



**HAL**  
open science

## Hybrid giant lipid vesicles incorporating a PMMA-based copolymer

Ylenia Miele, Anne-Françoise Mingotaud, Enrico Caruso, Miryam Malacarne,  
Lorella Izzo, Barbara Lonetti, Federico Rossi

► **To cite this version:**

Ylenia Miele, Anne-Françoise Mingotaud, Enrico Caruso, Miryam Malacarne, Lorella Izzo, et al.. Hybrid giant lipid vesicles incorporating a PMMA-based copolymer. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 2020, pp.129611. 10.1016/j.bbagen.2020.129611 . hal-02990072

**HAL Id: hal-02990072**

**<https://hal.science/hal-02990072>**

Submitted on 14 Dec 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Hybrid giant lipid vesicles incorporating a PMMA-based copolymer

*Ylenia Miele<sup>a</sup>, Anne-Françoise Mingotaud<sup>b</sup>, Enrico Caruso<sup>c</sup>, Miryam C. Malacarne<sup>c</sup>, Lorella Izzo<sup>\*c</sup>, Barbara Lonetti<sup>\*b</sup>, Federico Rossi<sup>d</sup>*

<sup>a</sup> Department of Chemistry and Biology, University of Salerno, Via Giovanni Paolo II, 132, 84084 Fisciano – Italy.

<sup>b</sup> Laboratoire des IMRCP, Université de Toulouse, CNRS UMR 5623, Université Toulouse III - Paul Sabatier, 118 Rte de Narbonne, F-31062 Toulouse cedex 9 – France.

<sup>c</sup> Dipartimento di Biotecnologie e Scienze della Vita, Università degli Studi dell'Insubria, via J. H. Dunant, 3, 21100 Varese – Italy.

<sup>d</sup> Department of Earth, Environmental and Physical Sciences – DEEP Sciences – Pian dei Mantellini 44, 53100 Siena – Italy.

\* Corresponding Author: L.I. [lorella.izzo@uninsubria.it](mailto:lorella.izzo@uninsubria.it)

B.L. [lonetti@chimie.ups-tlse.fr](mailto:lonetti@chimie.ups-tlse.fr)

1 STATISTICAL SUMMARY

2 Words: 6494

3 Figures/Tables: 5

4 HIGHLIGHTS

5 • Methacrylate based polymer is mixed with a lipid to produce giant vesicles

6 • Electroformation is not a suitable method to obtain such hybrid systems

7 • Phase transfer method is used to obtain hybrid giant vesicles

8 • Phase transfer method useful when the polymer has a relatively high  $T_g$

9

# 1 ABSTRACT

## 2 Background

3 In recent years, there has been a growing interest in the formation of copolymer-lipid hybrid self-  
4 assemblies, which allow combining and improving the main features of pure lipid-based and  
5 copolymer-based systems known for their potential applications in the biomedical field. As the  
6 most common method used to obtain giant vesicles is electroformation, most systems so far used  
7 low  $T_g$  polymers for their flexibility at room temperature.

## 8 Methods

9 Copolymers used in the hybrid vesicles have been synthesized by a modified version of the  
10 ATRP, namely the Activators ReGenerated by Electron Transfer ATRP and characterized by  
11 NMR and DSC. Giant hybrid vesicles have been obtained using electroformation and droplet  
12 transfer method. Confocal fluorescence microscopy was used to image the vesicles.

## 13 Results

14 Electroformation enabled to obtain hybrid vesicles in a narrow range of compositions (15 mol %  
15 was the maximum copolymer content). This range could be extended by the use of a droplet  
16 transfer method, which enabled obtaining hybrid vesicles incorporating a methacrylate-based  
17 polymer in a wide range of compositions. Proof of the hybrid composition was obtained by  
18 fluorescence microscopy using labelled lipids and copolymers.

## 19 Conclusions

20 This work describes for the first time, to the best of our knowledge, the formation of giant hybrid  
21 polymer/lipid vesicles formed with such a content of a polymethylmethacrylate copolymer, the  
22 glass temperature of which is above room temperature.

## 23 General Significance

1 This work shows that polymer structures, more complex than the ones mostly employed, can be  
2 possibly included in giant hybrid vesicles by using the droplet transfer method. This will give  
3 easier access to functionalized and stimuli-responsive giant vesicles and to systems exhibiting a  
4 tunable permeability, these systems being relevant for biological and technological applications.

5 KEYWORDS liposomes; polymersomes; hybrid GUVs; self-assembly; surfactant; polymer;  
6 phase transfer method

## 7 1. INTRODUCTION

8 Liposomes and polymersomes are self-assembled vesicles formed by lipids and  
9 copolymers respectively and characterized by the presence of a hydrophobic double layer  
10 delimitating an internal water pool. Their structure allows the encapsulation of both hydrophobic  
11 and hydrophilic components with high loading efficiency, which mainly boosted the research in  
12 applications related to drug delivery [1–4]. Besides, the last 10 years of research witnessed a  
13 growing interest in polymersomes used as reactors [5]. For this particular application, the low  
14 bilayer permeability of the high molecular weight constituents represents a drawback. Block  
15 copolymers forming intrinsically permeable polymersomes [6,7], polymersomes whose  
16 permeability can be modulated by a stimulus, i.e. pH [8], light [9], CO<sub>2</sub> [10], and self-adaptive  
17 polymersomes [5] have been proposed. A further, less explored possibility is the use of hybrid  
18 lipid/polymer vesicles, which could exploit the complementary properties of liposomes and  
19 polymersomes, i.e. biocompatibility, biodegradability and permeability of the former and high  
20 mechanical stability and chemical versatility of the latter. These hybrid vesicles have higher  
21 permeability to small dyes like carboxyfluorescein or calcein with respect to polymersomes [11–  
22 13] and it has been recently shown that the hybrid vesicles permeability could be increased by

1 adding phospholipase A2 that can selectively degrade lipids [14]. Examples of hybrid nanoscale  
2 polymersomes have been proposed for applications in nanomedicine and seemed to improve the  
3 targeting efficiency [15,16]. Interestingly, hybrid vesicles at the micron scale are valid  
4 alternatives to giant liposomes as simplified cell models for a deeper understanding of  
5 fundamental biological processes [17,18]. They have indeed the potential for mimicking the  
6 membrane functionalities like the presence of raft-like domains [19,20], membrane asymmetry  
7 [21] transport or recognition properties thanks to the insertion of proteins in the membrane [22–  
8 24] and even chemical energy-driven ATP production [25] or cell compartmentalization [26]. To  
9 date, the potential responsiveness of block copolymers has not yet been exploited for hybrid  
10 vesicles. Indeed, only examples of temperature-responsive hybrid vesicles are reported in the  
11 literature, exploiting the phase transition of dioleoylphosphatidyl choline based lipids [27].

12 One of the reasons of the lack of polymer/lipid hybrid systems is the limited choice of the  
13 amphiphilic copolymers which were generally restricted to a small group based on the  
14 combination of poly(ethylene oxide) or poly(oxazoline) as hydrophilic unit and either  
15 poly(dimethyl siloxane) (PDMS) [28,29], poly(butadiene) (PBD) [30,31], polyisobutylene (PIB)  
16 [32] and very recently butylacrilate [33] as hydrophobic moiety.

17 Different methodologies have been used to form polymer based giant unilamellar vesicles  
18 (GUVs). They have been mainly inspired from those developed for lipids [34]. Electroformation  
19 method is the most employed one. It is a solvent-free method where a dry amphiphilic thin film  
20 is hydrated in the presence of an alternating current probably inducing fluctuations and interlayer  
21 repulsions, which favours the swelling and release of giant vesicles [35]. It was applied to block-  
22 copolymers for the first time in the case of poly(ethylene oxide)-*block*-poly(ethyl ethylene) [36].  
23 Electroformation can be applied to a limited class of block-copolymers [33,37,38], as the choice

1 of the nature of the block copolymer is usually dictated by the hydrophobic block glass transition  
2 temperature, since this should be as low as possible in order to guarantee a sufficient flexibility  
3 and mobility of the polymer chains. Gentle hydration has been described even earlier than  
4 electroformation for brain phospholipids [39] and is quite difficult to be applied to block-  
5 copolymers as witnessed by the very limited number of publications [40–42]. Gel assisted  
6 hydration using polyvinyl alcohol (PVA) [43] or agarose [44] have been proposed for lipid  
7 vesicles and PVA was also used for polymer-based vesicles [45]. In this case, the presence of  
8 unwanted impurities in the bilayer or lumen cannot be excluded.

9 Emulsion phase (or droplet) transfer has been developed for lipids [46], improved in  
10 microfluidic devices [47,48] or special centrifugation set-ups [49–51] (continuous droplet  
11 interface crossing encapsulation, i.e. cDICE) and has been rarely used for block copolymers  
12 [21,52]. This method is based on a water-in-oil emulsion where water droplets are coated with  
13 the amphiphilic species. These droplets are allowed to cross the interface of an oil-on-water  
14 biphasic system stabilized by an amphiphilic monolayer, thus forming the vesicles. A known  
15 drawback of this method is the presence of residual oil between the lipid leaflets [53]. However,  
16 membrane bending rigidity analysis revealed that mineral oil doesn't affect the mechanical  
17 properties of the membrane [54].

18 Because hybrid GUVs are essential tools for developing synthetic reactors or biomimetic cells  
19 and are so far very limited, in this work we propose to use block copolymers based on  
20 methylmethacrylate (MMA) and N,N-dimethylaminoethyl methacrylate (DMAEMA) mixed  
21 with palmitoyl oleyl phosphatidyl choline (POPC) in order to obtain giant hybrid vesicles. The  
22 presence of pH-sensitive DMAEMA unit could provide the access to systems with pH-  
23 responsive permeability. In the literature MMA/DMAEMA based block-copolymers have been

1 mainly used to develop nano-sized polymersomes. Polymeric nano-vesicles based on butyl-  
2 methacrylate (BMA) and DMAEMA were developed as reactors for the urea-urease system, for  
3 example [55]. We recently showed that the copolymer poly(ethylene glycol monomethyl ether)-  
4 *block*- poly( methyl methacrylate –*random*- N,N-dimethyl amino ethyl methacrylate), mPEG-  
5 *block*-P(MMA-*ran*-DMAEMA), was able to form nano-vesicles in a wide range of DMAEMA  
6 chemical compositions and architectures (linear and branched) [56]. Furthermore, they exhibited  
7 a pH-dependent swelling characterized by a strong increase in size, up to 10 times passing from  
8 pH 7.4 to pH 4.4. This swelling was attributed to electrostatic repulsions at low pH, linked to the  
9 concurrent increase of protonated DMAEMA units.

10 As for giant vesicles, which were not necessarily unilamellar ones, poly(methacrylic acid)-  
11 *block*-poly(methylmethacrylate-*random*-methacrylic acid), PMAA-*block*-P(MMA-*ran*-MAA)  
12 could form giant vesicles in a photo-polymerization induced self-assembly process in an aqueous  
13 methanol solution [57,58]. The authors investigated the pH-responsive behaviour of the vesicles  
14 showing that they were disrupted in basic environment (pH 12) and they could be reversibly  
15 reconstructed at neutral pH. Yoshida also characterized polymeric giant vesicles with  
16 copolymers containing DMAEMA units inserted in the P(MMA-*ran*-MAA) block [59]. These  
17 vesicles were unstable and had many holes on the surface. When quaternized DMAEMA was  
18 used the vesicles were stable, but they had much smaller size (below 1  $\mu\text{m}$ ).

19 Hybrid GUVs made of poly(cholesteryl methacrylate)-*block*-poly(2-(dimethylaminoethyl  
20 methacrylate) (pCMA-*block*-pDMAEMA) were characterized with confocal microscopy by W.  
21 Zong et al. [16] to confirm the presence of both phospholipids and the block copolymer in the  
22 same membrane, pCMA constitutes the hydrophobic block, while the pH-sensitive pDMAEMA  
23 constitutes the hydrophilic block. These GUVs were electroformed and the maximum polymer



1 content was 70% w/w (corresponding to 3.6 mol % using  $M_n=47.5$  kD as reported in Table 1 of  
2 the cited work). Also, hybrid nano-vesicles were formed by this copolymer mixed with  
3 phospholipids containing palmitoyl and oleyl as fatty acyl groups and different charged  
4 headgroups (i.e. phosphatidylcholine, ethylphosphocholine and phosphatidylserine) showed a  
5 prevalent cytosolic localization when incubated with mouse macrophages, indicating a good  
6 potential for applications in drug delivery [16].

7 In this paper, we want to show that a block copolymer with high molecular weight and high  $T_g$   
8 can be successfully incorporated into hybrid giant vesicles. We propose to use mPEG-*block*-  
9 P(MMA-*grad*-DMAEMA) in combination with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-  
10 phosphocholine (POPC) to prepare vesicles by means of two formation methods, namely  
11 electroformation and droplet transfer; it will be shown that the high  $T_g$  polymer diminishes the  
12 efficiency of the electroformation method even at low ( $\sim 15$  mol %) polymer content, whilst the  
13 droplet transfer method allows the formation of hybrid vesicles for a wide range of polymer/lipid  
14 ratios. With this approach, the polymer content in the hybrid GUVs has been sensibly increased  
15 with respect to that already reported in the literature ( $\sim 4$  mol %).

## 16 2. MATERIALS AND METHODS

### 17 *Materials*

18 Copper bromide ( $\text{CuBr}_2$ ), 2,2'-bipyridine (bpy), tin (II) 2-ethylhexanoate ( $\text{Sn}(\text{EH})_2$ ), 2,4-  
19 dimethyl-3-ethylpyrrole, trifluoroacetic acid (TFA), dichlorodicyanoquinone (DDQ),  
20 triethylamine ( $\text{Et}_3\text{N}$ ), boron trifluoride etherate ( $\text{BF}_3\cdot\text{Et}_2\text{O}$ ), butylated hydroxytoluene (BHT),  
21 basic alumina, Sephadex LH-20, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, Mineral oil  
22 M5904, toluene, methanol, dimethylformamide (DMF), n-hexane, poly(ethylene glycol)

1 monomethylether (mPEG,  $M_n = 2000 \text{ g mol}^{-1}$ ,  $M_w/M_n = 1.16$  and  $M_n = 5000 \text{ g mol}^{-1}$ ,  $M_w/M_n =$   
2 1.02) and pyranine were purchased from Sigma-Aldrich and used without further purification.  
3 18:1 Liss Rhodamine PE was used as received by Avanti Polar Lipids. Methylmethacrylate  
4 (MMA) and N,N-(dimethylamino)ethylmethacrylate (DMAEMA) (Sigma-Aldrich) were purified  
5 prior copolymerizations by means of a column filled with basic alumina to remove the inhibitors.  
6 Dichloromethane (Sigma-Aldrich) used for BODIPY synthesis was distilled over  $\text{CaCl}_2$ .

7 All manipulations involving air-sensitive compounds were carried out under nitrogen  
8 atmosphere using Schlenk techniques.

9 *Synthesis of the fluorescent-labeled DMAEMA monomer 2,6-diethyl-1,3,5,7-tetramethyl-8-[4-*  
10 *[8-(2-methacryloyloxyethyl)dimethylammoniumbromideoctyloxy]phenyl]-4,4'-difluoroboradiaza*  
11 *indacene (BODIPY-DMAEMA)*

12 As a first step, the precursor 2,6-diethyl-1,3,5,7-tetramethyl-8-[4-(8-bromooctyloxy)phenyl]-4,4'-  
13 difluoroboradiaza indacene was synthesized. 4-(8-bromooctyloxy)benzaldehyde was prepared as  
14 previously described [60]. 720  $\mu\text{L}$  of 4-(8-bromooctyloxy)benzaldehyde (2.3 mmol) and 756  $\mu\text{L}$   
15 of 2,4-dimethyl-3-ethylpyrrole (5.6 mmol) were then dissolved in 50 mL of absolute  $\text{CH}_2\text{Cl}_2$   
16 under  $\text{N}_2$  atmosphere, ten drops from a Pasteur pipette of TFA were added and the solution was  
17 stirred at RT overnight or until TLC analysis showed complete consumption of the aldehyde. At  
18 this time, 785 mg of DDQ (3.45 mmol) were added and stirring continued for 20 min. Then, 5 mL  
19 of  $\text{Et}_3\text{N}$  and 5 mL of  $\text{BF}_3 \cdot \text{OEt}_2$  were added. The mixture was stirred for 12 h and the organic layer  
20 containing the crude product was subsequently washed three times with water; the organic  
21 solution was dried over  $\text{Na}_2\text{SO}_4$ , and evaporated to dryness. The raw material was purified by  
22 chromatography ( $\text{SiO}_2$ , petroleum ether -  $\text{CH}_2\text{Cl}_2$ , 1:1) to afford 246 mg of product (yield: 18.2%)

1 in the form of orange needles.  $C_{31}H_{42}BBrF_2N_2O$ , MM = 587.39  $g\text{mol}^{-1}$ , UV-vis ( $CH_2Cl_2$ ): 528 nm  
2 ( $\epsilon = 92600\text{ M}^{-1}\text{ cm}^{-1}$ ). Quantum efficiency of fluorescence  $\Phi_{\text{fluo}}$  (in  $CH_2Cl_2$ ): 0.45 (544 nm) was  
3 calculated according to the following equation:

$$4 \quad \Phi_{\text{fluo}}^{\text{sample}} = \Phi_{\text{fluo}}^{\text{standard}} \times (I_{\text{fluo}}^{\text{sample}} / I_{\text{fluo}}^{\text{standard}}) \times (\text{Abs}^{\text{standard}} / \text{Abs}^{\text{sample}})$$

5 where  $I_{\text{fluo}}$  is the fluorescence intensity at the specific excitation wavelength, Abs denotes the  
6 absorbance at the excitation wavelength.

7  $^1\text{H}$  NMR ( $CDCl_3$ )  $\delta$ : 1.00 (t, 6H,  $2 \times CH_3$ ); 1.46 (s, 6H,  $2 \times CH_3$ ); 1.45-1.55 (m, 8H,  $4 \times CH_2$ );  
8 1.82-1.93 (m, 4H,  $2 \times CH_2$ ); 2.32 (q, 4H,  $2 \times CH_2$ ); 2.58 (s, 6H,  $2 \times CH_3$ ); 3.45 (t, 2H,  $CH_2Br$ ); 4.03  
9 (t, 2H,  $CH_2O$ ); 6.00 (s, 2H,  $2 \times CH$ ); 7.02 (d, 2H,  $2 \times CH$ ); 7.18 (d, 2H,  $2 \times CH$ ).

10 To obtain the fluorescent-labeled DMAEMA monomer, 10 mg (0.045 mmol) of 2,6-diterbutyl-  
11 4-methylphenol (BHT) were added to a mixture of 236 mg of 2,6-diethyl-1,3,5,7-tetramethyl-8-  
12 [4-(8-bromooctyloxy)phenyl]-4,4'-difluoroboradiazindacene (0.402 mmol) in 3 mL of  
13 DMAEMA (17.75 mmol), reaction scheme and chemical structures are reported in Figure S1 of  
14 the supporting information. The mixture was kept at 70  $^{\circ}\text{C}$  for 24 h. During the reaction time, a  
15 solid product was formed. After filtration, the product was washed 4 times with 20 mL of diethyl  
16 ether affording 214 mg (71.4%) of product.  $C_{39}H_{57}BBrF_2N_3O_3$ , MM = 744.60  $g\text{mol}^{-1}$  (ESI-MS  
17 spectrum is reported in Figure S2 of the supporting information).  $^1\text{H}$  NMR ( $CDCl_3$ )  $\delta$ : 0.97 (t,  
18 6H,  $2 \times CH_3$ ); 1.33 (s, 6H,  $2 \times CH_3$ ); 1.40 (m, 8H,  $4 \times CH_2$ ); 1.80 (m, 4H,  $2 \times CH_2$ ); 1.95 (s, 3H,  
19  $CH_3$ ), 2.25-2.33 (q, 4H,  $2 \times CH_2$ ); 2.51 (s, 6H,  $2 \times CH_3$ ); 3.56 (s, 6H,  $2 \times CH_3$ ); 3.61-3.65 (t, 2H,  
20  $CH_2$ ); 3.97-4.02 (t, 2H,  $CH_2$ ); 4.15 (t, 2H,  $CH_2$ ); 4.65 (t, 2H,  $CH_2$ ); 5.68 (s, 1H, CH); 6.15 (s, 1H,  
21 CH); 6.96-6.99 (d, 2H,  $2 \times CH$ ); 7.13-7.15 (d, 2H,  $2 \times CH$ ). Details of  $^1\text{H}$  peak assignment and  
22 HSQC-NMR spectra are reported in Figures S3 – S5 of the supporting information.

23 *Synthesis of mPEG-block-P(MMA-grad-DMAEMA) copolymers.*

1 Macroinitiator mPEG-Br was synthesized according to the procedure reported in literature. [61]  
2 In a typical procedure, the copolymerization of MMA/DMAEMA was carried out in a 50 mL  
3 glass flask charged, under nitrogen atmosphere, with 0.1 g of mPEG-Br macroinitiator in 5 mL  
4 of toluene. Subsequently, 100  $\mu$ L of CuBr<sub>2</sub> ( $1 \times 10^{-3}$  M in DMF,  $10^{-4}$  mmol), 100  $\mu$ L of bpy ( $1 \times$   
5  $10^{-2}$  M in DMF,  $10^{-3}$  mmol), 100  $\mu$ L of Sn(EH)<sub>2</sub> ( $1 \times 10^{-2}$  M in toluene,  $10^{-3}$  mmol), 2-4 mL of  
6 MMA (18.8-37.6- mmol) and 1.2 – 2.5 mL of DMAEMA (7.10 – 14.8 mmol) were added (the  
7 volumes of MMA and DMAEMA were varied to change the final composition in the  
8 copolymer). The mixture was thermostated at 60 °C and magnetically stirred. The reaction was  
9 stopped after 4h by adding *n*-hexane. The copolymer was recovered by filtration, washed with  
10 cold methanol (~ 4 °C) and dried in vacuum at 40 °C. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  0.87-1.03  
11 (-CH<sub>3</sub> main chain), 1.83-1.91 (-CH<sub>2</sub>- main chain), 2.30 (-N(CH<sub>3</sub>)<sub>2</sub>), 2.58 (-OCH<sub>2</sub> CH<sub>2</sub> N(CH<sub>3</sub>)<sub>2</sub>),  
12 3.61 (-OCH<sub>3</sub>), 3.66 (-OCH<sub>2</sub>CH<sub>2</sub>-), 4.08 (-OCH<sub>2</sub> CH<sub>2</sub> N(CH<sub>3</sub>)<sub>2</sub>). <sup>13</sup>C-NMR(400 MHz, CDCl<sub>3</sub>):  $\delta$   
13 16.9-19.1 (-CH<sub>3</sub> main chain), 44.9-45.3 (quaternary carbon in the main chain), 46.2 (-N(CH<sub>3</sub>)<sub>2</sub>),  
14 52.2 (-OCH<sub>3</sub>, MMA), 54.6 (-CH<sub>2</sub>- main chain), 57.6 (-OCH<sub>2</sub> CH<sub>2</sub> N(CH<sub>3</sub>)<sub>2</sub>), 63.5 (-OCH<sub>2</sub> CH<sub>2</sub>  
15 N(CH<sub>3</sub>)<sub>2</sub>), 70.9 (-OCH<sub>2</sub> CH<sub>2</sub>-), 176.3-178.2 (-C=O). Details of <sup>1</sup>H peak assignment and <sup>13</sup>C NMR  
16 spectra are reported in Figure S6 of the supporting information.

17 Chemical composition and the molecular weight ( $M_n$ ) were evaluated via <sup>13</sup>C NMR. The molar  
18 fractions of the components in the final copolymers were evaluated through the equations:

$$19 \quad X_{\text{mPEG}} = \frac{I_{\text{mPEG}}}{I_{\text{mPEG}} + 2I_{\text{MMA}} + I_{\text{DMAEMA}}} \quad (1)$$

$$20 \quad X_{\text{MMA}} = \frac{2I_{\text{MMA}}}{I_{\text{mPEG}} + 2I_{\text{MMA}} + I_{\text{DMAEMA}}} \quad (2)$$

$$X_{\text{DMAEMA}} = \frac{I_{\text{DMAEMA}}}{I_{\text{mPEG}} + 2I_{\text{MMA}} + I_{\text{DMAEMA}}} \quad (3)$$

where  $I_{\text{mPEG}}$  is the integration of the signal relative to mPEG units:  $-\text{OCH}_2\text{CH}_2-$ ,  $I_{\text{MMA}}$  is the signal corresponding to the units  $-\text{OCH}_3$  of MMA and  $I_{\text{DMAEMA}}$  integrates the two carbons of the amine group of DMAEMA ( $-\text{N}(\text{CH}_3)_2$ ).  $M_n$  was calculated from the following equation:

$$M_n = \text{DP}_{\text{mPEG}} \text{MM}(\text{EO}) + \text{DP}_{\text{MMA}} \text{MM}(\text{MMA}) + \text{DP}_{\text{DMAEMA}} \text{MM}(\text{DMAEMA}) \quad (4)$$

where:

$$\text{DP}_{\text{mPEG}} = M_{\text{nPEG}}/44$$

$$\text{DP}_{\text{MMA}} = \text{DP}_{\text{mPEG}} (2I_{\text{MMA}}/I_{\text{mPEG}})$$

$$\text{DP}_{\text{DMAEMA}} = \text{DP}_{\text{mPEG}} (I_{\text{DMAEMA}}/I_{\text{mPEG}})$$

The synthesis of the fluorescent-labelled copolymer was carried out under the same experimental conditions described above by adding 2 mL of MMA (18.8 mmol), 1.2 mL of DMAEMA (7.10 mmol), 2 mL of DMF and 5 mg of the fluorescent monomer BODIPY-DMAEMA ( $6.7 \times 10^{-3}$  mmol) to the reaction mixture. The applied procedure of purification [62,63] was based on the use of lipophilic Sephadex LH-20 to separate molecules with different molecular weights. A mini column (Pasteur pipette) was loaded with the lipophilic stationary phase ( $\sim 1.5$  g of Sephadex previously swollen for one hour in 10 mL of methanol) and filled with methanol to elute the samples.  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  0.87-1.04 ( $-\text{CH}_3$  main chain), 1.27 ppm (6  $\text{CH}_2$  of the alkyl chain of the BODIPY-DMAEMA), 1.83-1.91 ( $-\text{CH}_2-$  main chain), 2.30 ( $-\text{N}(\text{CH}_3)_2$ ), 2.59 ( $-\text{OCH}_2 \text{CH}_2 \text{N}(\text{CH}_3)_2$ ), 3.61 ( $-\text{OCH}_3$ ), 3.65 ( $-\text{OCH}_2\text{CH}_2-$ ), 4.08 ( $-\text{OCH}_2 \text{CH}_2 \text{N}(\text{CH}_3)_2$ ).  $^{13}\text{C-NMR}$  (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  16.5-18.7 ( $-\text{CH}_3$  main chain), 29.7 ppm (6  $\text{CH}_2$  of the

1 alkyl chain of the BODIPYDMAEMA), 44.6-44.9 (quaternary carbon in the main chain), 45.8 (-  
 2 N(CH<sub>3</sub>)<sub>2</sub>), 51.8 (-OCH<sub>3</sub>, MMA), 54.4 (-CH<sub>2</sub>- main chain), 57.2 (-OCH<sub>2</sub> CH<sub>2</sub> N(CH<sub>3</sub>)<sub>2</sub>), 63.1 (-  
 3 OCH<sub>2</sub>CH<sub>2</sub> N(CH<sub>3</sub>)<sub>2</sub>), 70.6 (-OCH<sub>2</sub>CH<sub>2</sub>-), 176.3-178.2 (-C=O). Details of <sup>1</sup>H peak assignment,  
 4 <sup>13</sup>C NMR and HSQC-NMR spectra are reported in Figures S7 and S8 of the supporting  
 5 information.

6 Chemical composition and the molecular weight (M<sub>n</sub>) were evaluated via <sup>13</sup>C NMR through  
 7 the equations:

$$8 \quad X_{\text{mPEG}} = \frac{I_{\text{mPEG}}}{I_{\text{mPEG}} + 2I_{\text{MMA}} + I_{\text{DMAEMA}} + \frac{I_{\text{BODIPYDMAEMA}}}{2}} \quad (5)$$

$$9 \quad X_{\text{MMA}} = \frac{2I_{\text{MMA}}}{I_{\text{mPEG}} + 2I_{\text{MMA}} + I_{\text{DMAEMA}} + \frac{I_{\text{BODIPYDMAEMA}}}{2}} \quad (6)$$

$$10 \quad X_{\text{DMAEMA}} = \frac{I_{\text{DMAEMA}}}{I_{\text{mPEG}} + 2I_{\text{MMA}} + I_{\text{DMAEMA}} + \frac{I_{\text{BODIPYDMAEMA}}}{2}} \quad (7)$$

$$11 \quad X_{\text{BODIPYDMAEMA}} = \frac{\frac{I_{\text{BODIPYDMAEMA}}}{2}}{I_{\text{mPEG}} + 2I_{\text{MMA}} + I_{\text{DMAEMA}} + \frac{I_{\text{BODIPYDMAEMA}}}{2}} \quad (8)$$

12 where  $I_{\text{mPEG}}$  is the integration of the signal relative to mPEG units: -OCH<sub>2</sub>CH<sub>2</sub>-;  $I_{\text{MMA}}$  is the  
 13 integration of the signal relative to MMA units: -OCH<sub>3</sub>;  $I_{\text{DMAEMA}}$  is the integration of methyl  
 14 group of the signal relative to DMAEMA units;  $I_{\text{BODIPYDMAEMA}}$  are the 4 CH<sub>2</sub> of the fluorescent  
 15 moiety (signals labeled i<sub>1-4</sub> in the NMR spectra, figures S7-S8 of the supporting info). The

1 degrees of polymerization and hence the molecular weights were calculated from the following  
2 equation:

$$3 \quad M_n = DP_{mPEG} (MM_{EO}) + DP_{MMA} (MM_{MMA}) + \quad (9)$$
$$+ DP_{DMAEMA} (MM_{DMAEMA}) + DP_{BODIPYDMAEMA} (MM_{BODIPYDMAEMA})$$

4 where:

$$DP_{mPEG} = M_{n(mPEG)} / 44$$
$$5 \quad DP_{MMA} = DP_{mPEG} (2I_{MMA} / I_{mPEG})$$
$$DP_{DMAEMA} = DP_{mPEG} (I_{DMAEMA} / I_{mPEG})$$
$$DP_{BODIPYDMAEMA} = DP_{mPEG} (I_{BODIPYDMAEMA} / 2I_{mPEG})$$

6 *NMR Analysis.*

7  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded at 25 °C in  $\text{CDCl}_3$  using a Bruker Avance 400 MHz  
8 spectrometer ( $D_1 = 5$  s for  $^{13}\text{C}$  NMR). Samples were prepared by introducing 20 mg of  
9 copolymer and 0.5 mL of  $\text{CDCl}_3$  into a tube (5 mm outer diameter). Chemical shifts ( $\delta$ ) are listed  
10 as parts per million:  $^1\text{H}$  NMR spectra are referenced using the residual solvent peak at  $\delta = 7.26$   
11 ppm, in  $^{13}\text{C}$  NMR spectra the residual solvent peak is at  $\delta = 77.2$  ppm.

12

13

14 *DSC.*

15 The glass transition temperatures were measured with Dynamic Scanning Calorimetry (Mettler  
16 Toledo DSC1 STAR SYSTEM FRS5). About 10 mg of sample were inserted in a 100  $\mu\text{L}$   
17 aluminum pan and heating/cooling cycles were registered at 20 K/min. In the first run, the

1 sample was heated from 25 °C to 165 °C, followed by cooling to -60 °C. In the other two runs,  
2 the sample was heated from -60 °C to 165 °C and cooled from 165 °C to -60 °C. The  $T_g$  was  
3 measured on the second heating run where the inflection point is generally more evident.

#### 4 *Microscopy.*

5 Images were acquired on an inverted optical microscope (Eurotek Orma INV100TFL) using a  
6 20× objective and then analysed with the software ImageJ. The mixed membranes were  
7 characterized with a confocal microscope (Leica TCS SP2 and TIRF OLYMPUS FV1000). The  
8 fluorescent polymer and the fluorescent lipid were excited respectively at 488 nm, with an Ar  
9 laser, and at 561 nm, with a DPSS 561 laser and the fluorescence was collected with  
10 photomultiplier tubes PMTs in the wavelength ranges 498 – 530 nm and 571 – 630 nm,  
11 respectively.

#### 12 *Preparation of Giant Vesicles.*

13 *Droplet Transfer Method.* Stock solutions of [POPC] = 3 mM (2.28 mg mL<sup>-1</sup>) and [copolymer]  
14 = 5 mg mL<sup>-1</sup> (0.36 mM by using the copolymer average molecular weight  $M_n = 13850$  g mol<sup>-1</sup>)  
15 were prepared in mineral oil. The fluorescent probes, being less soluble in mineral oil, were  
16 dissolved in CHCl<sub>3</sub> at the concentration [fluo-polymer]<sub>CHCl<sub>3</sub></sub> = 10 mg mL<sup>-1</sup> (0.027 mM using an  
17 average molecular weight  $M_n = 370928$  g mol<sup>-1</sup>) and [18:1 Liss Rhod PE]<sub>CHCl<sub>3</sub></sub> = 0.77 mM (1 mg  
18 mL<sup>-1</sup>). Giant Unilamellar vesicles were prepared with the Droplet Transfer Method described by  
19 Pautot et al. for lipid-based GUVs [46] using lipid:copolymer molar ratios 93:7, 85:15 and 60:40  
20 (corresponding to lipid:copolymer weight ratios 0.42:0.58, 0.24:0.76, 0.08: 0.92 by using the  
21 copolymer average molecular weight  $M_n = 13850$  g mol<sup>-1</sup>). An Eppendorf tube was filled with  
22 500 μL of an aqueous phase containing 200 mM of glucose (O-solution), then 300 μL of a



1 hydrophobic interfacial phase containing lipids and polymers at various ratios (obtained by  
2 mixing the POPC and polymer stock solution) was poured on top of the O-solution. A second  
3 Eppendorf tube was used to prepare a water/oil microemulsion: 20  $\mu\text{L}$  of an aqueous solution  
4 containing 200 mM of sucrose (inner solution, I-solution), were mixed by pipetting with 600  $\mu\text{L}$   
5 of an oil phase containing the same amount of lipids and polymers as the interfacial phase and 5  
6  $\mu\text{L}$  of chloroform solutions of fluorescent probes (chloroform was firstly evaporated in argon  
7 stream ~~before~~ and then oil containing other amphiphiles was added). The microemulsion was  
8 then poured over the interfacial phase and vesicles were formed by centrifuging the tube at 6000  
9 rpm for 10 minutes at room temperature. Vesicles were collected as a pellet at the bottom of the  
10 aqueous phase and gently washed with 100  $\mu\text{L}$  of O-solution and finally observed at the  
11 microscope. In these experimental conditions, however, pure polymeric vesicles could not be  
12 formed. When POPC was not present in the initial oil solution, the precipitation of a drop of  
13 coloured polymer (i.e. containing the fluorescent polymeric probe or pyranine when employed)  
14 was always detected at the bottom of the Eppendorf tube. Problems might be related with  
15 difference in density between the I- and O-solution that could be overcome by employing a  
16 different nonpolar solvent or by changing the concentrations of the sugars.

17 *Electroformation.* GUVs were prepared on a *Vesicles prep pro* instrument produced by Nanion  
18 through the electroformation method described by *Angelova et al.* [64] for lipid-based giant  
19 vesicles. Stock solution of [POPC] = 1.32 mM (1 mg mL<sup>-1</sup>) and [copolymer] = 1 mg mL<sup>-1</sup> (0.072  
20 mM if we consider a copolymer average weight of 13850 g/mol) and POPC were prepared at 1  
21 mg mL<sup>-1</sup> (both polymer and lipid) in chloroform and [copolymer] = 1 mg mL<sup>-1</sup> (0.072 mM by  
22 using the copolymer average molecular weight  $M_n = 13850 \text{ g mol}^{-1}$ ). For electroformation, they  
23 were mixed in different ratios so that the total final concentration of the polymer plus POPC was

1 0.25 mg mL<sup>-1</sup> and the total deposited amount was fixed in order to avoid the formation of a thick  
2 film. The POPC concentration was 0.136 mM, 0.08 mM and 0.05 mM in the samples with  
3 POPC:copolymer molar ratios equal to 93:7, 85:15 and 70:30 respectively (corresponding to  
4 lipid:copolymer weight ratios 0.42:0.58, 0.24: 0.76, 0.11: 0.89 by using the copolymer average  
5 molecular weight  $M_n = 13850 \text{ g mol}^{-1}$ ).

6 5  $\mu\text{L}$  of the obtained chloroform solutions of the polymer and lipid at different molar ratios  
7 were spread onto ITO-coated glass slide and dried under vacuum for at least 3 hours. The dry  
8 film was surrounded with a 1 mm O-ring in order to delimit the electroformation chamber which  
9 was subsequently filled with 250  $\mu\text{L}$  of a 240 mM sucrose solution. Another ITO-coated glass  
10 slide was used to close the chamber, which was further connected to an alternate voltage  
11 generator. A peak-to-peak voltage of 3 V and a frequency of 10 Hz were applied at 65 °C for at  
12 least 2 hours to form the GUVs. The temperature and the voltage were then slowly decreased and  
13 the vesicles were collected with a pipette.

### 14 3. RESULTS AND DISCUSSION

15 A mandatory condition that has to be satisfied to control the formation, the stability and the  
16 characteristics of mixed polymer/lipid vesicles is the similarity of the solubility parameter ( $\delta$ )  
17 [65,66] of the phospholipids' fatty acids chains with the hydrophobic part of the amphiphilic  
18 synthetic copolymer. In this respect, the comparable values of PMMA ( $\delta = 18.7 \text{ MPa}^{1/2}$  [67])  
19 with that of the fatty acid chains ( $\delta = 18.2 \text{ MPa}^{1/2}$  [20]), allows us to consider as good the  
20 compatibility between an amphiphilic block copolymer containing PMMA and the phospholipid  
21 chosen for the formation of the hybrid vesicles (POPC).

1     However, a rational design in terms of molecular weight, composition and  
2     hydrophilic/hydrophobic ratio is also needed since one of the main issue for the formation of  
3     hybrid vesicles also includes the similitude or the discrepancy of the size of the hydrophobic  
4     segments in the copolymers and phospholipids, other than a proper glass transition temperature  
5     ( $T_g$ ) of the synthetic copolymer [20].

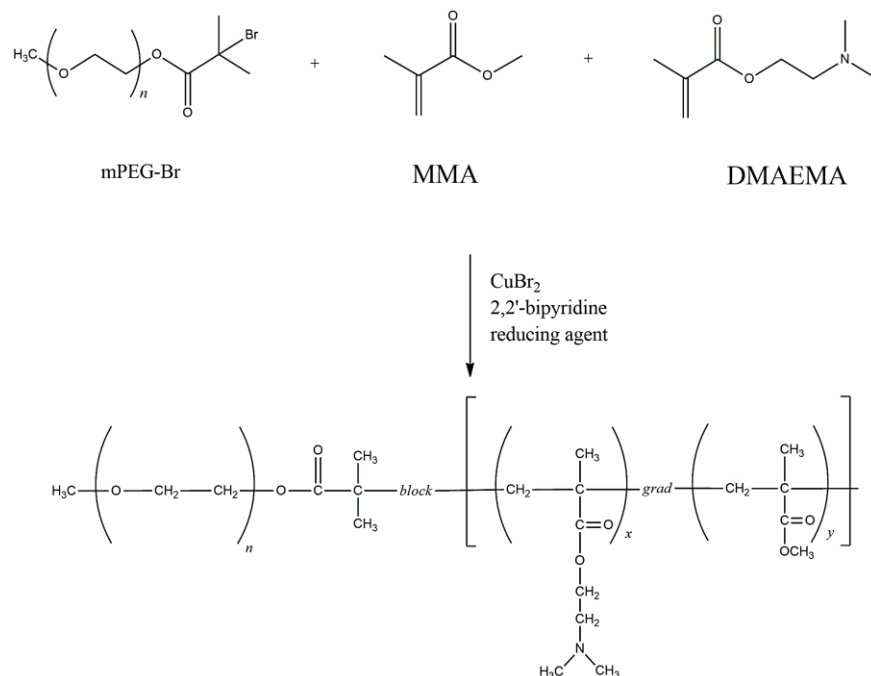
6     To this end, several MMA-based block copolymers having different properties ( $M_n$ ,  
7     Hydrophilic-Lipophilic Balance HLB, etc.) were synthesized by a modified version of the  
8     ATRP, namely the Activators ReGenerated by Electron Transfer ATRP (ARGET-ATRP)  
9     [68,69].

10    The copolymers have a linear, *A-block*-(BC), architecture and consist of a hydrophilic block  
11    (A) based on poly(ethylene glycol) monomethylether (mPEG), and a hydrophobic block (BC)  
12    made of a copolymer chain containing methyl methacrylate (MMA, B) and N,N-  
13    (dimethylamino)ethyl methacrylate (DMAEMA, C) as represented in Figure 1.

14    The DMAEMA monomer was introduced both to modulate the PMMA  $T_g$ , and to give  
15    potential pH-sensitivity for further applications to the hybrid vesicles [56,70–72]. However,  
16    though the chain based on MMA/DMAEMA has an increasing hydrophilicity with decreasing  
17    pH, at pHs ranging from neutrality to basic conditions, the hydrophobicity, and consequently the  
18     $\delta$  value, remains compatible with the POPC fatty acid chain.

19    Copolymers were thus synthesized using mPEG-Br ( $M_n = 2000 \text{ g mol}^{-1}$  and  $M_n = 5000 \text{ g mol}^{-1}$ )  
20    as macroinitiator in presence of  $\text{CuBr}_2/\text{bpy}$  and  $\text{Sn}(\text{EH})_2$  as reducing agent in toluene at  $60 \text{ }^\circ\text{C}$   
21    (Figure 1). The experimental conditions, meaning relative amount of MMA and DMAEMA in

- 1 the feed, were systematically changed in order to find the most suitable microstructure for the  
 2 formation of mixed vesicles with POPC.



3  
 4 **Figure 1. Reaction scheme for the synthesis of mPEG-*block*-P(MMA-*grad*-DMAEMA) copolymers**

5 The large difference in the reactivity ratios found between MMA and DMAEMA  
 6 suggested that the “BC” block is characterized by a *gradient-like* composition with the initial  
 7 part of the chain (i.e. the one closer to the mPEG block) richer in the monomer DMAEMA, that  
 8 smoothly changes towards a prevalent MMA composition [73]. This microstructure in principle  
 9 could favour the formation of blended vesicles allowing the insertion of the hydrophobic moiety  
 10 of the copolymer within the phospholipid carbon chains. The characteristics of all copolymers  
 11 synthesized are reported in Table 1.

12 **Table 1. Chemical composition, molar masses, HLB and  $T_g$  of the copolymers synthesized**

Sample	$M_n$ mPEG	$X_{\text{mPEG}}$ (mol)	$X_{\text{MMA}}$ (mol)	$X_{\text{DMAEMA}}$ (mol)	$M_n^a$ ( $\text{kg mol}^{-1}$ )	HLB <sup>b</sup>	$T_g^c$ ( $^{\circ}\text{C}$ )
--------	------------	----------------------------	---------------------------	------------------------------	-------------------------------------	------------------	-----------------------------------

1	2000	0.24	0.76	---	16.3	2.45	97
2	5000	0.14	0.51	0.35	62	1.61	85
3	5000	0.44	0.35	0.21	23	4.35	50
4	5000	0.50	0.27	0.23	19.4	5.15	55
5	2000	0.34	0.26	0.40	13.8	2.90	44

1 <sup>a</sup> Determined by <sup>13</sup>C-NMR using equation (4)

2 <sup>b</sup> Calculated by Griffin method:  $HLB = 20(M_h/M)$  where  $M_h$  is the molar mass of the hydrophilic portion (mPEG)  
3 and  $M$  is the molar mass of the copolymer

4 <sup>c</sup> heating rate 20 K/min

5

6 Considering that the  $T_g$  of the homopolymers is  $\sim -65$  °C for PEG,  $\sim 105$  °C for PMMA  
7 and  $\sim 20$  °C for PDMAEMA, data reported in Table 1 show that the lower is the content of  
8 MMA and the value of molecular weight (e.g. samples 3 – 5), the lower is the  $T_g$  of the  
9 corresponding copolymer. Among the copolymers in Table 1, we selected the one with a molar  
10 composition of 34% – 26% – 40% of mPEG – MMA – DMAEMA, respectively and  $M_n = 13.8$   
11  $\text{kg mol}^{-1}$  (sample 5) to produce hybrid GUVs using the electroformation method. This copolymer  
12 has the lowest  $T_g$  and average molecular weight together with a HLB (hydrophilic-lipophilic  
13 balance) value similar to the one of POPC (3.98), it then appears the most suitable for the  
14 preparation of hybrid vesicles. It is reasonable that the HLB value together with a low steric  
15 mismatch between copolymers and POPC molecular weight should ensure a major compatibility  
16 with the lipid. Besides, as it has the lowest  $T_g$  value is the best candidate for electroformation  
17 where the higher the polymer chain mobility the higher the yield of vesicle formation.

18 Giant vesicles, containing different percentages of lipid and copolymer were produced by  
19 using two different processes: an electroformation and a phase transfer method.

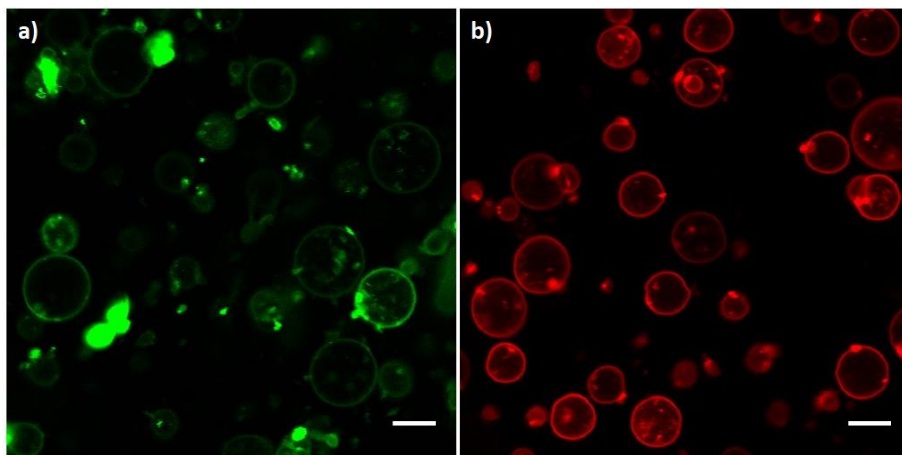
20 As copolymer 5 did not form pure GUVs by electroformation, we decided to explore  
21 compositions with high molar amount of POPC. A large number of electroformed POPC giant

1 vesicles could be easily obtained within 30 minutes at room temperature, as reported in Figure  
2 S9 a) of the Supporting Information. Even a small amount of copolymer (< 5 mol%) added to  
3 POPC inhibited the vesicle formation at 25 °C and the temperature had to be increased till 65 °C  
4 in order to be able to obtain a few vesicles after 2 hours, as shown in Figure S9 b) in the  
5 Supporting Information. The maximum initial copolymer content clearly allowing vesicles  
6 formation was 15 mol%, though the typical microscopy images showed few electroformed  
7 aggregated vesicles (often containing solid residues of presumably precipitated polymers and/or  
8 lipids), which hardly detached from the film. In the presence of higher initial percentage of  
9 polymer, only scant irregular objects could be detected.

10 These results clearly show that the presence of the polymer, even at low percentage (< 15  
11 mol%), with a high glass transition temperature hinders POPC vesicles electroformation. For this  
12 reason we decided to use the phase transfer method, developed for giant liposomes and  
13 previously employed for the synthesis of asymmetric hybrid polymer-lipid giant vesicles [21].  
14 With this method, the same amount of copolymer and lipid was dispersed in both the interfacial  
15 phase and in the emulsion in order to favour a symmetric composition of the two leaflets of the  
16 bilayer. In order to get insight into the nature of the formed vesicles, two fluorescent probes were  
17 employed to highlight the possible partitioning of the two different components, lipid and  
18 copolymer, within the hybrid GUVs. A fluorescent lipid, 18:1 Liss Rhod PE, known for its  
19 affinity for the liquid disordered domains in phase separated model membranes [74] and also  
20 used as a marker for the lipid-rich region in hybrid vesicles [75–78], was chosen. To stain the  
21 polymer-rich domains, a modified DMAEMA with a pendant group consisting of a BODIPY-  
22 based fluorescent dye was synthesized and used to obtain a labeled mPEG-*block*-P(MMA-*grad*-  
23 DMAEMA) copolymer (composition:, mPEG = 4.4%, MMA = 51%, DMAEMA = 38%,

1 BODIPY = 6.2%, indicated as BODIPY-copolymer). The chemical structure of this BODIPY-  
2 copolymer should guarantee a preferential partition of the probe in the polymer-rich region. The  
3 affinity between the BODIPY-copolymer probe and the copolymer 5 has been checked by DSC  
4 measurements (Figure S10 in the Supporting Information). The presence of a single  $T_g$  transition,  
5 different from the ones of neat copolymers, clearly indicates that they are molecularly miscible.  
6 On the other hand, blends of the BODIPY-copolymer and POPC did not show changes in phase  
7 transitions (Figure S11 in the Supporting Information).

8 Because of the difficulties related to the phase transfer method in determining the final  
9 composition of vesicles [79–81], the percentages of lipids and polymers reported hereon refer to  
10 the initial amount of the components in the solutions prior the centrifugation phase. In a first  
11 step, lipid vesicles stained with the fluorescent dye were prepared at pH ~ 7 to characterize the  
12 absorption and emission spectra useful for the observation at the confocal microscope. Figure 2  
13 a) and b) show confocal images, respectively, at the characteristic emission wavelengths for the  
14 fluorescence of BODIPY ( $498 < \lambda < 530$  nm) and for 18:1 Liss Rhod PE ( $571 < \lambda < 630$  nm),  
15 when blended in the double layer of POPC vesicles. Figure 2 a) clearly shows that a very low  
16 amount of the BODIPY-copolymer = 0.02 mol% with respect to the total amount of amphiphiles,  
17 can be homogeneously dispersed into the POPC phospholipid membranes and confirms that the  
18 solubility parameter of the synthesized copolymer is close to that of pure PMMA.



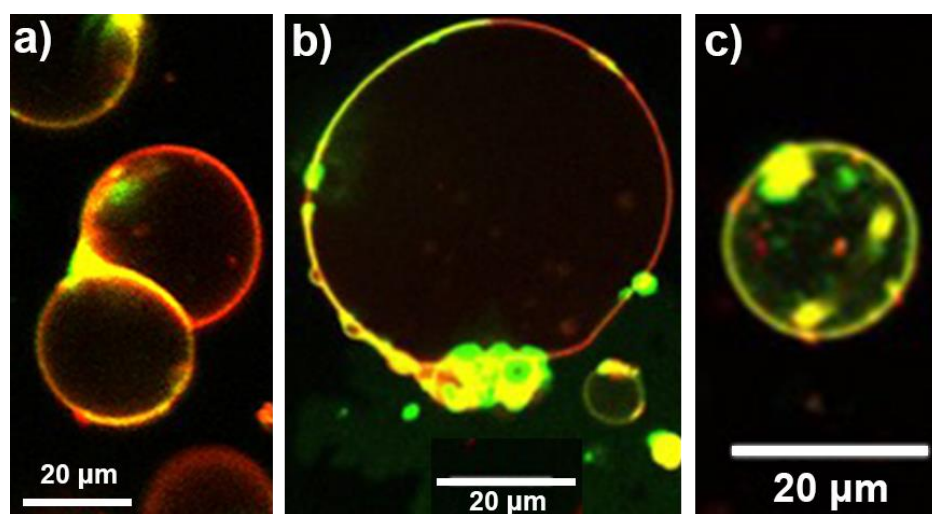
1  
2 **Figure 2** POPC vesicles labelled with fluorescent probes. a) POPC and BODIPY-copolymer 99.98 %: 0.02 %;  
3 b) POPC and 18:1 Liss Rhod PE 99 %: 0.8%. Small round spots in a) and b) are probably due to mineral oil  
4 residues. White bar scales 10  $\mu\text{m}$ .

5 Mixed vesicles were successfully prepared at  $\text{pH} = 7$ , where 50% of the copolymer is  
6 protonated ( $\text{pK}_a \sim 7$ , see Figure S12 in Supporting Information), by varying the unlabeled  
7 copolymer mole percentage in the range 8 – 40 % (58 – 89 wt%), higher concentration of  
8 copolymer hindered the self-assembly of the amphiphiles and the formation of a pellet at the end  
9 of the phase transfer procedure was not obtained. Furthermore, we could not observe the  
10 formation of pure polymer GUVs.

11 Vesicles with the compositions mol % POPC – mol % copolymer 5: 92% – 8%, 85% – 15%,  
12 60% – 40% labeled with 18:1 Liss Rhod PE and BODIPY-copolymer are shown in Figure 3 a) –  
13 c), respectively. In each figure, the green channel represents the fluorescence of the copolymer,  
14 the red channel corresponds to the fluorescence of the lipid and the yellow channel represents  
15 their overlap. When the concentration of the copolymer in the vesicles was below  $\sim 20$  mol% the  
16 mixed membranes were mainly heterogeneous, with small regions where copolymer blended into  
17 the lipid layers. Interestingly, the presence of the copolymer in the membranes promoted the



1 fusion of single vesicles into budded structures, as shown in Figure 3 a), or large aggregates of  
2 fused vesicles. The welding surface between vesicles was indeed characterized by a large  
3 concentration of copolymer, highlighted by the intense yellow colour, which probably favours  
4 electrostatic interaction between the outer leaflets of the membranes, as previously observed in  
5 the presence of charged fatty acids incorporated in the double layer of POPC vesicles [82,83].



6  
7 **Figure 3 Mixed POPC- copolymer vesicles having molar composition: a) 92% – 8%; b) 85% – 15%; c) 60% –**  
8 **40%. All systems were stained with 18:1 Liss Rhod PE (0.8%) and BODIPY-copolymer (0.02%). More**  
9 **pictures are reported in the Supporting Information (Figure S13).**

10 When the percentage of copolymer increased, the length of the domains where it was present  
11 seemed to increase accordingly (Figure 3 b), until a homogeneous distribution of lipids and  
12 copolymers in the membrane was attained when the concentration of latter was around 40 mol%,  
13 as shown in Figure 3 c). It is worth noting that mixed vesicles were obtained in presence of the  
14 same amount of Liss Rhod PE (0.8%) and BODIPY-copolymer (0.02%) for all the investigated  
15 compositions. At very low concentration of copolymer 5 (8%), image 3 a) shows the prevailing  
16 presence of POPC as stained by Liss Rhod PE while, increasing the copolymer amount, the  
17 images 3 b) and 3 c) show an increase in homogeneity of the membrane with consequent

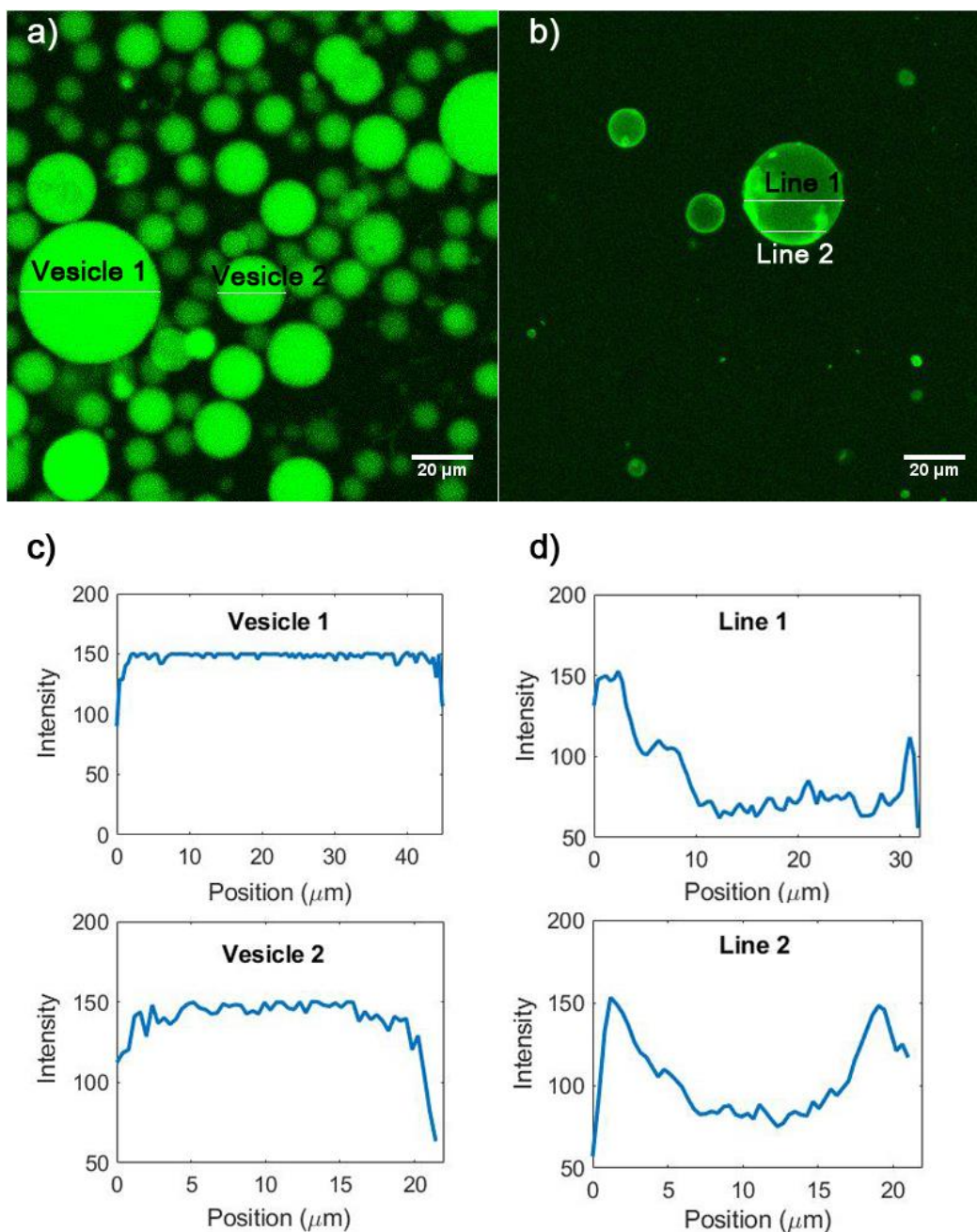
1 overlapping of both the probes. The increasing spreading of BODIPY-copolymer in the  
2 membrane, despite its fixed concentration, demonstrates both the presence of the copolymer into  
3 the membrane and the prevailing affinity of the fluorescent copolymer for copolymer-rich  
4 regions. Although the membrane composition became more homogeneous at higher copolymer  
5 percentages, the total number of stable vesicles detected in the samples drastically diminished,  
6 until amphiphilic structures could not be observed at copolymer amount  $> \sim 40$  mol%. The  
7 concentration dependence of both the lipid/copolymer distribution in the membrane and the  
8 successful formation of vesicles might depend on the complex attractive interactions taking place  
9 between the two molecules, as observed for a DPPC/PIB<sub>37</sub>-b-PEO<sub>48</sub> system [32].

10 It is interesting to compare our results with those previously reported in the literature  
11 concerning POPC-based giant vesicles blended with PBD or PDMS. POPC is a lipid in the fluid  
12 state at room temperature and in the case of PBD [13,31], homogeneous hybrid GUVs are  
13 formed in a restricted range of composition (i.e. in the region of low and high mole polymer  
14 content). In these homogeneous vesicles lipid molecules are dispersed in a “sea” of copolymers  
15 and vice versa. This situation is the most energetically favoured. Indeed, apart from the chemical  
16 affinity, also the structural compatibility has to be taken into account when dealing with  
17 lipid/copolymer GUVs. The thickness of polymer bilayers varies between 5 and 50 nm, while the  
18 lipid bilayer is 3-4 nm [20,84], i.e. there is a so called hydrophobic mismatch. As a consequence  
19 of this hydrophobic mismatch, in phase separated systems, the copolymer has to distort in order  
20 to adjust to the lipid bilayer and avoid as much as possible the contacts with water. This has a  
21 high energy cost corresponding to high line tension at lipid-polymer boundary. Then, domain  
22 formation is most of the time energetically disfavoured. Interestingly in the case of PDMS  
23 triblock copolymers [29], hybrid GUVs were formed in all the weight concentration range and

1 were homogeneous in the polymer-rich region (till 85 wt % polymer content for 1.5k  
2 copolymers, 50 wt% for 3k copolymers), while domain formation was observed at lower  
3 polymer content. This phase separation was accompanied by budding and the phase-separated,  
4 budded vesicles were not stable and budding degenerated in vesicle fission in the lipid rich  
5 region. For molecular weight as high as 5k stable phase separated vesicles could not be observed  
6 and pure lipid or pure polymer vesicles were present together with homogeneous structures.  
7 Budding phenomena can evolve in fission when the line tension at boundary is higher than the  
8 curvature energy associated to bending. The authors concluded that line tension at the lipid-  
9 polymer boundary were too high in the case of the 5k copolymers. Besides, the authors stressed  
10 that block copolymers with the same molecular weight showed even higher instability towards  
11 fission than the triblock structures. As observed for PDMS based copolymers, the copolymer  
12 presented here forms homogeneous GUVs at high polymer content. On the contrary domain  
13 formation is observed without fission between 24 w % and 42 w % POPC despite their higher  
14 hydrophobic mismatch (13.8k compared to 5k in the literature). At these compositions the  
15 bending costs associated with budding and fission are still higher than the boundary line energy,  
16 so phase separation is still energetically favoured to fission. We speculate that our polymer has a  
17 higher  $T_g$  than PDMS based ones, and consequently a higher curvature energy.

18 In a different set of experiments, we investigated the influence of the copolymer on the  
19 distribution of the water-soluble fluorescent probe pyranine (structure reported in Figure S14 in  
20 the Supporting Information) that was dissolved in the I-solution. This probe is membrane  
21 impermeable and in pure POPC vesicles it is firmly confined into the aqueous lumen [85,86] as  
22 highlighted in Figure 4 a). However, when the copolymer was present, the pyranine tended to  
23 accumulate along the membrane (Figure 4 b). The intensity profiles shown in Figure 4 c) and 4

- 1 d) highlight the different distribution of pyranine. In pure POPC vesicles the fluorescence is
- 2 constant along the line and decreases at the border, in the mixed vesicle, in contrast, two peaks
- 3 appear in proximity of the membrane where pyranine accumulates.



1  
 2 **Figure 4 Vesicles made of POPC - copolymer (93% - 7% mol/mol) and pyranine 50  $\mu$ M. a) Pure POPC**  
 3 **vesicles; b) Mixed vesicles; c) Intensity profile taken along a line for vesicles 1 and 2 of Panel a); d) Intensity**  
 4 **profile taken along line 1 and line 2 for the vesicle of Panel b).**  
 5 This behaviour could be attributed to a tendency of pyranine to accumulate in copolymer-rich  
 6 domains, possibly due to the interaction of its OH moieties with the pH-sensitive units of the

1 DMAEMA group. This different behaviour observed for pyranine in the absence and in the  
2 presence of the copolymer is another proof that the latter is indeed present in the POPC double  
3 layer.

#### 4 CONCLUSION

5 Hybrid lipid vesicles incorporating a copolymer with a  $T_g$  above room temperature have  
6 been presented. Our study shows that electroformation cannot be used for these polymers to  
7 efficiently obtain giant vesicles, probably linked to a low mobility in the electroformation  
8 conditions. However, a method based on an emulsion phase transfer, so far used for lipids or  
9 polymers such as PDMS or PBD has been successfully adapted. This can be preferably applied  
10 to polymers compatible with lipids based on their solubility and affinity. For this, the solubility  
11 parameter and the HLB value can be used to select appropriate candidates. The method presented  
12 here will open up new possibilities to develop hybrid systems with a much larger chemical  
13 structure variety, leading to new types of synthetic micro reactors or biomimetic compartments.  
14 Possible future developments will therefore be adaptive micro-reactor with catalyst groups fixed  
15 to the membrane, or pH-sensitive micro- or nano-reactors or synthetic cells with a controlled  
16 permeability versus an external stimulus.

1 ACKNOWLEDGMENTS

2 The authors thank COST ACTION CM1304 and ERASMUS+ exchange program for funding  
3 the mobility of Y.M. from the University of Salerno to the University of Toulouse “Paul  
4 Sabatier” and the EU (FEDER-35477 “Nano-objets pour la biotechnologie”) for financial support.  
5 Thanks are due to Dr.Patrizia Iannece (University of Salerno) for ESI-MS spectral measurements  
6 and Dr. Patrizia Oliva (University of Salerno) for the bidimensional NMR spectral measurements  
7 and Dr Stephanie Dauvillier (IPBS, University of Toulouse and CNRS) for assistance with  
8 confocal microscope. L.I. thanks Università degli Studi dell’Insubria for the funding granted via  
9 “Fondo dell’Ateneo per la Ricerca” (FAR 2018).

10

## 1 LIST OF ABBREVIATIONS

Monomethoxypoly(ethyleneglycol)	mPEG
Poly(ethylene glycol) methyl ether 2-bromoisobutyrate	mPEG-Br
2,2'-Bipyridyl	bpy
1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine	POPC
1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)	18:1 Liss Rhod PE
Methylmetacrylate	MMA
N,N-(dimethylamino)ethyl methacrylate	DMAEMA
2,6-diethyl-1,3,5,7-tetramethyl-8-[4-[8-(2-methacryloylolethyl)dimethylammoniumbromideoctyloxy]phenyl]-4,4'-difluoroboradiaza indacene	BODIPY-DMAEMA
fluorescent mPEG- <i>block</i> -P(MMA- <i>grad</i> -DMAEMA)	BODIPY-copolymer

2

## 3 ASSOCIATED CONTENT

4 Supplementary information is available. It describes the characterization of the products in more  
5 details and report more pictures about hybrid GUVs.



## 1 REFERENCES

- 2 [1] F. Meng, Z. Zhong, J. Feijen, Stimuli-responsive polymersomes for programmed drug  
3 delivery, *Biomacromolecules*, 10 (2009) 197–209.
- 4 [2] M. Huo, J. Yuan, L. Tao, Y. Wei, Redox-responsive polymers for drug delivery: from  
5 molecular design to applications, *Polym. Chem.*, 5 (2014) 1519–1528.
- 6 [3] L. Messenger, J. Gaitzsch, L. Chierico, G. Battaglia, Novel aspects of encapsulation and  
7 delivery using polymersomes, *Curr. Opin. Pharmacol.*, 18 (2014) 104–111.
- 8 [4] M. Li, C. Du, N. Guo, Y. Teng, X. Meng, H. Sun, S. Li, P. Yu, H. Galons, Composition  
9 design and medical application of liposomes, *Eur. J. Med. Chem.*, (2019).
- 10 [5] H. Che, J.C.M. van Hest, Adaptive Polymersome Nanoreactors, *ChemNanoMat*, 5 (2019)  
11 1092–1109.
- 12 [6] I. Louzao, J.C. van Hest, Permeability effects on the efficiency of antioxidant nanoreactors,  
13 *Biomacromolecules*, 14 (2013) 2364–2372.
- 14 [7] K. Langowska, C.G. Palivan, W. Meier, Polymer nanoreactors shown to produce and  
15 release antibiotics locally, *Chem. Commun.*, 49 (2013) 128–130.
- 16 [8] X. Liu, D. Appelhans, B. Voit, Hollow Capsules with Multiresponsive Valves for  
17 Controlled Enzymatic Reactions, *J. Am. Chem. Soc.*, 140 (2018) 16106–16114.
- 18 [9] O. Rifaie-Graham, S. Ulrich, N.F. Galensowske, S. Balog, M. Chami, D. Rentsch, J.R.  
19 Hemmer, J. Read de Alaniz, L.F. Boesel, N. Bruns, Wavelength-selective light-responsive  
20 DASA-functionalized polymersome nanoreactors, *J. Am. Chem. Soc.*, 140 (2018) 8027–  
21 8036.

- 1 [10] Q. Yan, J. Wang, Y. Yin, J. Yuan, Breathing polymersomes: CO<sub>2</sub>-tuning membrane  
2 permeability for size-selective release, separation, and reaction, *Angew. Chem. Int. Ed.*, 52  
3 (2013) 5070–5073.
- 4 [11] M. Mohammadi, S. Taghavi, K. Abnous, S.M. Taghdisi, M. Ramezani, M. Alibolandi,  
5 Hybrid Vesicular Drug Delivery Systems for Cancer Therapeutics, *Adv. Funct. Mater.*, 28  
6 (2018) 1802136.
- 7 [12] W. Shen, J. Hu, X. Hu, Impact of amphiphilic triblock copolymers on stability and  
8 permeability of phospholipid/polymer hybrid vesicles, *Chem. Phys. Lett.*, 600 (2014) 56–  
9 61.
- 10 [13] S.K. Lim, H.-P. de Hoog, A.N. Parikh, M. Nallani, B. Liedberg, Hybrid, Nanoscale  
11 Phospholipid/Block Copolymer Vesicles, *Polymers*, 5 (2013) 1102–1114.
- 12 [14] M. Mumtaz Virk, E. Reimhult, Phospholipase A<sub>2</sub>-induced degradation and release from  
13 lipid-containing polymersomes, *Langmuir*, 34 (2017) 395–405.
- 14 [15] Z. Cheng, D.R. Elias, N.P. Kamat, E.D. Johnston, A. Poloukhine, V. Popik, D.A. Hammer,  
15 A. Tsourkas, Improved Tumor Targeting of Polymer-Based Nanovesicles Using Polymer–  
16 Lipid Blends, *Bioconjug. Chem.*, 22 (2011) 2021–2029.
- 17 [16] W. Zong, B. Thingholm, F. Itef, P.S. Schattling, E. Brodzkij, D. Mayer, S. Stenger, K.N.  
18 Goldie, X. Han, B. Städler, Phospholipid–Block Copolymer Hybrid Vesicles with  
19 Lysosomal Escape Ability, *Langmuir*, 34 (2018) 6874–6886.
- 20 [17] E. Rideau, R. Dimova, P. Schwille, F.R. Wurm, K. Landfester, Liposomes and  
21 polymersomes: a comparative review towards cell mimicking, *Chem. Soc. Rev.*, 47 (2018)  
22 8572–8610.

- 1 [18] C. G. Palivan, R. Goers, A. Najer, X. Zhang, A. Car, W. Meier, Bioinspired polymer  
2 vesicles and membranes for biological and medical applications, *Chem. Soc. Rev.*, 45  
3 (2016) 377–411.
- 4 [19] M. Schulz, W.H. Binder, Mixed Hybrid Lipid/Polymer Vesicles as a Novel Membrane  
5 Platform, *Macromol. Rapid Commun.*, 36 (2015) 2031–2041.
- 6 [20] J.-F. Le Meins, C. Schatz, S. Lecommandoux, O. Sandre, Hybrid polymer/lipid vesicles:  
7 state of the art and future perspectives, *Mater. Today*, 16 (2013) 397–402.
- 8 [21] A. Peyret, E. Ibarboure, J.-F. Le Meins, S. Lecommandoux, Asymmetric hybrid polymer–  
9 lipid giant vesicles as cell membrane mimics, *Adv. Sci.*, 5 (2018) 1700453.
- 10 [22] M. Bieligmeyer, F. Artukovic, S. Nussberger, T. Hirth, T. Schiestel, M. Müller,  
11 Reconstitution of the membrane protein OmpF into biomimetic block copolymer–  
12 phospholipid hybrid membranes, *Beilstein J. Nanotechnol.*, 7 (2016) 881–892.
- 13 [23] M. Schulz, S. Werner, K. Bacía, W.H. Binder, Controlling molecular recognition with  
14 lipid/polymer domains in vesicle membranes, *Angew. Chem. Int. Ed.*, 52 (2013) 1829–  
15 1833.
- 16 [24] J. Kowal, D. Wu, V. Mikhalevich, C.G. Palivan, W. Meier, Hybrid Polymer–Lipid Films as  
17 Platforms for Directed Membrane Protein Insertion, *Langmuir*, 31 (2015) 4868–4877.
- 18 [25] L. Otrin, N. Marušič, C. Bednarz, T. Vidaković-Koch, I. Lieberwirth, K. Landfester, K.  
19 Sundmacher, Toward artificial mitochondrion: mimicking oxidative phosphorylation in  
20 polymer and hybrid membranes, *Nano Lett.*, 17 (2017) 6816–6821.
- 21 [26] R.J.R.W. Peters, M. Marguet, S. Marais, M.W. Fraaije, J.C.M. van Hest, S.  
22 Lecommandoux, Cascade Reactions in Multicompartmentalized Polymersomes, *Angew.*  
23 *Chem. Int. Ed.*, 53 (2014) 146–150.

- 1 [27] J. Nam, T. Kyle Vanderlick, P. A. Beales, Formation and dissolution of phospholipid  
2 domains with varying textures in hybrid lipo-polymersomes, *Soft Matter*, 8 (2012) 7982–  
3 7988.
- 4 [28] T.P.T. Dao, F. Fernandes, M. Fauquignon, E. Ibarboure, M. Prieto, J.F.L. Meins, The  
5 combination of block copolymers and phospholipids to form giant hybrid unilamellar  
6 vesicles (GHUVs) does not systematically lead to “intermediate” membrane properties,  
7 *Soft Matter*, 14 (2018) 6476–6484.
- 8 [29] T.P.T. Dao, F. Fernandes, E. Ibarboure, K. Ferji, M. Prieto, O. Sandre, J.-F.L. Meins,  
9 Modulation of phase separation at the micron scale and nanoscale in giant polymer/lipid  
10 hybrid unilamellar vesicles (GHUVs), *Soft Matter*, 13 (2017) 627–637.
- 11 [30] C. Magnani, C. Montis, G. Mangiapia, A.-F. Mingotaud, C. Mingotaud, C. Roux, P. Joseph,  
12 D. Berti, B. Lonetti, Hybrid vesicles from lipids and block copolymers: Phase behavior  
13 from the micro-to the nano-scale, *Colloids Surf. B Biointerfaces*, (2018).
- 14 [31] J. Nam, P.A. Beales, T.K. Vanderlick, Giant Phospholipid/Block Copolymer Hybrid  
15 Vesicles: Mixing Behavior and Domain Formation, *Langmuir*, 27 (2011) 1–6.
- 16 [32] M. Schulz, D. Glatte, A. Meister, P. Scholtysek, A. Kerth, A. Blume, K. Bacia, W.H.  
17 Binder, Hybrid lipid/polymer giant unilamellar vesicles: effects of incorporated  
18 biocompatible PIB–PEO block copolymers on vesicle properties, *Soft Matter*, 7 (2011)  
19 8100–8110.
- 20 [33] A. Kubilis, A. Abdulkarim, A.M. Eissa, N.R. Cameron, Giant polymersome protocells dock  
21 with virus particle mimics via multivalent glycan-lectin interactions, *Sci. Rep.*, 6 (2016)  
22 32414.

- 1 [34] E. Rideau, F.R. Wurm, K. Landfester, Self-Assembly of Giant Unilamellar Vesicles by  
2 Film Hydration Methodologies, *Adv. Biosyst.*, 3 (2019) 1800324.
- 3 [35] M.I. Angelova, D.S. Dimitrov, Liposome electroformation, *Faraday Discuss. Chem. Soc.*,  
4 81 (1986) 303–311.
- 5 [36] B.M. Discher, Y.-Y. Won, D.S. Ege, J.C.-M. Lee, F.S. Bates, D.E. Discher, D.A. Hammer,  
6 Polymersomes: Tough Vesicles Made from Diblock Copolymers, *Science*, 284 (1999)  
7 1143–1146.
- 8 [37] M. Dionzou, A. Morere, C. Roux, B. Lonetti, J.-D. Marty, C. Mingotaud, P. Joseph, D.  
9 Goudouneche, B. Payre, M. Leonetti, A.-F. Mingotaud, Comparison of methods for the  
10 fabrication and the characterization of polymer self-assemblies: what are the important  
11 parameters, *Soft Matter*, 12 (2016) 2166–2176.
- 12 [38] A.M. Eissa, M.J. Smith, A. Kubilis, J.A. Mosely, N.R. Cameron, Polymersome-forming  
13 amphiphilic glycosylated polymers: Synthesis and characterization, *J. Polym. Sci. Part*  
14 *Polym. Chem.*, 51 (2013) 5184–5193.
- 15 [39] J.P. Reeves, R.M. Dowben, Formation and properties of thin-walled phospholipid vesicles,  
16 *J. Cell. Physiol.*, 73 (1969) 49–60.
- 17 [40] R. Dimova, U. Seifert, B. Pouligny, S. Förster, H.-G. Dobereiner, Hyperviscous diblock  
18 copolymer vesicles, *Eur. Phys. J. E*, 7 (2002) 241–250.
- 19 [41] Y. Zhou, D. Yan, Real-Time Membrane Fission of Giant Polymer Vesicles, *Angew. Chem.*  
20 *Int. Ed.*, 44 (2005) 3223–3226.
- 21 [42] E. Rideau, F.R. Wurm, K. Landfester, Giant polymersomes from non-assisted film  
22 hydration of phosphate-based block copolymers, *Polym. Chem.*, 9 (2018) 5385–5394.

- 1 [43] A. Weinberger, F.-C. Tsai, G.H. Koenderink, T.F. Schmidt, R. Itri, W. Meier, T. Schmatko,  
2 A. Schröder, C. Marques, Gel-Assisted Formation of Giant Unilamellar Vesicles, *Biophys.*  
3 *J.*, 105 (2013) 154–164.
- 4 [44] K.S. Horger, D.J. Estes, R. Capone, M. Mayer, Films of Agarose Enable Rapid Formation  
5 of Giant Liposomes in Solutions of Physiologic Ionic Strength, *J. Am. Chem. Soc.*, 131  
6 (2009) 1810–1819.
- 7 [45] T.P.T. Dao, M. Fauquignon, F. Fernandes, E. Ibarboure, A. Vax, M. Prieto, J.-F. Le Meins,  
8 Membrane properties of giant polymer and lipid vesicles obtained by electroformation and  
9 PVA gel-assisted hydration methods, *Colloids Surf. Physicochem. Eng. Asp.*, 533 (2017)  
10 347–353.
- 11 [46] S. Pautot, B.J. Frisken, D.A. Weitz, Production of Unilamellar Vesicles Using an Inverted  
12 Emulsion, *Langmuir*, 19 (2003) 2870–2879.
- 13 [47] C. Martino, A.J. deMello, Droplet-based microfluidics for artificial cell generation: a brief  
14 review, *Interface Focus*, 6 (2016) 20160011.
- 15 [48] S. Deshpande, C. Dekker, On-chip microfluidic production of cell-sized liposomes, *Nat.*  
16 *Protoc.*, 13 (2018) 856–874.
- 17 [49] M. Abkarian, E. Loiseau, G. Massiera, Continuous droplet interface crossing encapsulation  
18 (cDICE) for high throughput monodisperse vesicle design, *Soft Matter*, 7 (2011) 4610–  
19 4614.
- 20 [50] M.C. Blosser, B.G. Horst, S.L. Keller, cDICE method produces giant lipid vesicles under  
21 physiological conditions of charged lipids and ionic solutions, *Soft Matter*, 12 (2016) 7364–  
22 7371.

- 1 [51] K. Dürre, A.R. Bausch, Formation of phase separated vesicles by double layer cDICE, *Soft*  
2 *Matter*, (2019).
- 3 [52] J. Petit, I. Polenz, J.-C. Baret, S. Herminghaus, O. Bäumchen, Vesicles-on-a-chip: A  
4 universal microfluidic platform for the assembly of liposomes and polymersomes, *Eur.*  
5 *Phys. J. E*, 39 (2016) 59.
- 6 [53] K. Kamiya, R. Kawano, T. Osaki, K. Akiyoshi, S. Takeuchi, Cell-sized asymmetric lipid  
7 vesicles facilitate the investigation of asymmetric membranes, *Nat. Chem.*, 8 (2016) 881–  
8 889.
- 9 [54] A. Moga, N. Yandrapalli, R. Dimova, T. Robinson, Optimization of the Inverted Emulsion  
10 Method for High-Yield Production of Biomimetic Giant Unilamellar Vesicles,  
11 *ChemBioChem*, 20 (2019) 2674–2682.
- 12 [55] H. Che, S. Cao, J.C.M. van Hest, Feedback-Induced Temporal Control of “Breathing”  
13 Polymersomes To Create Self-Adaptive Nanoreactors, *J. Am. Chem. Soc.*, 140 (2018)  
14 5356–5359.
- 15 [56] S. Villani, R. Adami, E. Reverchon, A.M. Ferretti, A. Ponti, M. Lepretti, I. Caputo, L. Izzo,  
16 pH-sensitive polymersomes: controlling swelling via copolymer structure and chemical  
17 composition, *J. Drug Target.*, 25 (2017) 899–909.
- 18 [57] E. Yoshida, pH response behavior of giant vesicles comprised of amphiphilic poly  
19 (methacrylic acid)-block-poly (methyl methacrylate-random-methacrylic acid), *Colloid*  
20 *Polym. Sci.*, 293 (2015) 649–653.
- 21 [58] E. Yoshida, Giant vesicles comprised of mixed amphiphilic poly (methacrylic acid)-block-  
22 poly (methyl methacrylate-random-methacrylic acid) diblock copolymers, *Colloid Polym.*  
23 *Sci.*, 293 (2015) 3641–3648.

- 1 [59] E. Yoshida, Preparation of giant vesicles containing quaternary ammonium salt of 2-  
2 (dimethylamino) ethyl methacrylate through photo nitroxide-mediated controlled/living  
3 radical polymerization-induced self-assembly, *J. Polym. Res.*, 25 (2018) 109.
- 4 [60] K. Sreenath, R.J. Clark, L. Zhu, Tricolor emission of a fluorescent heteroditopic ligand over  
5 a concentration gradient of zinc (II) ions, *J. Org. Chem.*, 77 (2012) 8268–8279.
- 6 [61] S. Matrella, C. Vitiello, M. Mella, G. Vigliotta, L. Izzo, The Role of Charge Density and  
7 Hydrophobicity on the Biocidal Properties of Self-Protonable Polymeric Materials,  
8 *Macromol. Biosci.*, 15 (2015) 927–940.
- 9 [62] M. Manunta, L. Izzo, R. Duncan, A.T. Jones, Establishment of subcellular fractionation  
10 techniques to monitor the intracellular fate of polymer therapeutics II Identification of  
11 endosomal and lysosomal compartments in HepG2 cells combining single-step subcellular  
12 fractionation with fluorescent imaging, *J. Drug Target.*, 15 (2007) 37–50.
- 13 [63] M. Berna, D. Dalzoppo, G. Pasut, M. Manunta, L. Izzo, A.T. Jones, R. Duncan, F.M.  
14 Veronese, Novel monodisperse PEG- dendrons as new tools for targeted drug delivery:  
15 synthesis, characterization and cellular uptake, *Biomacromolecules*, 7 (2006) 146–153.
- 16 [64] M.I. Angelova, S. Soléau, Ph. Méléard, F. Faucon, P. Bothorel, Preparation of giant  
17 vesicles by external AC electric fields Kinetics and applications, in: C. Helm, M. Lösche,  
18 H. Möhwald (Eds.), *Trends Colloid Interface Sci. VI*, Steinkopff, Darmstadt, 1992: pp.  
19 127–131.
- 20 [65] J.H. Hildebrand, R.L. Scott, *The Solubility of Non-electrolytes*, third edition, Reinhold,  
21 New York, 1950.
- 22 [66] J.H. Hildebrand, R.L. Scott, *Regular Solutions*, Prentice-Hall Inc., New Jersey, 1962.



- 1 [67] M. Gilbert, Chapter 5 - Relation of Structure to Chemical Properties, in: M. Gilbert (Ed.),  
2 Brydsons Plast. Mater. Eighth Ed., Butterworth-Heinemann, 2017: pp. 75–102.
- 3 [68] K. Min, H. Gao, K. Matyjaszewski, Use of Ascorbic Acid as Reducing Agent for Synthesis  
4 of Well-Defined Polymers by ARGET ATRP, *Macromolecules*, 40 (2007) 1789–1791.
- 5 [69] H. Dong, K. Matyjaszewski, ARGET ATRP of 2-(Dimethylamino)ethylMethacrylate as an  
6 Intrinsic Reducing Agent, *Macromolecules*, 41 (2008) 6868–6870.
- 7 [70] A. Tagliabue, L. Izzo, M. Mella, Impact of Charge Correlation, Chain Rigidity, and  
8 Chemical Specific Interactions on the Behavior of Weak Polyelectrolytes in Solution, *J.*  
9 *Phys. Chem. B*, 123 (2019) 8872–8888.
- 10 [71] M. Mella, L. Izzo, Modulation of ionization and structural properties of weak  
11 polyelectrolytes due to 1D, 2D, and 3D confinement, *J. Polym. Sci. Part B Polym. Phys.*, 55  
12 (2017) 1088–1102.
- 13 [72] M. Mella, L. Izzo, Structural properties of hydrophilic polymeric chains bearing  
14 covalently-linked hydrophobic substituents: Exploring the effects of chain length,  
15 fractional loading and hydrophobic interaction strength with coarse grained potentials and  
16 Monte Carlo simulations, *Polymer*, 51 (2010) 3582–3589.
- 17 [73] M. Mella, M.V.L. Rocca, Y. Miele, L. Izzo, On the origin and consequences of high  
18 DMAEMA reactivity ratio in ATRP copolymerization with MMA: An experimental and  
19 theoretical study#, *J. Polym. Sci. Part Polym. Chem.*, 56 (2018) 1366–1382.
- 20 [74] A.S. Klymchenko, R. Kreder, Fluorescent Probes for Lipid Rafts: From Model Membranes  
21 to Living Cells, *Chem. Biol.*, 21 (2014) 97–113.

- 1 [75] M. Chemin, P.-M. Brun, S. Lecommandoux, O. Sandre, J.-F. Le Meins, Hybrid  
2 polymer/lipid vesicles: fine control of the lipid and polymer distribution in the binary  
3 membrane, *Soft Matter*, 8 (2012) 2867–2874.
- 4 [76] T. Ruysschaert, A.F.P. Sonnen, T. Haefele, W. Meier, M. Winterhalter, D. Fournier, Hybrid  
5 Nanocapsules: Interactions of ABA Block Copolymers with Liposomes, *J. Am. Chem.*  
6 *Soc.*, 127 (2005) 6242–6247.
- 7 [77] A. Somasundar, S. Ghosh, F. Mohajerani, L.N. Massenburg, T. Yang, P.S. Cremer, D.  
8 Velegol, A. Sen, Positive and negative chemotaxis of enzyme-coated liposome motors, *Nat.*  
9 *Nanotechnol.*, 14 (2019) 1129–1134.
- 10 [78] R. Dimova, C. Marques, C. Marques, *The Giant Vesicle Book*, CRC Press, 2019.
- 11 [79] P. Stano, T.P. de Souza, P. Carrara, E. Altamura, E. D’Aguanno, M. Caputo, P.L. Luisi, F.  
12 Mavelli, Recent Biophysical Issues About the Preparation of Solute-Filled Lipid Vesicles,  
13 *Mech. Adv. Mater. Struct.*, 22 (2015) 748–759.
- 14 [80] P. Stano, Minimal cells: relevance and interplay of physical and biochemical factors,  
15 *Biotechnol. J.*, 6 (2011) 850–859.
- 16 [81] E. Altamura, P. Stano, P. Walde, F. Mavelli, Giant Vesicles as Micro-Sized Enzymatic  
17 Reactors: Perspectives and Recent Experimental Advancements, *Int. J. Unconv. Comput.*,  
18 11 (2015) 5–21.
- 19 [82] L. Jin, N.P. Kamat, S. Jena, J.W. Szostak, Fatty Acid/Phospholipid Blended Membranes: A  
20 Potential Intermediate State in Protocellular Evolution, *Small*, 14 (2018) 1704077.
- 21 [83] P. Carrara, P. Stano, P.L. Luisi, Giant Vesicles “Colonies”: A Model for Primitive Cell  
22 Communities, *ChemBioChem*, 13 (2012) 1497–1502.

- 1 [84] J.F. Nagle, S. Tristram-Nagle, Structure of lipid bilayers, *Biochim. Biophys. Acta*, 1469  
2 (2000) 159–195.
- 3 [85] Y. Miele, T. Bánsági, A.F. Taylor, P. Stano, F. Rossi, Engineering Enzyme-Driven  
4 Dynamic Behaviour in Lipid Vesicles, in: F. Rossi, F. Mavelli, P. Stano, D. Caivano (Eds.),  
5 *Adv. Artif. Life Evol. Comput. Syst. Chem.*, Springer International Publishing, Cham,  
6 2016: pp. 197–208.
- 7 [86] Y. Miele, Z. Medveczky, G. Hollo, B. Tegze, I. Derenyi, Z. Horvolgyi, E. Altamura, I.  
8 Lagzi, F. Rossi, Self-Division of Giant Vesicles Driven by an Internal Enzymatic Reaction,  
9 *Chem. Sci.*, in press (2020).
- 10