation precedes the amyloid fibril formation and oligomers may serve as nuclei for fibrillar growth. It has also been suggested that oligomers, rather than the apparently inert amyloid fibrils are major cytotoxic agents in amyloid diseases. Both α -lactalbumins and lysozymes form amyloids *in vitro* [28,29] and the lysozymes amyloid formation is associated with systemic amyloidosis in the body [30]. Under EL self-assembly both amyloid oligomers and ELOA become well populated, providing a unique opportunity to compare them directly.

Both ELOA and EL amyloid oligomers exhibited very similar stoichiometry with 4 to 20-30 EL molecules involved [4,22,24]. Both ELOA and the amyloid species of corresponding size display the cytotoxic apoptotic activity, clearly absent in EL itself. ELOA and EL amyloid oligomers were characterized by spherical morphology examined by AFM and both tended to self-assemble into donut-like circular structures with very similar diameters of *ca.* 30 nm as measured by AFM [4,31]. In addition, ELOA and EL amyloid oligomers possess characteristic amyloid tinctorial properties such as binding of thioflavin-T dye, which is known as an amyloid specific marker. Thus, ELOA has some common structural and cytotoxic features with both HAMLET and amyloid oligomers and their further studies may shed light on both these phenomena and potential link between them. It is important that ELOA complexes are stable enough to be amenable for structural characterization at atomic resolution, whereas the amyloid oligomers are often transient in nature and tend to associate into larger aggregates or split into monomers. Amyloid oligomers are also not well populated and attempts have been made to stabilize them by using fatty acids and surfactants [32-35], which extend further the comparison between HAMLET-type complexes and amyloid species.

7. Live cell study of ELOA interaction with the plasma membrane

Molecular mechanisms of protein complexes interaction with living cells and their primary targets at the cell surface remain largely unknown and disputed [20,36]. Methods with

single molecule sensitivity, Fluorescence Correlation Spectroscopy (FCS) and Confocal Laser Scanning Microscopy (CLSM) imaging by avalanche photodiodes (APD), so called APD imaging [37], which enable quantitative and nondestructive studies of molecular interactions and mobility in living cells, revealed that ELOA primarily acts on the plasma membrane of PC12 cells, inflicting damage and eventually causing plasma membrane rupture (Figure 2 A and B) followed by a rapid influx and distribution of ELOA inside the already dead cell (Figure 2 C) [21].

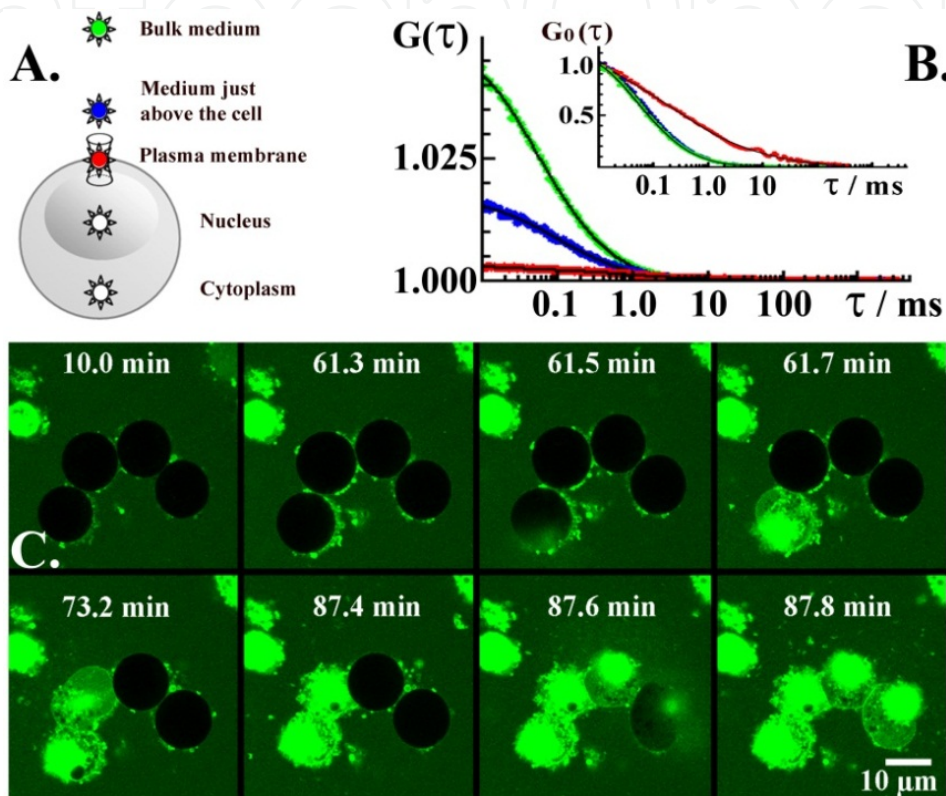


Figure 2. ELOA interaction with live PC12 cells studied by Fluorescence Correlation Spectroscopy (FCS) and APD imaging. (A) Schematic presentation of different locations – the cell culturing medium, PC12 cell plasma membrane and nucleus, at which FCS measurements were performed. (B) FCS measurements show that the concentration of ELOA in the bulk medium ($c_{medium}^{bulk} = 240$ nM) is lower than in the immediate vicinity of the cell ($c_{medium}^{cell\ surr.} = 670$ nM) and the plasma membrane ($c_{medium}^{plasma\ membrane.} = 2.5$ μM). **Insert:** Autocorrelation curves normalized to the same amplitude show that lateral mobility of ELOA in the plasma membrane (red) is significantly slower than in the medium (blue and green), as evident from the shift of the autocorrelation curve recorded at the plasma membrane (red) towards longer characteristic times. ELOA was neither detected in the cell nucleus nor in the cytoplasm. FCS measurements were taken 40–45 min after exposing PC12 cells to fluorescently labeled ELOA. (C) APD imaging shows that fluorescently labeled ELOA complexes are not gradually taken up by PC12 cells. Instead, the concentration of ELOA complexes in the immediate cellular surroundings progressively increases, reaching a local concentration that is several times higher than the concentration in the bulk medium. At a critical time-point (61.5 min), the plasma membrane ruptures. Only then the ELOA complexes “stream in” and swiftly distribute in the cellular interior, preferring particularly the cell nucleus. The scale bar is 10 μm .

8. Putative mechanism of ELOA-induced cellular toxicity

Local rearrangements of lipid organization in the plasma membrane of PC12 cells (Figure 3) observed using a general lipophilic marker that differently partitions between the ordered and disordered phase of the lipid bilayer (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate dye, DiIC18(5)) [21,38], are consistent with the hypothesis that ELOA may form transient pores in the plasma membrane.

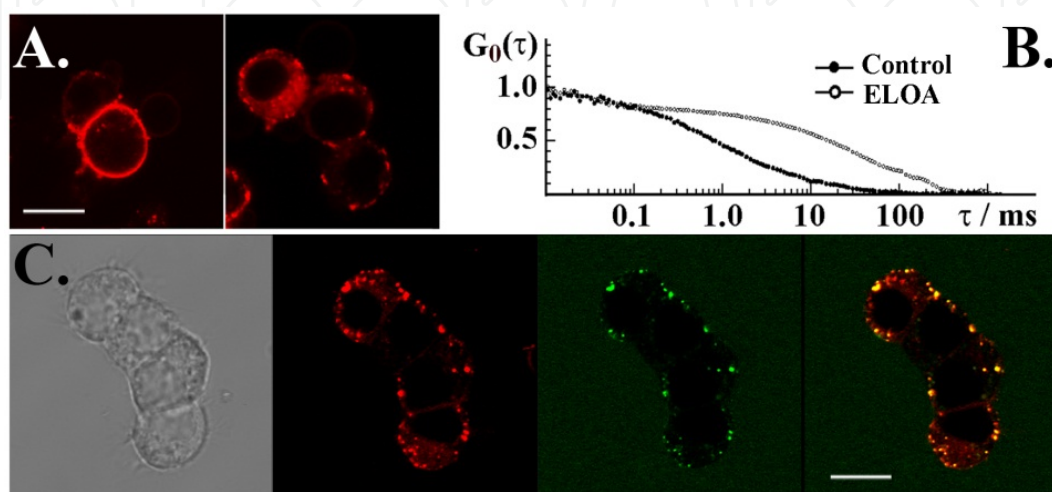


Figure 3. Lipid marker distribution in the plasma membrane of live PC12 treated with ELOA. (A) Representative image showing uniform distribution of the fluorescent lipid marker DiIC₁₈(5) in PC12 cells not exposed to ELOA (left). In cells exposed to ELOA the distribution of DiIC₁₈(5) becomes patchy, and regions of local accumulation could be observed (right). (B) DiIC₁₈(5) partitioning between different regions in the plasma membrane is also affected, as evident from the shifting of the autocorrelation curve to longer characteristic times in cells treated with ELOA. (C) Transmitted-light and APD images of PC12 cells taken 40 min after exposure to ELOA show that the plasma membrane marker DiIC₁₈(5) (red) colocalizes with the fluorescently labeled ELOA complexes (green). The scale bar is 10 μ m.

9. Future development and prospective applications

Recently, complexes of bovine β -lactoglobulin and pike parvalbumin with OA were produced and classified as HAMLET-type complexes [39]. These proteins are neither structurally related to α -lactalbumins nor to lysozymes. Nevertheless, their complexes with OA displayed cytotoxic activity that bears a resemblance to the cytotoxic activity of HAMLET [39]. This suggests that protein self-assembly may be mediated by oleic acid and more oleic acid-protein complexes can be discovered in future. Their putative ability to eliminate specifically rapidly divided cells, such as cancer cells, has a significant therapeutic potential. The mechanisms of their toxic activity are still debated. Our research provides first insight at a single cell level that ELOA interactions with the cellular membrane play critical role in cytotoxicity, leading to membrane permeability and even rupture. There are obvious differences in the composition and structure of protein-oleic acid complexes arising due to differences in the structure and dynamics of the protein component and differences in the conditions of complex formation. The common feature of these complexes can be

related to the fact that they all serve as cargo vessels delivering oleic acid to the cells and facilitating its penetration into cell membrane and cell interior. HAMLET is the first example of proteinaceous complexes with oleic acid effectively used in combating various cancer conditions and other complexes can be also potentially used for this purpose if their properties will be well-understood and controlled.

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