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Small extracellular vesicles impact on target membrane structure and dynamics

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Summary. — Extracellular vesicles (EVs) are a main intercellular communication system. To deepen their cell internalization mechanisms, we applied scanning calorimetry to EVs mixed with model membranes of variable complex composition. Our results contribute to the description of EVs mixing mechanisms showing their impact on lipid ordering and target membrane structuring.

1. – Introduction

Extracellular vesicles (EVs) are an emerging player in intercellular communication, being able to transport macromolecules, proteins, and genetic material between cells and different organs. They contain specific characteristics of the cell of origin and can strongly influence the fate of the target cell [1]. A wide variety of routes for cellular uptake is described in literature [2], such as surface binding mediated endocytosis [3] or fusion, depending on the composition of the cell membrane [4] and on EVs physico-chemical properties [5]. Their internalization mechanism may also depend on their size [6], on the type of cell from which they are produced and whether it is healthy or pathological [7]. However, a complete understanding of the process of EVs internalization is still lacking [2, 8], as well as their influence on the physico-chemical properties of the target cell membrane after internalization. In living cells, membrane lipids are organized in clusters of dynamic liquid ordered (Lo) domains, called "rafts", which are phase-separated from the surrounding more fluid disordered domains. This liquid-liquid phase separation is a fundamental feature of membranes, regulating, among other processes, the important one of protein sorting [9]. Rafts are also claimed to be among the platforms of EVs interaction with cells [3]. With the present study, following our previous investigation [10], we exploited differential scanning calorimetry (DSC) on large unilamellar vesicles (LUVs) of variable composition, including raft models, to investigate the effect of the fusion of dopant incremental amounts of EVs derived from mesenchymal stem cells. In fact, lipids

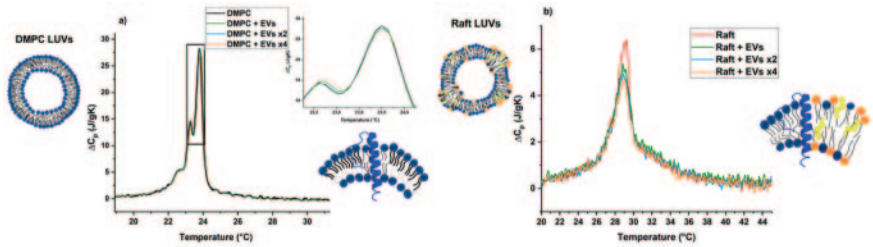

Fig. 1. – $C_p(t)$ for DMPC (panel (a)) and raft (panel (b)) LUVs before and after the interaction with incremental amounts of EVs.

organized in the membranes of about 5 nm thickness show a melting phase transition with several features of a first-order transition, presenting a broad enthalpic peak, which shape depends on the degree of cooperativity of the transition. Membrane curvature, domain parcellation and inhomogeneities affect the shape of specific heat C_p(T) in the transition temperature interval. The melting/gelling process involves the formation of growing domains of the emerging phase inside the original one, with different structural features such as thickness and lipid mobility [11]. From a cell membrane perspective, such lateral domains segregation can be to in-plane fluctuations of raft-like ordered (thicker) and fluid disordered (thinner) domains, widening the significance of exploiting DSC to investigated EVs in lipid membrane.

2. – Materials and methods

2˙1. *Materials*. – Mesenchymal stem cells derived EVs have been isolated and purified by filtration and ultracentrifugation according to the protocol described in [10]. 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), sphingomyelin (SM) and cholesterol (CHOL) have been purchased by Avanti Polar Lipids (Alabama) and used without any further purification. LUVs were prepared by a standard procedure [12], based on thin film deposition, hydration and extrusion on polycarbonate filters with pores of 80 nm. Samples were suspended in 60 mM ringer buffer. The molar proportion of components in the raft model was: DMPC : SM : CHOL = 2 : 1 : 0.15. Mixed EVs-LUVs systems were obtained by mixing EVs and LUVs solutions to the final EVs : LUVs ratios of the order of $1 : 10^8$.

2˙2. *Methods*. – Calorimetric measurements were performed with a non-commercial calorimeter [13], with temperature sensitivity of 0.002 K. Samples were submitted to temperature cycles from 10 °C to 45 °C, with a scan rate of 1 °C/3 min, both in heating and cooling modes. After each cycle, an isotherm of 1800 s was imposed. EVs were not submitted to DSC investigation, being the accessible mass too low to give a detectable signal. Dynamic light scattering (DLS) measurements were performed at 20 °C and 40 °C with a non-commercial apparatus [14].

3. – Results

In order to deepen the details of fusion between EVs and cells, two membrane models were chosen, as for previous studies [10]: a) LUVs composed of DMPC phospholipids and b) LUVs composed of mixed DMPC, SM and CHOL, addressed to as the "raft" model. While pure phospholipids are the simplest system which can be exploited as target membrane, rafts show more complex behaviour during the broader transition process, associated with phase separation of more-ordered domains enriched in SM and CHOL, with respect to less-ordered SM and CHOL poor domains [15]. In our study, DSC and DLS on EVs doped systems allow disclosing the impact of EVs fusion on membrane features.

TABLE I. – *Thermodynamical parameters of the different systems investigated by DSC. The overall cooperative units were calculated as overall* $n = \Delta H_{VH,approx} / \Delta H_{calorimetric}$.

	DMPC	DMPC + EVs	DMPC + EVs ×2	DMPC + EVs ×4
$\Delta H_{calorimetric}$ [J/g]	25.08	25.21	25.15	25.48
overall n	187	180	178	174
	Raft	Raft + EVs	Raft + EVs ×2	Raft + EVs ×4
$\Delta H_{calorimetric}$ [J/g]	23.53	23.46	21.50	21.45
overall n	52	44	50	48
$FWHM_{sharp}$ [°C]	2.0	2.4	2.5	2.7

Figure 1 shows the thermograms obtained for the melting transition of the two model LUVs before and after the addition of incremental amounts of EVs. DMPC in vesicular shape presents two main enthalpic peaks, interpreted as contributions of domains of the two membrane leaflets, convex/inner and concave/outer, having different packing [16-18]. On the other side, in raft models, the transition occurs on a wider temperature interval, composed of a sharper part at lower temperature followed by a very broad one, associated with the membrane portions poor and rich in SM and CHOL, respectively. Panels (a) and (b) of fig. 1 report the thermograms of the two systems after EVs addition and confirm [10] that fusion occurs in both systems. EVs components affect the thermotropic behaviour of the target LUVs in opposite ways, slightly "ordering" decreasing the mobility of DMPC (T_m and ΔH slightly increased), while altering the shape of ΔC_p(T) in the raft model. By applying a Van't Hoff's two-state transition model [19] the degree of cooperativity of the overall transition in the different systems can be estimated, as reported in table I. Significant are the alterations induced by EVs fusion on raft transition (see fig. 1(b)), visible in the sharper peak component referred to the poor SM and CHOL portion of the membrane. Such a cooperativity decrease of the sharper component, which can be measured by the FWHM increase in the presence of EVs (table I), is consistent with an augmented parcellation of the coexisting gel and fluid domains in the "mosaic like" membrane during melting/gelling. In our previous work [10] it was observed by AFM that EVs fusion in phase-separated bilayers preferentially occurred at the phase domain borders and, remarkably, EVs fusion induced an increase in the border length. This phenomenon was ascribed to a decrease of the domain rim energy produced by the EVs that eventually partially recover the thickness mismatch between the two liquid phases. This effect of rim lengthening can also be caused by modified cholesterol content/distribution within domains [18] after EVs inclusion. A decrease of the border tension is usually associated with a decreased cooperativity of the transition. Our hypothesis is that in the DSC study, the counterpart of the morphological event is disclosed inside the not entirely first-order transition, by a clear cooperativity decrease of the sharpest event. It is worth recalling that a dopant amount of EVs is used but, acting along a line border, a major effect is induced.

DLS measurements allowed investigating the effect of EVs on the final aggregate size. EVs were sized 120 ± 50 nm [10], while data referred to LUVs are reported in table II. All LUVs increase their size above T_m, due to an increase in membrane fluctuations [20]. EVs fusion influences the size of LUVs, the effect being more visible at low temperatures. The addition of EVs to DMPC leads to a stiffening of the membrane, while in the raft model, they allow LUVs to close up on smaller radii, that is with an augmented curvature, symptom of a lowered membrane stiffness.

TABLE II. – *Hydrodynamic diameters (D_h) of LUVs before and after EVs fusion at the investigated temperatures.*

	DMPC	DMPC + EVs	Raft	Raft + EVs
D_h [nm], $T = 20$ °C	88 var 1.11%	117 var 0.5%	96 var 4.1%	86 var 2.4%
D_h [nm], $T = 40$ °C	118 var 2.75%	134 var 0.5%	127 var 0.5%	131 var 0.4%

4. – Conclusions

Our results indicate that EVs fusion occurs with both DMPC and raft model membranes. EVs effects on lipid ordering and membrane curvature depend on membrane complexity. EVs, by altering the structure of target membranes undertaking the melting transition, influence the lateral dynamics of components and of organized domains (line tension decrease). Membrane lateral structuring associated with phase transition can be akin to cell membrane in-plane fluctuations; therefore, scaling these findings to the cell, the relevance of EVs impact on the features and dynamics of functional domains, can be speculated. For a future perspective it is important to underline that these results can strongly depend on the specific EVs of different origin.

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