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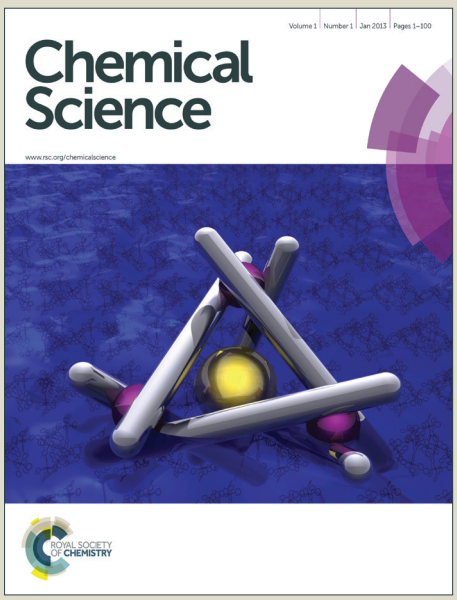

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ARTICLE TYPE

Chemical Communication Between Liposomes Encapsulating a Chemical Oscillatory Reaction

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Long-range chemical communication takes place over micrometer distance within different biological organisms and biomimetic chemical micro-compartments). A proper model for studying this phenomenon could rely on three features, namely *i*) the compartmentalization of chemical information (using microfluidics), *ii*) a stable emitter of periodic chemical signals inside compartments (Belousov-Zhabotinsky oscillating reaction) and *iii*) a suitable spatio-temporal monitoring of the emitted chemical signal. In this paper we study chemical transmission across the interface between two immiscible liquids, eventually in the presence of lipid, by local electrochemical probing. We show that chemical information is transmitted either by direct transfer of redox active species and or by interfacial electron transfer. Insights obtained by electrochemical measurements, together with numerical simulations, are then used to transpose the communication across a phospholipid bilayer among oscillators compartmentalized in liposomes and dispersed in a water medium. The procedure for the successful generation of these cell-like compartments through microfluidics is reported here for the first time.

The generation, propagation and reception of (bio-)chemical information between individual organisms are the keystones of many intelligent systems. At the level of unicellular organisms, chemical communication is based on a chemical messenger diffusion/reaction and it spans over a wide range of time and length-scales. Inside a cell, within trans-membrane protein machines chemical communication is restricted to the nanometer scale. However, in many other examples, chemical reagents are able to freely cross biological membranes and be transported to a target by diffusion over larger distances in an aqueous environment. For instance, the nervous system involves micron-scale chemical communication during synaptic communication from neurons to axons. Diffusion of chemical reagents over even longer distances (hundreds of micrometers and more) in the extra-cellular solution is believed to be responsible of the large-scale collective behaviours of colonies of unicellular organisms.[1] Apprehending such long-range chemical communication is also important for the development of biomimetic networks,[2] new forms of chemical computing[1c, 3] or attempts toward artificial human nervous system.[4] In this field of research, the biomimetic approach often concerns artificial compartments emitting chemical signals.

Droplet-based microfluidics is a powerful tool for encapsulating biological entities and chemical reagents in artificial micro-compartments with monodisperse size, mostly made of water in oil microdroplets.[5] They are generally used for the high throughput screening of reactions for their ability to compartmentalize materials in libraries of isolated chemical micro-reactors. As in nature, chemical communication plays an essential role in these artificial micro-reactors and is manifested in various ways, either within the encapsulated entities (such as cell colonies),[1a] or between the interior and outside of the compartment (partition) or between neighbouring compartments.[3a] The leakage (or partitioning) of chemical information from the micro-reactor to its external environment has been detected,[5-6] and modeled.[7] It may be a drawback[6a] in screening tests, but can be exploited in extraction or delivery processes,[6b-6d, 6f, 7] or for inducing collective chemical behaviours between micro-reactors.[1b, 1c, 6e, 6g]

A biomimetic model of chemical and electrical activity in neurons can be found in non-equilibrium chemical systems such as the Belousov-Zhabotinsky (BZ) oscillating reaction.[8] Due to the chromogenic and redox nature of its ferroin catalyst ($Fe(phen)_3^{2+}$, named here Fe^{II}), the ferroin-catalyst BZ reaction is a pertinent model to illustrate the complexity of communication in chemical systems. The compartmentalization of the BZ reaction in individual reactors,[2b] microparticles,[2a] or aqueous microdroplets[6e, 9] eventually stabilized by a lipid surfactant,[3c, 10] dispersed in an organic phase, described short and long-range chemical communication between neighbouring BZ-encapsulated micro-reactors. The communication between compartments can be explained by the generation of intermediates that allow for the spatio-temporal propagation of chemical information.[3a-3c] This phenomenon eventually leads to the production of collective behaviours such as coupling and synchronization[2] which is promising for the development of complex communication droplet-networks. However, as in many other droplet-based systems a fully aqueous environment of the water cell-like compartments is preferred.

Giant phospholipid vesicles (or liposomes), where water droplets are compartmentalized by phospholipid membranes and dispersed in a water medium provide a more reliable and promising artificial cell-like platform.[11, 12] With such, not only the mode of encapsulation but also the permeability properties of the membrane and inter-liposomes chemical communication will differ. Size monodispersity is also a key criterion in our experimental and theoretical studies. Bulk techniques such as swelling and electro-formation, have important limitations such as lack of size control, poor encapsulation and, compared to microfluidic techniques, they afford limited process control and are often poorly reproducible.[13] Moreover, droplet-based microfluidic systems to form liposomes provide a high control over space and time of the encapsulation process and the resulting liposomes. Indeed using microfluidics allows to monitor on-line (herein optically) the local redox state inside the formed liposomes and to manipulate continuously the liposomes under flow.

Herein we address the transmission of chemical information between liposomes. The BZ reaction is chosen as the encapsulated chemical information. It is indeed pertinent as it provides stable oscillating source of redox and photoactive signals. First, the interfacial transmission of a redox chemical signal across a lipid barrier is probed at the liquid-liquid interface by the scanning electrochemical microscopy (SECM). The SECM, or equivalently a microelectrode, provides an ultimate tool for the local probing of the chemical signal transmission at micrometric distances from an interface.[14] Then, for the first time, a microfluidic approach is successfully used to encapsulate the BZ reaction in liposomes and to transpose the transmission of chemical signal between neighbouring liposomes.

The coupling of neighbouring chemical oscillators, important for the network collective behaviour, was shown to rely on the extent of the exchange (permeation/partitioning) of chemical reagents between the chemical oscillators and the surrounding solution.[2a, 2b, 6e] In particular, from the reduction of the complex kinetic scheme of the BZ reaction (FKN model), three key intermediates were recognized as responsible for the onset of oscillations, namely the inhibitor Br^-, the autocatalytic activator $HBrO_2$ and the oxidized form of the catalyst, Fe^{III}.[15] Depending on which of the key intermediates acts as the messenger molecule, the behaviour of the network can be sensibly different.[16]

The chemical signal transmitted through the emulsion interface may be of redox nature and imply mass, charge and electron (ET) transfers. The process of chemical communication in droplet-based microfluidics could be then apprehended from electrochemical measurements at a planar interface between two immiscible electrolyte solutions (ITIES), a model of the micro-droplet emulsion. A similar approach has also been used for investigating chemical communication among liquid membrane oscillators on mm-scale and the influence of the interfacial conduction (i.e. electrolyte concentration) on chemical signal propagation rate was proven.[17] Here, a microelectrode is used to get monitor the communication process at the micrometer-scale in the vicinity of the l/l interface. Such strategy of local (electrochemical) probing has been successfully applied to identify the transfer of molecules, ions and electrons across model soft interfaces, such as the ITIES[18] eventually modified with lipid layers,[19] or biomimetic giant liposomes.[20]

The electrochemical measurement of interfacial mass transfer can be made either by moving the microelectrode toward the interface (Scanning electrochemical microscopy – SECM configuration) or at static electrode imprinted in the microfluidic chamber as recently developed for water in oil micro-droplets.[18b, 18c, 21]

As successful transmission of BZ oscillations was observed in emulsions, we first propose to probe by SECM the interfacial processes at play in such liquid-liquid systems from its modelling by an ITIES, an aqueous phase containing BZ reactants and an upper organic benzonitrile, BENZ, phase. The propagation of BZ oscillations from the aqueous BZ phase to the BENZ phase was detected locally in the vicinity of the ITIES by the microelectrode tip of the SECM, as described in the following.

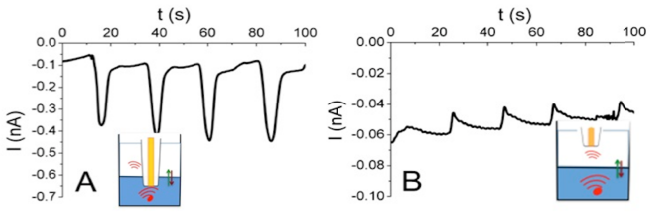

Figure 1. Oscillations of the BZ reaction recorded in (A) aqueous and (B) benzonitrile (BENZ) phases at a Pt tip polarized at 0.2V vs Ag/AgCl. A: aqueous solution 1M H_2SO_4 + 60 mM MA + 2 mM Fe^{II} + 180 mM BrO_3^-. B: BENZ + 0.25 mM Fe^{II} + 0.1M NBu_4BF_4.

Figure 1 presents the oscillations recorded at the SECM tip detecting, at a distance ~50 µm from the liquid/liquid interface, the oxidized catalyst, Fe^{III}, in both an aqueous oscillating BZ phase (Figure 1A) and a benzonitrile (BENZ) phase (Figure 1B) containing only 0.25mM of Fe^{II}. Oscillations were observed in both phases, validating the transmission of chemical information across the ITIES. The oscillations are at the same frequency (T = 20 s) but with 20 times lower intensity in BENZ (ΔI_{BENZ}= 1.5 × 10^{-2} nA) with respect to the aqueous phase (ΔI_{H2O}= 30 × 10^{-2} nA).

Deeper mechanistic insight is also drawn from Figure 1. As the SECM microelectrode tip is polarized at 0.2V vs Ag/AgCl, it detects the oxidized form of the catalyst, Fe^{III}, in both phases even if it had initially been introduced as Fe^{II}. The oxidized form, Fe^{III}, is obviously possible in the aqueous because of the presence of the intermediate oxidants, BrOx (BrOx= H^++ BrO_3^-, $HBrO_2$, HBrO or Br_2). As BrO_3^- was not introduced in BENZ, the detection of Fe^{III} suggests ET reactions (1)[22] occurring either at the ITIES interface or in the BENZ phase after permeation of oxidants from water.

$$Fe^{II} + BrOx \rightarrow Fe^{III} + BrRed \qquad (1)$$

Similarly, Fe^{II}/Fe^{III} oscillations in the BENZ phase also suggests the transmission of the catalytic BZ process from the aqueous to the BENZ phase initially devoid of its constituents. Besides the Fe^{II} oxidation steps (1), oscillations in BENZ far from the interface reveals the Fe^{III} reduction by the

intermediate bromomalonic acid, BrMA.

$$Fe^{III} + BrMA \rightarrow Fe^{II} + product \quad (2)$$

In addition to the transmission (permeation) of $HBrO_2$ and other BrOx across the interface, the permeation of BrMA was indirectly detected here confirming an organophilic nature of the brominated organic substrate respect to the more hydrophilic MA.[23] SECM examination of the proposed mass and electron transfers across the liquid/liquid interface during BZ reaction was prevented here because of CO_2 bubbles frequently disrupting the interface. These CO_2 bubbles also prevent the examination of the interface at closer microelectrode-interface distances and the transposition of these local electrochemical measurements on micro-droplets. This could be circumvented in future experiments by replacing MA with cyclohexanedione.

Along with the direct transmission of chemical species, interfacial change of redox state or transmission of electrons is also a source of chemical signal transmission indirectly demonstrated in Fig 1. In liquid/liquid systems, chemical communication can be facilitated by using permeable (redox inactive) ions increasing the interface conduction.[17] These permeable ions, also named potential determining ions, allows ion transfer that accompany interfacial ET between two redox species constrained in two different liquid phases even though the redox species are not directly crossing the interface. A strategy to indirectly improve the chemical communication between micro-reactors in droplet-based microfluidics relies on adding in the different liquid phase some permeable ions (here for example BF_4^-, Br^-). The occurrence of such indirect chemical communication through interfacial ET processes occurring at the aqueous/BENZ interface was tested by SECM approach curves (Figure 2) in the absence of BZ oscillations (absence of BrO_3^-, details in ESI). A strong oxidant, the anthracene cation radical A^+ ($E^0_{A/A+}$ = 1.3 V vs Ag/AgCl), was generated in the BENZ phase, at the tip approached to the ITIES, to trigger interfacial ETs, as any BrOx would do.

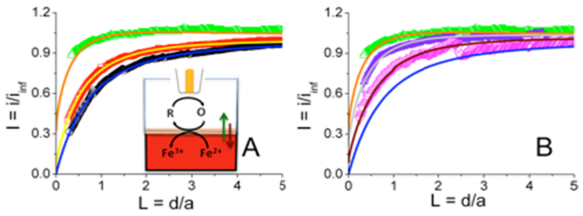

Figure 2. SECM approach curves of the ITIES between BENZ and H_2SO_4 1M. Upper phase: BENZ + 1mM Anthracene + 0.1M NBu_4BF_4 and (B) DMPC. A: Lower phase: (O) H_2SO_4 1M; (□) + 60mM MA; (△) + 2mM Fe^{II}. B: Effect of DMPC: (△) no lipid at 21°C; + 100 µM DMPC at (+) 21°C, (▽) 17°C. Lines are theoretical fits. With RG = 3.5 and k_{het} values specified in text.

Approach to the aqueous 1 M H_2SO_4 phase reveals the negative feedback behaviour (k_{het}=0), there is no communication between the two phases. After addition of MA, the increased feedback curve suggests an interfacial charge transfer at the ITIES. The insolubility of anthracene in the aqueous phase and of MA in BENZ, supports that a chemical signal is transmitted across the interface albeit none of A^+ and MA species is transmitted across the interface. The efficient oxidation of MA by A^+ at the ITIES is in agreement with reported data for similar systems, as oxalic acid.[24] The chemical communication between the two redox species constrained in their respective phases, is rationalized by an interfacial ET of apparent rate constant $k_{het} = 2.4 \times 10^{-4}$ cm s^{-1} (details in ESI).

The subsequent addition of ferroin (2 mM) results in an increased feedback curve, increased communication rate, with apparent $k_{het} = 1.6 \times 10^{-3}$ cm s^{-1}. This k_{het} increase is consistent with the large driving force for the ET ($\Delta E_{1/2}$ = 0.5 V, see ESI). The measured rate is expected to be in the diffusion-limited regime or in the inverted Marcus region, as for other L/L systems.[19a, 25] However, the absolute value of the interfacial bimolecular ET rate was not estimated, since the observed feedback also reflects some permeation of Fe^{II} in BENZ (details in ESI).

Finally, the effect of the phospholipid presence at the L/L interface on the inter-phases chemical communication was inspected (Figure 2B). As discussed later, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was chosen to build the lipid interface. The approach curves recorded at 21°C before and after adding DMPC 100µM in BENZ show that DMPC slightly decreased the ET rate, $k_{het} = 10^{-3}$ cm s^{-1}. Similar conclusions were previously drawn[19a, 26] in agreement with the phospholipid assembly at the ITIES. This rate was even slower (k_{het}=4 10^{-4} cm s^{-1}) when the temperature was decreased to 17°C in the presence of DMPC. The temperature variation of k_{het} is stronger than expected from activation driving force relationships, whereas no significant change in k_{het} was observed in the absence of DMPC at the BENZ/ water ITIES (see ESI), in agreement with experiments described at other ITIES.[27] This suggests that a primary role is played by the lipid barrier at the interface.

Electrochemical investigations at the L/L interface pointed the importance of both permeation and interfacial ET as potent chemical signals crossing the ITIES; these phenomena were retarded but not stopped by a lipid layer. They give confidence for the possibility of mass/charge transfer at an organized layer such as vesicle membranes. As a proof of concept, the BZ reaction was encapsulated inside phospholipid vesicles and the chemical communication between such compartments was studied experimentally. The results were also confronted to theoretical simulations. The next step concerns the transposition of signal transmission to lipid layer and BZ encapsulating liposomes.

We then had recourse to microfluidic encapsulating strategies. Recently, microfluidic devices have been introduced as a reliable and easy method to synthesize liposomes with controllable size, monodispersity and encapsulation.[12-13] To generate liposomes loaded with BZ, a coaxial flow microfluidic device that has been reported in details by D. Weitz et *al*.[25] was used in this work (Figure 3, top). The initial step in the process of liposome preparation is the formation of W/O/W double emulsions. The mixture of BZ reactants was injected in the inner phase, while the middle phase consisted of DMPC dissolved in choloroform/cyclohexane, that are suitable and volatile organic solvents. DMPC was chosen to form the liposomes since previous results showed that

saturated phosphatidylcholines do not undergo decomposition in the presence of the highly acidic and oxidizing BZ reaction on the time scale of our experiments (<1h).[28] A property of the DMPC bilayers, which affect the permeability of chemical intermediates across the vesicle membrane, is the gel or liquid crystalline status of its aliphatic chains.[11a] In pure water the transition temperature of DMPC is 23.5 °C, but differential scanning calorimetry (DSC) experiments showed that in our system this value is shifted upward by ~ 5 °C (see ESI, Figure S2), implying that the DMPC bilayer was in the gel status during the experiments with microfluidics performed in this work. The external phase was a viscous aqueous solution of polyvinyl alcohol (PVA). Inner drops containing the BZ reaction were formed in the dripping regime from the small injection tube while the middle oil stream containing the drops was flow-focused by the outer continuous phase. Hydrodynamically focused inner and middle fluid streams break up at the orifice of the outer capillary to form monodisperse BZ/O/W double emulsion drops. Figure 3a and b show the continuous generation and formation of monodisperse BZ/O/W double emulsion droplets (diameter ≈ 300 μm) with a thin layer of organic solvent, at the entrance and exit of the collection capillary tube in the micro-device. As it can be seen, during the formation of the double emulsion, the oxidation of the catalyst occurs in the core of the droplet. This process is reflected by the change in colour from the red ferroin, Fe^{II}, (at the entrance, dark colour in the picture) to the blue ferriin, Fe^{III} (at the exit, bright colour in the picture) and can be attributed to flow recirculation inside the droplets that efficiently mix the reagents and homogenize the mixture. At the outlet of the micro-device the solvent quickly evaporated and the formed liposomes could freely float in a water solution containing $NaBrO_3$. This salt was added to balance the osmotic pressure inside liposomes to prevent them from popping up quickly. It may also be a source of potential determining ion to facilitate the interfacial chemical signal transmission.

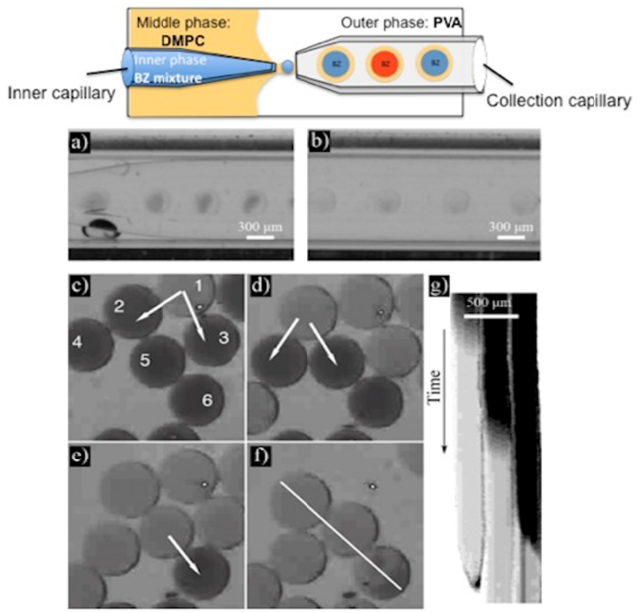

Figure 3. Top: cartoon showing the coaxial microfluidic device and schematizing the encapsulation process. Down: formation of the BZ/O/W double emulsions in the microfluidic device: a) at the entrance of the collection capillary, b) at the exit of the collection capillary, time elapsed 80 seconds. Panels c) – f) show the pulses transmission across the touching liposomes after solvent evaporation. White arrows indicate the direction of pulse propagation. Panel g) shows the space-time plot of liposomes 2, 5, 6 along the white bar in panel f).

After solvent evaporation the droplets reached a stable, well-defined size of 275 μm, and, as showed in Figure 3c, the kinetics of the system brought them back to the reduced form, Fe^{II} (dark colour). In the collection vessel, liposomes eventually formed clusters of several units, which could touch each other without coalescing, due to the good stability of their lipid membranes. Panels c) to f) in Figure 3 show an example of a typical liposome island. The sequence of the four snapshots also showed an interaction pattern among different units in the cluster. After about 20 s an oxidation wave propagated from the upper to the bottom part of liposome 1 (Figure 3c). Interestingly, once the oxidation pulse reached the lipid membrane, it was propagated through this barrier, after a short delay and new waves started in neighbour liposomes, along the direction of the white arrows. The signal transmission sequence among liposomes 2, 5 and 6 was analysed by means of the space-time (ST) plot reported in Figure 3g. Thin slices cut from each frame along the white line in Figure 3f were vertically stacked, so that the horizontal axis represent the actual space spanned by the chemical wave (≈ 820 μm) and the vertical axis represents the time elapsed from the generation of the first pulse in liposome 2 to the end of the last pulse in liposome 6 (≈ 23 s). The reciprocal of the slope of the diagonal borders between dark and bright areas represents the speed (v) at which the chemical pulses travel inside the water compartment of the liposome and was calculated to be in the range 110 - 150 μm/s. This is in line with the theoretical wave propagation speed, inside a single compartment, given in our experimental conditions by $v = (4k_aD_a)^{1/2}([BrO_3^-][H^+])^{1/2} = 107$ μm/s, where $k_a = 42$ $M^{-2}s^{-1}$ is the kinetic constant of the autocatalytic step and $D_a = 1.8 \times 10^{-5}$ cm^2/s is the diffusion coefficient in water for the autocatalytic species $HBrO_2$.[29] From the ST plot we could also quantify the lag time to about 5 s (solid bright area), during which a liposome remains in an oxidized state before transmitting the impulse to its neighbour. Local electrochemical probing of the interfacial processes occurring at the BZ encapsulated liposomes could however not be obtained owing to their actual low stability (< 10 min). The ITIES local electrochemical inspection (Figs 1 and 2) suggests that a mechanism based on mass and charge transfer, such as the permeation of $HBrO_2$, could explain the observed signal transmission between neighbouring liposomes.

The modelling of the chemical transmission between liposomes detected in Fig 3, is complex, first because of the kinetic complexity of the BZ reaction, and also because different chemical signals/messengers have been identified. In a simplified approach we may consider that the process will be ruled by the activator transport. We then performed some

simulations with the *oregonator* model (see ESI) to obtain the minimal concentration of the activator that should cross the membrane to excite the system ($[HBrO_2]_{ex}$ = 1.1 × 10^{-6} M) and the concentration of the residual $HBrO_2$ in the transmitting liposome after oxidation of the catalyst ($[HBrO_2]_{tr}$ = 7.4 × 10^{-5} M). Considering that small polar molecules, such as $HBrO_2$, can cross phospholipids bilayers through passive diffusion, we calculated the time needed to reach $[HBrO_2]_{ex}$ in the excitable droplet as 2 – 9 s, which is in excellent agreement with the delay time of our experiments. Simulations with two communicating homogeneous liposomes, coupled through the autocatalytic species, were also performed, as detailed in SI. The impulse transmission was clearly reproduced on a timescale comparable with the experiments (~ 4 s).

Conclusions

In summary, this work reports the first experimental and theoretical study on the propagation of BZ reaction as a source of chemical information encapsulated in giant phospholipid vesicles, chosen as a model of cell like micro-compartments and separated by a water medium. Regardless of the harsh chemical conditions of the BZ mixture, successful encapsulation was possible using a microfluidic strategy. Chemical waves propagating and travelling inside or among different liposomes were observed, which may reflect communication between liposomes.

Various sources of chemical signals transmitted across the interface can be proposed. In principle, direct transmission (permeation) of redox active species is possible but indirect chemical signal transmission, such as interfacial ET, can also play a significant role. Several sources of chemical signals were identified from preliminary insights on mass and ET processes across interfaces local electrochemical probing (SECM) on a bulk liquid/liquid interface model.

At this stage we cannot exclude the migration of other chemical intermediates across the phospholipid bilayers (e.g. un-dissociated MA, Br_2, etc.); however, simulations of the process with the *oregonator* model were in agreement with the experiments and supported the hypothesis of $HBrO_2$ as a messenger for transmitting chemical information. Respect to previous works, where coupling among oscillators occurs either *via* all chemical species[2c, 30] or through inhibitory coupling,[6e, 9] we can tune our system to have liposomes separated by an aqueous phase so that the activator species can be selectively exchanged among droplets and new dynamical scenarios can be explored.

Notes and references

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