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Abstract

Single liposome electrochemistry is a very sensitive and versatile electroanalytical tool for understanding biological processes and fundamental mechanisms occurring at the lipid membrane / electrode interface. The extension of the electrochemistry of single redox liposome impact for sensing applications represents a great opportunity for detecting various biotargets in a short time of analysis with a high sensitivity. Both the electrochemistry of individual vesicle impact onto a surface of ultramicroelectrode and the detection of the electroactive content encapsulated in a giant unilamellar vesicle are reported in this review and illustrated with recent examples. The limitations, applications, perspectives and challenges of single liposome electrochemistry are briefly discussed based on the last published studies.

Keywords

Single liposome electrochemistry, Vesicle lipid membrane, Encapsulated electroactive content, Redox liposome impact, Chronoamperometry.

Introduction

Electrochemistry of single liposome and vesicle is a powerful tool for rapid and sensitive detection because of the versatility of these micro- and nano-objects in term of lipid membrane composition and redox probe content [1,2]. Even if several techniques such as cryo-transmission electron microscopy, atomic force microscopy, dynamic light scattering and nanoparticle tracking analysis have been developed for liposomes analysis, the main advantages of single entities label-free electrochemical analysis nevertheless remain the high time resolution and sensitivity that allow to characterize several properties (size distribution, surface charge, and liposomes concentration) [3,4]. Since 2016, the single liposome electrochemistry has been increasingly studied and its applications highly extended [5–8]. Beyond the electrochemical analysis at the single-liposome scale, electrochemical methods such as amperometry have indeed high temporal resolution (sub-millisecond) and low limits of detection (down to a few thousand molecules) [9]. Because of the ability of liposomes to encapsulate an electroactive probe, the chronoamperometry (*i*-*t* curve) has been the most used electrochemical technique for not only detecting and quantifying the liposome redox content in real time, but for also understanding how liposomes open during collision with an ultramicroelectrode (UME) surface [9]. Chronoamperometry technique also allows to bring a better understanding of biological and chemical mechanisms occurring through the lipid bilayer or the cell membrane, based on the charge transfer reaction at the liposome / electrode interface. The use of synthetic liposomes and vesicles for making micro- and nano-reactors able to interact with various targets through their lipid membrane and release their electroactive probe for detection at the single-entity scale is an attractive way for several fundamental purposes and sensing applications. The release process of the vesicle electroactive content in single liposome electrochemistry is dependent on the type of vesicles

and especially their lipid membrane composition, which leads to different membrane permeation mechanisms [9,10].

In the present review, we will focus on the recent advances in single liposome electrochemistry including single-vesicle electrochemical events with the exocytosis process [11–13], electrochemical single impacts of synthetic redox liposomes [7,14,15], and the electroanalysis of single giant unilamellar vesicle (GUV) [16,17]. We will present these three different electroanalytical techniques and discuss their current limitations and applications through different recent examples. In contrast to the last reviews dealing with nano-electrochemistry for studying single vesicles [4,8,10,18,19], here we will mainly discuss the last two years-published results which open the way to new applications and purposes for the electrochemistry of single liposomes. We will conclude this review by proposing some of the perspectives and challenges for this specific research field.

Single-vesicle electrochemical events: exocytosis process

The study of exocytosis process based on the detection of single-vesicle electrochemical events is a useful way that has widely been investigated and improved since thirty years [6,10–13,18–22]. Exocytosis is the fundamental process by which cells communicate with each other [23], consisting to the release of the vesicles cargo (signaling molecules as neurotransmitters) into the extra-cellular space [10]. Usually, a single cell is chemically stimulated by injecting cations such as potassium or calcium with a nanopipette in order to detect the exocytosis process and to quantify it via the vesicles content release at an ultramicroelectrode (UME) [6,11,12]. Neurotransmitter-filled vesicles encapsulate a high concentration of catecholamine such as 0.5 to 1 M which can be oxidized at a polarized UME [6,10,13]. A very complete review on the electrochemistry of single-vesicle events was published in 2020 [10] and hence, here we will mainly focus on the most recent and original

works. Especially, Ewing *et al.* have published numerous studies about the single-vesicle electrochemical events, focusing on the understanding of the exocytosis process in different model cells such as single chromaffin and pheochromocytoma (PC12) cells [12,24–31] and the mechanism of the vesicle pore-opening by vesicle impact electrochemical cytometry (VIEC) [32–35]. Two main electroanalytical techniques for probing single-vesicle events onto nano- and microelectrodes from in- and outside the cell are developed (Figure 1): single-cell amperometry (SCA) and intracellular vesicle impact electrochemical cytometry (IVIEC) [13,26,27,36]. SCA consists to place the microelectrode on top of a cell for measuring exocytotic release upon chemical stimulation (nanopipette injecting potassium cation). SCA is the only approach that allows the quantification of the number of released molecules [13,21]. IVIEC is a more recent technique which aims to penetrate the cell membrane with a nanotip electrode in the cytoplasm of a live cell in order to directly quantify vesicular content inside a single cell [27,37,38]. In the IVIEC technique the intracellular vesicles are adsorbed on the nanotip electrode surface and their rupture by electroporation (formation of pores in the vesicles membrane induced by the electric field of the polarized nano-electrode) leads to the release of their content, allowing to quantify storage in single vesicles [13,27]. The combination of these two electrochemical techniques allows to improve the understanding of the mechanism of exocytosis as well as cellular communication at the single cell scale, thanks to the analysis of the current spikes in chronoamperometry measurements (Figure 1) [27,37,38].

In Figure 1A, SCA and IVIEC techniques are performed for investigating the release mechanism of serotonin (5-hydroxytryptamine, 5-HT) in a human carcinoid BON cell, which is an in vitro human enterochromaffin cell model that synthesizes and secretes 5-HT [26]. For SCA (Figure 1Ai and ii), a disk carbon fibre UME polarized at + 0.65 V *vs.* Ag/AgCl is placed in close proximity to a BON cell and the cell is stimulated with 10 mM ionomycin

(Ca^{2+} ionophore used to increase intracellular calcium levels) in order to activate 5-HT secretion and exocytotic events (chronoamperometry current spikes recorded at the UME are displayed in Figure 1Aii) [26].

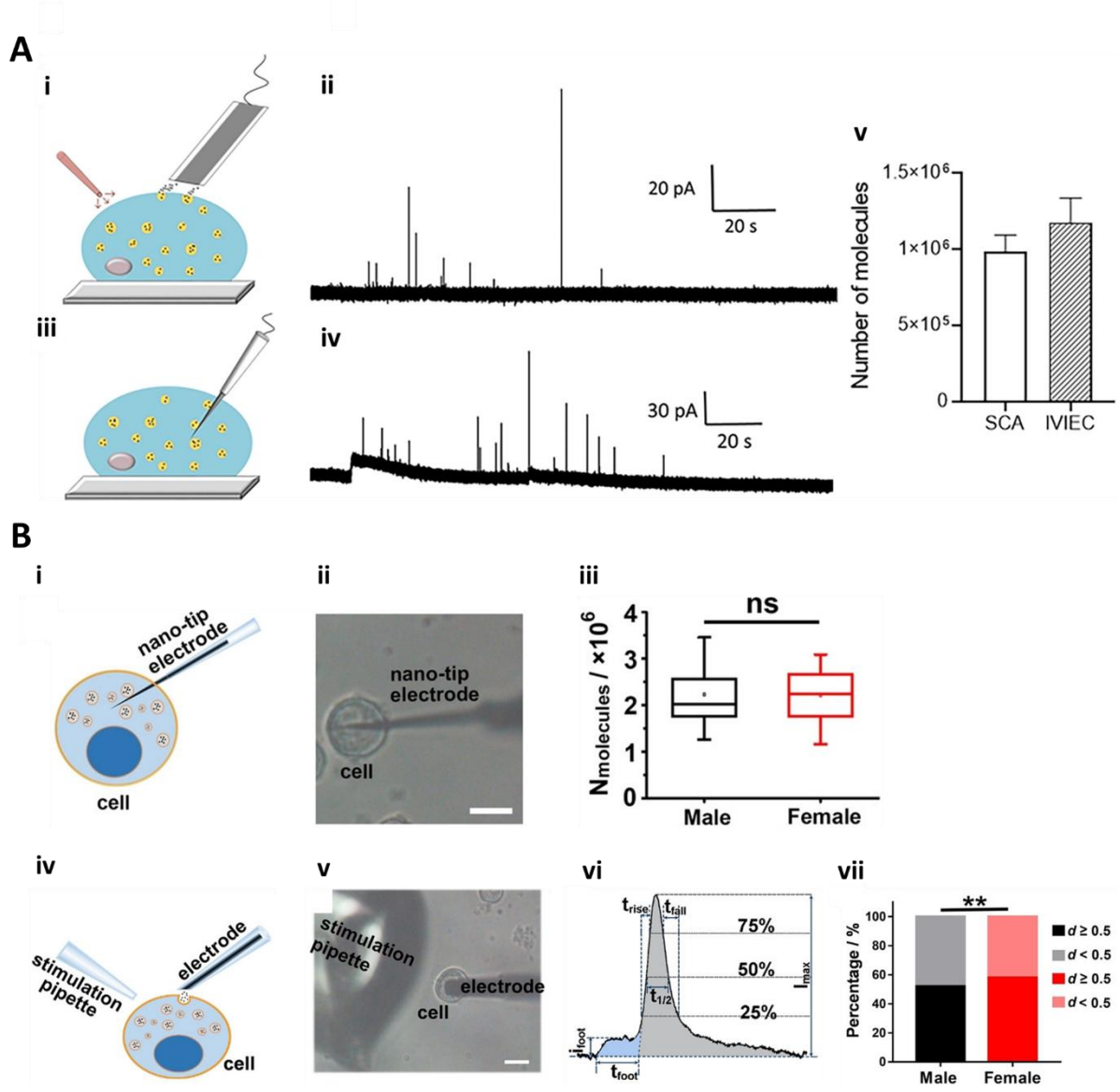

Figure 1. (A) Exocytosis and vesicular content in BON cells. Schematic of (i) single cell amperometry (SCA) and (iii) intracellular vesicle impact electrochemical cytometry (IVIEC). Typical amperometric traces obtained from (ii) SCA and (iv) IVIEC in BON cells. (v) Average number of molecules detected by SCA and IVIEC from BON cells (p = 0.23). n > 100 cells. Adapted with permission from the study by Ewing *et al.* [26]. Copyright 2021 Wiley Online Library. (B) TOP: Quantification of vesicular catecholamine content in chromaffin cells prepared from male and female rats with IVIEC. (i) Schematic diagram and (ii) bright-field photomicrograph of the experimental setup of IVIEC with nano-tip electrodes (scale bar = 10 μm). (iii) Box plots of catecholamine molecules stored in single vesicles in chromaffin cells prepared from male and female rats. BOTTOM: Electrochemical quantification of catecholamine released from chromaffin cells during single exocytotic events with SCA. (iv) Schematic diagram and (v) bright-field photomicrograph of the

experimental setup for SCA with a carbon-fibre disk microelectrode (scale bar = 10 μm). (vi) Scheme diagram showing the parameters used for data analysis of the single current spikes. (vii) Statistical comparison of *d* value for two groups. Adapted with permission from the study by Mao *et al.* [36]. Copyright 2022 Wiley Online Library.

For IVIEC (Figure 1Aiii and iv), a carbon fibre nanotip electrode is used to penetrate the cell membrane, and then intracellular vesicles adsorb and break themselves on the electrode surface, releasing their content (current spikes observed in Figure 1Aiv) [26]. The average number of 5-HT molecules (Figure 1Av) is quantified with Faraday's law ($N = Q/nF$) and the obtained values indicate that vesicles in BON cells release a large fraction of 5-HT (80%) during individual exocytosis events [26] contrary to previous work in pancreatic beta cells which only release about 34 % of 5-HT during exocytosis [39]. These fundamental results are very important for understanding the mechanisms operating the release of gut-derived 5-HT in the intestinal mucosal epithelium and for the development of effective therapeutic strategies to treat gastrointestinal diseases [26].

Single-vesicle electrochemical experiments (SCA and IVIEC) used for quantitative measurements of sex difference in rats are illustrated in Figure 1B [36]. The important result from the IVIEC analysis (Figure 1Bi and ii) is that there is no significant sex-based difference in vesicular catecholamine storage, as observed in Figure 1Biii. In contrast, the SCA analysis (Figure 1Biv and v) in this recent study of Mao *et al.* reveals that the duration of single exocytotic events of chromaffin cells prepared from male rats is statistically longer than that from female rats (Figure 1Bvi), leading to more neurotransmitter released in the male group [36]. In addition, a higher percentage of vesicles in the female group releases neurotransmitter (partial release) during exocytosis than that in male group (Figure 1Bvii) [36]. These observations are supported by the accurate analysis of the current spikes in the *i-t* curve including the maximum current (I_{max}), the half time ($t_{1/2}$), the rise time (t_{rise}) and the fall time (t_{fall}) related to the geometrical size of fusion pore, the duration of exocytosis and the time of

the opening and closing fusion pore, respectively (Figure 1Bvi). The analysis of these parameters do not show a significant difference in I_{max} between male and female groups (no difference in the geometry of fusion pore) but the characteristic peak times ($t_{1/2}$, t_{rise}, t_{fall}) of male group are significantly higher than those of female groups, suggesting that the exocytotic fusion pore formed in male group stays longer than female group [36]. In addition, the pre-spike foot characterized by t_{foot} and I_{foot} gives crucial information on the initial fusion pore size and shows that the size of initial exocytotic fusion pore in male group is smaller than that in female group (I_{foot}) [36]. This elegant work provides a new insight into the sex dimorphism of peripheral nervous system thanks to quantitative measurements at the single-vesicle level, demonstrating the power of single liposome electrochemistry for fundamental understanding of crucial biological processes.

Single-vesicle electrochemical events based on the combination of SCA and IVIEC techniques are very sensitive methods for understanding the cellular communication and chemical storage in biological cells. Nevertheless the related studies are still limited to few model cells due to the difficulty to maintain cells alive and stimulate a living cell on a substrate without damage for analysis [40]. Hence the VIEC technique, which consists to place directly the polarized UME into a suspension of isolated vesicles, is an interesting option for detecting single liposome impact events without the cell [12,24]. When the UME is polarized, the adsorbed vesicles rupture stochastically and the content of individual vesicles can be detected via an oxidation current corresponding to the catecholamine electrolysis [24]. This technique also called “reactor method” is more detailed in the next section.

Electrochemical single impacts of synthetic redox liposomes

The proof of concept of the electrochemical detection of pharmaceutical liposomes containing vitamin C which is oxidized at a carbon UME by single collision is first carried out in 2014

by Compton *et al.* [41].Then, single impacts of synthetic redox liposomes are reported in 2015 by Bard *et al.* with 120 µm diameter-DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) phospholipid vesicles containing a high concentration of ferrocyanide as an electroactive content [14]. In this study, two electrochemical single impact techniques are developed for characterizing the redox DMPC liposomes in terms of concentration and size: the blocking (Figure 2Ai) and the reactor (Figure 2Aii) methods, which both are well described in literature [4,42,43]. Briefly, the reactor method consists in detecting the oxidation of ferrocyanide encapsulated in redox liposomes during single collisions onto the polarized UME surface, corresponding to current spikes in the *i-t* curve [14,15]. The relevant result of the work reported in Figure 2A is that no current spike is observed in the chronoamperometry curve for the reactor method because the redox DMPC liposomes do not break during impact (or collision) onto the UME surface and hence the ferrocyanide content is not released and oxidized (blue curve in Figure 2Aii) [14]. The addition of an optimal concentration (0.2 mM) of Triton X-100 is required for weakening the liposome lipid bilayer in order to facilitate the breakdown of the liposome and the release / electrolysis of its encapsulated redox probe during the impact onto the UME (red curve in Figure 2Aii) [14].

In order to study the different parameters that can increase the permeability of the redox DMPC liposome lipid bilayer and hence allow the observation of current spikes in *i-t* curves, several external agents such as a surfactant (Triton X-100) and an increase of the solution temperature have been investigated by the reactor method [15]. The study of the increase of the solution temperature (Figure 2B) shows that in the same way than for the Triton X-100 concentration, there is an optimal temperature (60 °C) where the frequency of current spikes in the chronoamperometry measurement related to single liposome collisions is in agreement with the redox DMPC liposomes concentration in solution [15]. The liposome size distribution obtained from the charge by integration of the current spikes is also in agreement

with dynamic light scattering data, confirming that the observed events signal are related to single impacts of redox DMPC liposome onto UME [14,15].

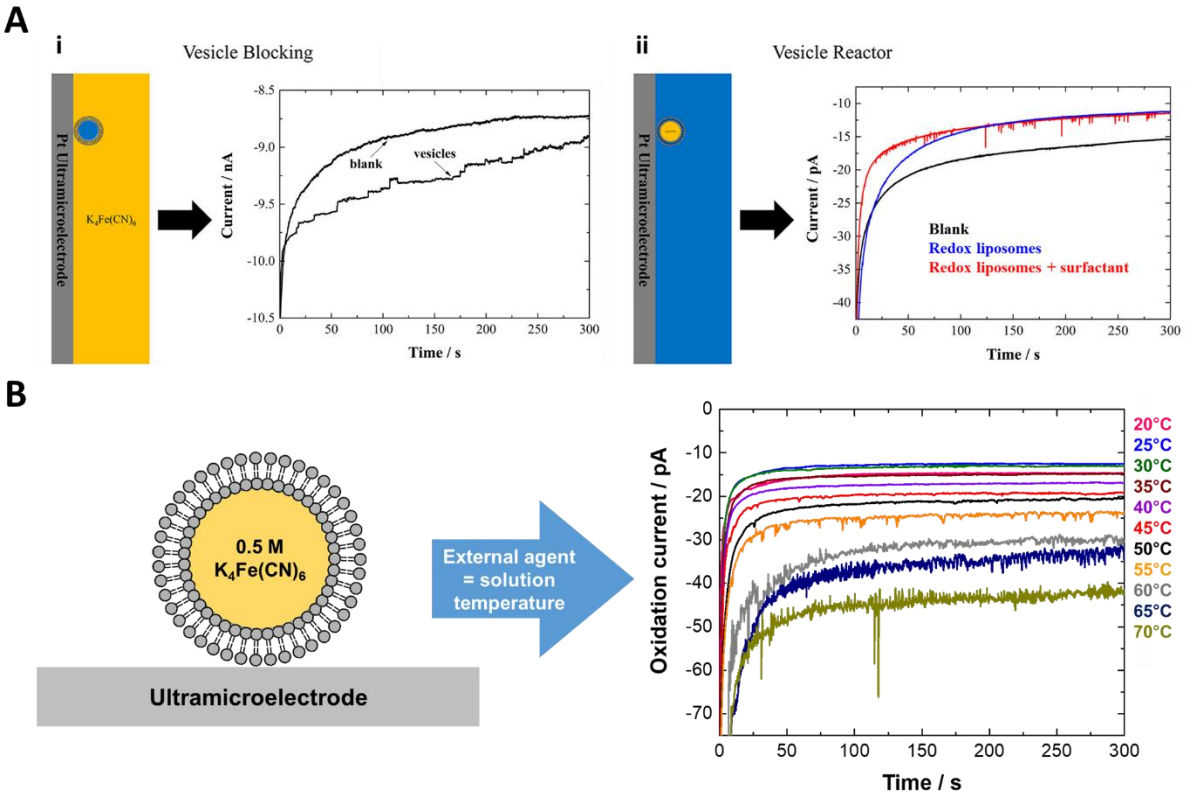

Figure 2. (A) Two vesicle collision techniques where the potential applied is at +0.6 V *vs* Ag/AgCl and the oxidation currents are plotted in the negative direction: (i) the electrochemical oxidation of $Fe(CN)_6^{4-}$ in aqueous solution (negative current) is partially blocked by single vesicle blocking which produces an anodic current step and (ii) the electrochemical oxidation of $Fe(CN)_6^{4-}$ encapsulated inside the vesicle reactor, which gives an anodic current spike in the presence of Triton X-100 as surfactant. Adapted with permission from the study by Bard *et al.* [14]. Copyright 2015 American Chemical Society. (B) Collision experiments based on *i*−*t* curves recorded at +0.6 V *vs* Ag/AgCl on 10 µm diameter Pt UME in potassium phosphate buffer aqueous solution in the presence of redox DMPC liposomes at different temperatures (from 20 °C to70 °C). Adapted with permission from the study by Bard *et al.* [15]. Copyright 2020 American Chemical Society.

This characteristic specific to the redox DMPC liposomes is recently used for detecting the lowest concentration of Rhamnolipid virulence factor produced by *Pseudomonas aeruginosa* pathogenic bacteria reported in literature, by using the ability of the toxin to act as a biosurfactant on the liposome lipid membrane [7]. In this proof of concept based on the redox

DMPC liposome single impact electrochemistry, the lowest limit of detection of Rhamnolipid toxin (500 nM) is detected in less than 30 minutes [7].

Although the blocking method is an efficient single collision technique for determining the frequency and the height of the current steps during the particle adsorption onto the UME surface [4,14,42,44,45], the reactor method brings more information on the mechanism of the electroporation process and the liposome content release [4,9,15,33,46]. The liposomes size distribution calculated from the charge passed in each current spike (by peak integration) in the *i-t* curve is a crucial information on the electrolysis process occurring upon impact onto the UME surface. In this way, several recent investigations deal with the permeability of the liposomes lipid membrane and the effect of the membrane tension on the liposomes content release based on single impact events [9,15,44,46]. Especially, Zhang *et al.* [9] have found that the liposomes electroporation process and the amount of released ferrocyanide content can be controlled by modifying the osmolarity of the buffer in which the 200 nm diameter-liposomes are suspended. Especially, they have observed a decrease of the current spikes magnitude associated with the increase of the buffer osmolarity suggesting that a partial release of ferrocyanide occurs during electroporation [9]. Furthermore, the development of resistive-pulse sensing method using nanopipettes and nanopore microelectrodes is an efficient strategy for improving control and sensitivity in the redox liposomes single impact electrochemistry [47–49]. Nowadays, the trend is to use the versatility of the redox liposomes properties in terms of lipid membrane composition and encapsulated content, added to the sensitivity of the reactor method for extending single vesicle impact electrochemistry to sensing purposes [7,46,50].

The method using electrochemical single impacts of synthetic redox liposomes is an attractive way for developing highly sensitive sensing strategies, especially because of the possibility to modulate the redox liposomes composition to reach the desired properties [7,46,50]. In

contrast, the mechanisms of the membrane electroporation process and the content release at the polarized UME surface are very dependent on the type of used liposomes (composition) and not fully understood yet [9,15,35]. Hence fundamental studies remain necessary for understanding and then controlling all these parameters which are essential for liposomes stability and single event signal. An interesting way is to couple electrochemical measurements to imaging techniques as presented in the next section.

Electroanalysis of single giant unilamellar vesicle

Single giant unilamellar vesicle (GUV) is a simple model membrane system (composed of only one lipid bilayer) of cell-size (from 1 to 100 µm diameter) [51], and used as an ideal micro-reactor mimicking the living cell membrane in order to study biological and biochemical reactions induced inside [52,53]. The GUV size allows to combine electrochemical and optical microscopy measurements such as chronoamperometry coupled to fluorescence in order to reach a high level of sensitivity and analysis of the vesicle content and its environment. In addition, contrary to living cells, the stability and easy preparation of GUVs are attractive advantages allowing to modulate the lipid membrane composition and the microinjection through it for detecting electrochemical reactions at the single vesicle scale [16,54]. The electroanalysis of a single GUV is usually performed by chronoamperometry recorded onto an UME polarized at the potential of the vesicle electrochemical content and placed in the vicinity of the GUV, close to the lipid membrane [16]. Because of the relative high permeability of the lipid membrane, the vesicle opening and the content release / electrolysis are fast mechanisms, which give the opportunity to encapsulate a large variety of different probes and to quickly analyse biochemical reactions induced following the injection (micropipette) of different species inside the GUV [16,54]. Thanks to the size of GUV and their ability to encapsulate a highly reactive-load, the electrochemical signal recorded at the

polarized UME corresponding to the content release / electrolysis is significantly and accurately detected (several pico-amperes) [16]. The main advantages of the electroanalysis of single GUV are probably the ability to make versatile micro-reactors with various contents allowing a high electrochemical sensing and to simultaneously combine with optical microscopy measurements. In this way, Arbault *et al.* [16,54] have recently investigated enzymatic activity via electrochemical and fluorescence measurements of single GUV content, illustrated in Figure 3.

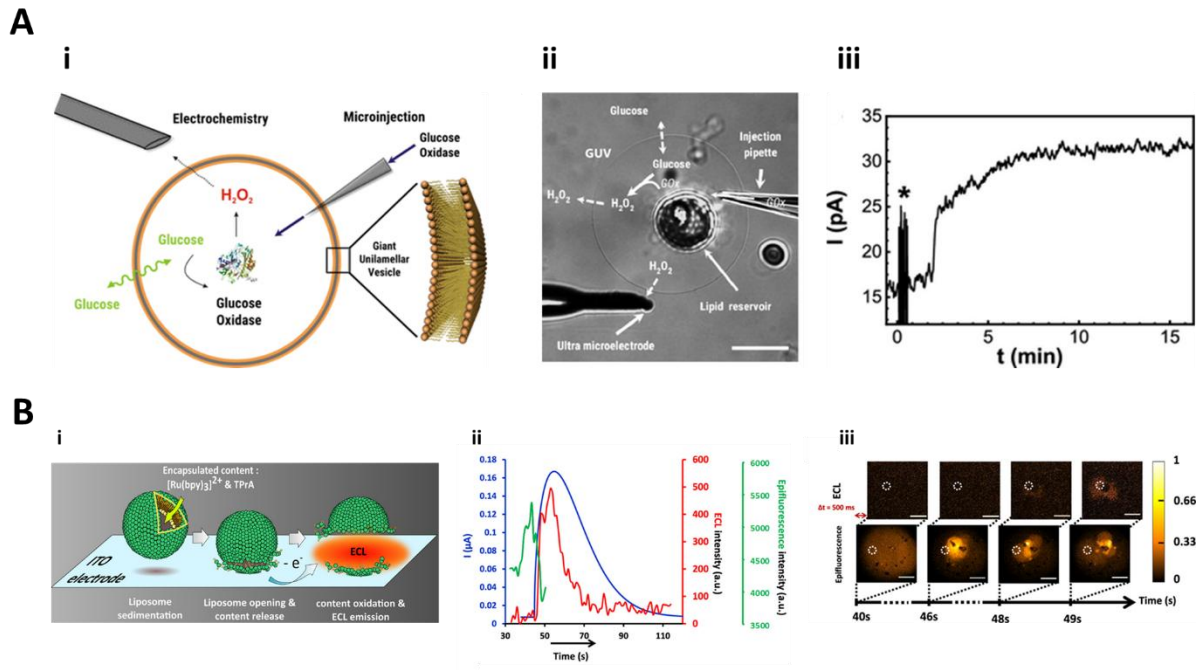

Figure 3. Single GUV analysis. (A) (i) Schematic representation of the methodological approach and (ii) microscopy image (scale bar = 25 µm): glucose oxidase (GOx) is microinjected (pipette) inside a GUV containing glucose, leading to the enzymatic production of H_2O_2. (iii) The produced flux is probed *in situ* by electrochemistry with an UME positioned in the vicinity of the GUV membrane and polarized at +0.35 V *vs* Ag/AgCl in the chronoamperometry measurement (star indicates the electric pulses used for electro-microinjection). Adapted with permission from the study by Arbault *et al.* [16]. Copyright 2021 American Chemical Society. (B) (i) ECL imaging of single giant liposome opening at a polarized indium tin oxide (ITO) electrode. Giant asymmetrical liposomes are mainly made of DOPG/DOPC for their lipid bilayer membrane and contain 250 µM $[Ru(bpy)_3]^{2+}$ and 100 mM TPrA. (ii) Amperometry (blue curve), photoluminescence (green curve) and ECL (blue curve) data are simultaneously obtained as a function of time for (iii) a single GUV opening at an ITO surface polarized at +1.2 V *vs* Ag/AgCl (scale bar = 200 µm). Adapted with permission from the study by Buriez *et al.* [17]. Copyright 2022 American Chemical Society.

On the one hand, the authors have reported the microinjection of glucose oxidase (GOx) inside single GUV containing 1 mM glucose and the successive enzymatic production of H_2O_2 diffusing across the lipid membrane toward the UME (polarized at +0.35 V *vs* Ag/AgCl) placed in the vicinity of the membrane for a direct electrochemical detection (Figure 3Ai and ii) [16]. In this work, electrochemistry of single GUV allows a highly sensitive-*in situ* detection of the H_2O_2 flux generated by the enzyme activity at the black platinum-modified carbon UME surface and selective and time-resolved analysis (chronoamperometry at a constant applied potential) over an hour duration without modifying the internal GUV medium (Figure 3Aiii) [16]. On the other hand, the production of resorufin is reported by the mean fluorescence intensity after injection of GOx and horseradish peroxidase (HRP) inside a GUV containing glucose and Amplex Red (AR) [54]. In this case, the bi-enzyme reaction is detected by laser scanning confocal microscopy (LSCM) [54].

Recently, the characterization of release events from GUVs has been addressed quantitatively by an electrochemiluminescence (ECL) imaging strategy (Figure 3B) [17]. Similarly to a previous study dealing with probing outside and inside single giant liposomes containing ECL reagents by scanning electrochemical microscopy [53], $[Ru(bpy)_3]^{2+}$ and tripropylamine are encapsulated in sealed giant asymmetrical liposomes (100 μm in diameter) made of DOPG/DOPC (1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)/1,2-dioleoyl-sn-glycero-3-phosphocholine) phospholipids (Figure 3Bi) [17]. The rupture of giant liposomes during the release of their content at a polarized indium tin oxide (ITO) electrode is monitored and imaged by amperometry, epifluorescence imaging, and ECL imaging, allowing a good spatial and temporal resolution combined to a quantification of the vesicle content (Figure 3Bii and iii) [17]. ECL is able to provide the image of the efflux of matter (luminophores) after liposome opening and this original imaging approach compares favourably with strictly

photoluminescent or electrochemical techniques and appears to be adapted for the investigation of membrane rupture / permeation events [17].

Single GUV is a micro-object able to mimic the living cell membrane and activity as an ideal micro-reactor which can encapsulate electrochemical probe allowing the electroanalysis of the release content. Its size is appropriate for coupling amperometry measurement and imaging, nevertheless the high membrane permeability of GUVs limits their ability for sensing applications where the content release must be more controlled and less spontaneous.

Conclusions and outlook

Single liposomes electrochemistry is a very attractive electroanalytical tool for studying biological processes occurring at the lipid membrane / electrode interface and for various sensing purposes because of the wide variety of vesicles in terms of size and composition. Both in single-vesicle electrochemical events for investigating the cellular exocytosis, electrochemical individual impacts of synthetic redox liposomes, and electroanalysis of single giant unilamellar vesicle as an electrochemical micro-reactor able to mimic a cell, the critical aspects which have to be understood and controlled are the parameters influencing the stability and the permeability of the lipid membrane. Because the liposomes redox content release process is strongly dependent on the vesicles nature and their membrane composition, in addition to the environment such as liposomes solution and microelectrode polarization, it is crucial to improve our understanding on these fundamental aspects. In the future, the coupling of electrochemistry and specific imaging techniques such as electrochemiluminescence imaging will be a very promising strategy to study in real time the membrane electroporation mechanism with a high spatial and temporal resolution, especially for observing the release of the liposome content into the extra-vesicular medium after opening. In addition to amperometry measurements, new individual particle detection

technique such as electrochemical impedance spectroscopy [55] should also be addressed in the future for single liposome electrochemistry.

As overall perspectives, single liposome electrochemistry is an easy handling and versatile technique for fundamental research aiming to solve chemical and biological reaction mechanisms and all permeation processes occurring at the lipid membrane in a confined and controlled environment. In our opinion, the next big challenge for single liposome electrochemistry is the detection of pathogens for medical purposes, especially biomolecules such as virulence factors and viruses able to destructively interact with lipid membrane. Beyond the detection, that will also provide insight into the interaction mechanisms during infection which lead to cell death. In this way, continuous improvement in temporal resolution and current spike analysis are necessary for making single liposome electrochemistry promising for biosensor applications with high selectivity and low detection limit.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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•• of outstanding interest

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The proof of concept of the Rhamnolipid virulence factor detection, a toxin secreted by Pseudomonas aeruginosa pathogenic bacteria, based on redox DMPC liposome single impact electrochemistry onto Pt ultramicroelectrode is reported with the lowest limit of detection (500 nM) reached in a short time of analysis (less than 30 minutes).

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The original result that no current spike is observed in the chronoamperometry curve because the redox DMPC liposomes do not break during collision onto the Pt ultramicroelectrode surface and hence the ferrocyanide content is not released and oxidized. The addition of an optimal concentration of surfactant Triton X-100 in solution is necessary for observing current peaks in the i-t curve.

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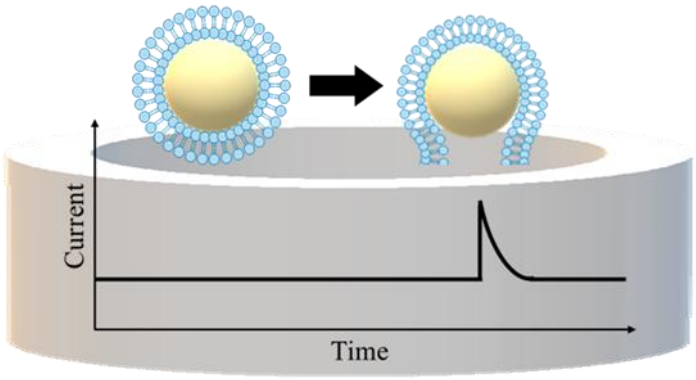
Graphical abstract



Highlights

- Single liposome electrochemistry for analyse the lipid membrane/electrode interface
- Electroactive content encapsulated in single vesicle is released and electrolyzed
- Single-vesicle electrochemical events for investigating cellular exocytosis process
- Rapid and sensitive sensing based on single redox liposome impact electrochemistry
- Giant unilamellar vesicle as an electrochemical micro-reactor for mimicking cell