Recent trends in the tuning of polymersomes' membrane properties.

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Colloquium

Recent trends in the tuning of polymersomes' membrane properties

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Abstract. "Polymersomes" are vesicular structures made from the self-assembly of block copolymers. Such structures present outstanding interest for different applications such as micro- or nano-reactor, drug release or can simply be used as tool for understanding basic biological mechanisms. The use of polymersomes in such applications is strongly related to the way their membrane properties are controlled and tuned either by a precise molecular design of the constituting block or by addition of specific components inside the membrane (formulation approaches). Typical membrane properties of polymersomes obtained from the self-assembly of "coil coil" block copolymer since the end of the nineties will be first briefly reviewed and compared to those of their lipidic analogues, named liposomes. Therefore the different approaches able to modulate their permeability, mechanical properties or ability to release loaded drugs, using macromolecular engineering or formulations, are detailed. To conclude, the most recent advances to modulate the polymersomes' properties and systems that appear very promising especially for biomedical application or for the development of complex and bio-mimetic structures are presented.

Introduction

In the late nineties hollow polymer structures, namely polymer vesicles, have emerged among the wide variety of morphologies that could be obtained from the self-assembly of block copolymers, thanks to their versatility in terms of chemical nature, flexibility and interaction offered by the recent developments in controlled polymer synthesis [1]. These polymer vesicles were commonly named polymersomes in analogy with liposomes obtained from the self-assembly of lipids. From a biophysical point of view liposomes can be considered as interesting cell membrane mimic. Their closed bilayer structure is a first step towards compartmentalization, which is one of the key architectural requirements to reproduce the natural environment of living cells. A lot of biological phenomena, for instance the exchange of nutrients or metabolite between compartments, imply a variety of processes including vesicle budding, membrane fusion and fission (*e.g.* endocytosis), protein-assisted membrane permeation and cell motility. The low molecular weight of lipids confers to the bilayer lateral fluidity and other "soft" properties that appear conducive to such cellular processes [2]. Besides,

liposomes are an interesting platform for drug delivery due to their biocompatible character and their ability to encapsulate hydrophobic and hydrophilic species. However, they suffer from a lack of mechanical and chemical stability rendering their experimentations and analysis difficult, limiting therefore their use as cellular model systems, drug carriers, or nano-reactor for (bio)chemical reactions.

Obtained from the self-assembly of block copolymers, polymersomes have been the subject of numerous studies because of their multiple interests. Different sizes, from a few hundred nanometers to a few hundred microns, can be obtained depending on the fabrication process and other molecular parameters. Compared to liposomes, they present a rather high mechanical stability and elastic (Hookean) behavior, a very high membrane viscosity and larger resistance to bending. As a consequence, they are also interesting tools for the understanding of various cellular processes. For instance, fusion and fission can occur in polymer vesicles but at a slower time scale compared to living cells or liposomes [3,4]. This was shown on polymer vesicles generated from the molecular self-assembly of an amphiphilic multi-arm copolymer with a hyper-branched poly(3-ethyl-3-oxetanemethanol) core and poly(ethylene oxide) arms (HBPO-star-PEO) in water [3], helping to distinguish the different stages of the phenomenon. Fusion and fission induced by temperature

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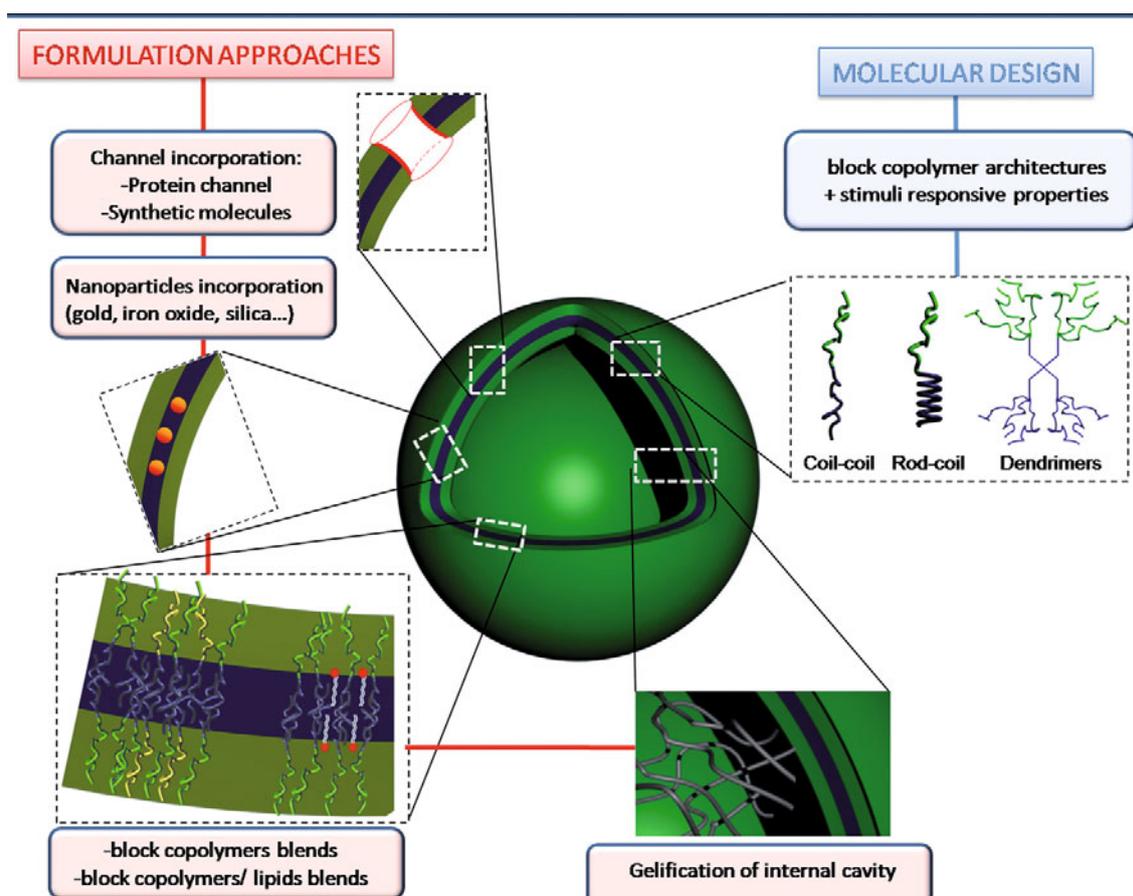[b] e-mail: lecommandoux@enscbp.fr

Fig. 1. The different routes to modulate polymersomes' membrane properties.

have been recently observed on giant vesicles made from the self-assembly of poly(trimethylene carbonate)-block-poly(L-glutamic acid) copolymer [4]. Polymersomes can be easily loaded with hydrophilic molecules in the internal cavity as well as hydrophobic ones in the membrane. They present a low passive permeability to low-molecular-weight solutes, allowing a controlled release by external triggers such as temperature, pH, or light [5].

All these properties make them excellent candidates for different applications such as nano- or micro-reactors, drug delivery systems or as fundamental tools for the understanding of biological mechanisms. Over the last five years, an increasing number of research teams worldwide are very active in developing their (bio) functionality, biomimetic character and modulating their properties.

In this article we are essentially focusing on vesicular structure obtained from the self-assembly of amphiphilic block copolymer (polymersomes). The aim is not to give an exhaustive overview of all the work done so far on polymersomes, but to emphasize recent promising advances with a specific attention on those aiming at modulating their membrane physical properties and/or developing their bio-mimetic character. For a more general reading on basic properties and applications of polymersomes, the reader can refer to recent reviews in the field [5–8]. It has

to be noted that the self-assembly of block copolymers is obviously not the only way to develop hollow vesicular or polymer architectures that can be also achieved using other methodologies such as layer-by-layer deposition [9] phase separation or template-assisted polymerization techniques [10,11]. These methods and the applications of the resulting vesicular structures as nano-reactors have been recently reviewed [12].

The article is divided into four parts. In the first one, the origin of the formation of vesicle as well as parameters that govern their size, size dispersity and shape will be detailed before describing the basic membranes properties of polymersomes, evidenced since the beginning of the 2000s. Their advantages and drawbacks compared to those of liposomes will be emphasized. Some aspects that are still under debate will be underlined and the very first approaches proposed to modulate physical properties of polymersomes will be reminded. The second part will be devoted to the different approaches to modulate the permeability of polymersomes membrane or their ability to release encapsulated species. The third part will discuss the tuning of their mechanical properties. Finally, we will present in the last part some recent approaches to modulate their physical and functional properties that appear to be promising for biomedical applications or in
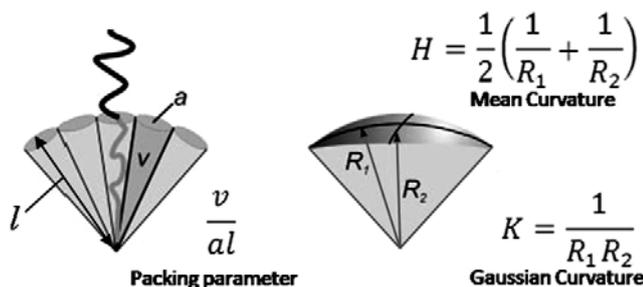
Fig. 2. Description of amphiphile shape in terms of surfactant packing parameter $v/(al)$ and its relation to the interfacial mean curvature (H) and Gaussian curvature (K) Adapted from [13].

the development of bio-mimetic structures. The different approaches able to tune polymersomes' membrane properties that will be described are schematically illustrated in fig. 1.

1 General parameters governing polymersome formation and properties

1.1 Thermodynamics of polymer vesicle

In certain conditions, the self-assembly of amphiphilic molecules and macromolecules can lead to the vesicular structure, *i.e.* a sac, with a membrane made of molecular layers —most often a bilayer— that is closed on itself and separates an internal aqueous compartment from the external aqueous solution. It has to be noted, that in the case of polymersomes the membrane is not really a bilayer (two monolayers constituting a bilayer) as entanglement and interdigitation can occur between hydrophobic blocks. In addition, in the specific case, for instance, of triblock copolymers (A-B-A), where B is the hydrophobic block, the concept of bilayer is not adapted, as the hydrophobic core of the membrane can consist of one copolymer block only. However in the following we will often use for convenience the term bilayer to describe the membrane of polymersomes. The formation of the bilayer, often belonging to a stack of many planar lamellae is controlled by the relative size (or weight fraction) of hydrophilic to hydrophobic segments. This parameter determines the curvature of the hydrophilic-hydrophobic interface. Two curvatures are commonly introduced to describe the membrane geometry in 3 dimensions: the Gaussian curvature K and the mean curvature H, given by the two principal radii of curvature of the interface R_1 and R_2 (fig. 2).

The hydrophobic volume, v, the interfacial area a, and the hydrophobic chain length l, normal to the interface, characterize a certain arrangement of the surfactant. These parameters are linked together via p, the surfactant packing parameter (or Israelachvili's parameter), linked to the curvature in the following manner [14]:

$$p = \frac{v}{al} = 1 - Hl + \frac{Kl^2}{3}. \qquad (1)$$

Depending on the p value, different morphologies can be predicted (micelles, cylinders, lamellae, ...). Vesicles characterized by a local lamellar order are thus obtained when the packing parameter is close to unity (*e.g.*, R_1 and $R_2 \sim \propto$).

This model, where pure geometrical considerations are taken into account, is not adequate to describe the self-assembly of amphiphilic macromolecules, where polymer chains entropy and entropy loss during vesicle formation can have a considerable effect on the resultant structure at the thermodynamic equilibrium. As a result, the evolution of the morphology with the hydrophilic-to-hydrophobic ratio of block copolymers is only in coarse qualitative agreement with packing parameter prediction. One of the first systematic studies showing this tendency was performed on a series of coil-coil poly(butadiene)-block-poly(ethylene oxide), PB-b-PEO block copolymers [13] and on dendron-based block copolymers combining well-defined polystyrene (PS) with poly(propylene imine) dendrimers [15].

For block copolymers, the obtained morphology not only results from geometrical aspects but also from the minimization of the free energy in which the interfacial energy of the hydrophobic-hydrophilic interface and the loss of entropy of the polymer chains are involved. Even if there is no valid theory able to fully predict the molecular requirements to obtain vesicles, Discher and Eisenberg collected the data obtained hitherto for coil-coil block copolymers and concluded that a hydrophilic weight fraction close to 35% ($\pm 10\%$) is required for vesicle formation [16]. On the contrary, for stiff polymer chains with low conformational entropy, the minimization of the interfacial area dominates due the low entropy loss during the segregation process. The amphiphilic copolymers will therefore self-assemble by minimizing the interfacial area per unit volume given by $Av = d\phi/l$, ϕ being the hydrophobic volume fraction, d being the dimensionality of the assembly [13]. The minimum value of Av occurs for a given ϕ and l at the lowest value of d ($d = 1$), corresponding to a planar interface. The range of stability of planar bilayer structures will therefore be extended. Consequently hydrophobic shape persistent blocks (*e.g.*, rod-like polymers) or block developing Coulombic, π-π interactions or H-bonding will have a larger tendency to form bilayers.

1.2 Polymersome formation

Theoretical calculations have been performed to predict the mechanism of formation of vesicles from the homogeneous state (*i.e.*, block copolymer initially fully dissolved as unimers). Two main situations arise from these calculations, as illustrated in fig. 3.

In mechanism 1, amphiphilic block copolymers rapidly self-assemble into small spherical micelles, which then slowly evolve into larger micelles such as cylindrical micelles, and open disc-like micelles by collision. The large disc-like micelles then slowly (relative to the first step) close up to form vesicles [17]. This mechanism has been predicted by several computer simulations based on the
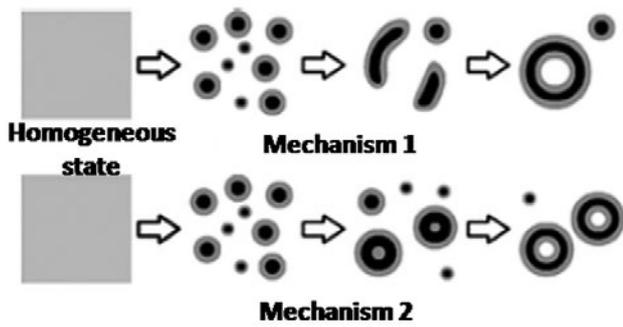
Fig. 3. Schematic representation of two vesicle formation mechanisms from the initial homogeneous state. Black and gray color correspond to hydrophobic and hydrophilic sub chains, respectively. Adapted from [17].

particle models (Brownian dynamics [18] dissipative particle dynamics [19], molecular dynamics [20]). This mechanism has also been shown more recently by numerical simulations based on density functional theory (DFT) for block copolymer solutions. It was also shown that this approach can reproduce morphological transitions (micelles to cylinders to vesicles) by changing the interaction Flory-Huggin's parameter χ in the simulation, which reflects the change in solvent quality during the experiment [17]. The transition states created by a change in polarity of the solvent have been experimentally quenched and observed by TEM for polystyrene-block-poly(acrylic acid) block copolymer [21] and poly(ethylene oxide)-b-poly(3-(trimethoxysilyl) propyl methacrylate) (PEO-b-PTMSPMA) [22]. In both cases the addition of a selective solvent into polymer solution led to vesicular self-assembly. It was observed for PEO-b-PTMSPMA that spherical micelles were the dominant morphology at low water content, while further addition of water led to rod-like micelles first and finally to vesicles at higher water content. For PS-b-PAA a rod-to-vesicle transition was also observed by increasing the water content. It was proposed that those experimental results support mechanism 1 to explain vesicle formation [21].

In mechanism 2, the initial stage is identical (spherical micelles). But rather than evolving toward other structures (cylindrical micelles and open-disk micelles), the copolymer chains remaining in solution aggregate to the micelles, increasing their size. The solvent then diffuses into the micelles to lower their bending energy by increasing the radius of curvature, leading to vesicles. Such a mechanism has been predicted by dynamic simulations using external potential dynamics (EPD), in which monomer concentration is a conserved quantity and the polymer dynamics is inherently of Rouse type [23]. In mechanism 2, micelles slowly evolve into vesicles, so their ability to trap hydrophilic molecules should be extremely reduced. This low loading efficiency of hydrophilic molecules has been experimentally observed on vesicles made from the self-assembly of poly(ethylene oxide)-block-poly(N,N-diethylaminoethyl methacrylate) (PEO-b-PDEAMA) by a pH switch [24].

It is interesting to note that Monte Carlo simulation and experiments on lipids system are in favor of the first mechanism, suggesting that the vesicle formation mechanism for lipid and polymersomes could be the same [25–27].

1.3 Control of the polymersome size

A basic view is to consider a disk resulting from the self-assembly of amphiphilic compounds (lipids, block copolymers, . . .). Because of the line energy $E_l = 2\pi R_d \gamma$ (due to line tension, γ) existing on the edges of the disk of radius R_d, the system takes benefit from closing into a vesicle, which in counterpart costs a bending energy $E_{\text{bend}} = 8\pi\kappa$, κ being the bending modulus, phasing out the line tension. If we assume that mechanism 1 previously described is correct, then for a given area the disk radius is twice the vesicle one, and the minimal vesicle size ($R_{\min}$) that could be achieved is obtained by balancing the line energy with the bending energy, which writes

$$R_{\min} = 2\kappa/\gamma, \qquad (2)$$

γ being the line tension and κ the bending modulus of the membrane. When the line tension is around $1\,\text{kT/nm}$ and the bending modulus is of the order of $\sim 40\,\text{kT}$ which is typical for block copolymer bilayer, the minimum vesicle sizes must exceed 80 nm. The equilibrium size of the vesicle depends, for a given number of amphiphilic molecules, on a balance between the mixing entropy which tends to favor many assemblies, and the molar bending energy which favors a small number of larger vesicles (also more difficult to bend). Apart from this size aspect, it has to be noted that an increase of the bending modulus seems to favor the formation of vesicle (for block copolymer presenting a hydrophilic ratio compatible with vesicle formation). The bending modulus is known to increase with molecular weight (see sect. 1.4). It has been shown by Eisenberg *et al.* in a systematic study focusing on the block length dependence of the morphological diagram performed on different polystyrene-block-poly(acrylic acid) copolymers (PS-b-PAA) with similar hydrophilic ratio that it was easier to form vesicles from block copolymers having larger molar masses [28].

In the case of polymersomes, the molar mass and polydispersity of the hydrophilic block play an important role on the vesicle size and size distribution. It has been shown that the curvature energy of the vesicles is lowered by segregation of the hydrophilic chains, the inner leaflet being enriched with the shorter ones while the outer leaflet is enriched with the longer ones [29]. This phenomenon is a particularity of the self-assembly of amphiphilic block copolymers even though it may occur also for pegylated phospholipids, having a macromolecular polar head. Moreover, Eisenberg *et al.* demonstrated, by blending a series of PS-b-PAA block copolymer presenting similar PS molecular weight but variable PAA molecular weight, that vesicle size tends to decrease when the polymolecularity of the hydrophilic chains increases, due to this segregation

Table 1. A comparison between liposomes and polymersomes membrane properties.

	Liposomes	Polymersomes	Scaling with membrane thickness
Bending modulus (kT)	11–30 [46]	40–460 [38,40]	$d^{\sim 2}$ [38]
Stretching modulus (mN/m)	250 ± 2 [46]	80–100 [36]	$d^{\sim 0}$ [36]
Lysis strain (%)	5 [36]	20–50 [36]	$d^{\sim 0.6}$ [36] (for $M < M_c$)
Membrane surface shear viscosity (mN/ms)	10^{-5} [47]	$15 \cdot 10^{-3}$ [40]	-
Membrane thickness (nm)	3–5 nm [48]	10–50 nm [36]	$M^{\sim 0.55}$ [36,49] or $M^{\sim 0.66}$ [50]
Water permeability (μm/s)	15–150 [51]	0.7–10 [51]	$d^{\sim -1}$ [52]
Lateral diffusion coefficient (μm^2/s)	3.8 [42]	0.12–0.0024 [42]	Rouse scaling

phenomenon that tends to increase the membrane curvature [30,31]. Interestingly the vesicle size polydispersity has also been reported to decrease in the meantime, leading to this counter-intuitive result that more polydisperse chains lead to more mono-disperse assemblies!

It is worthwhile to note that all these considerations are only valid at the thermodynamic equilibrium. However, it is well known that vesicles are experimentally often trapped or in non-equilibrium state (frozen state). Lipids and block copolymers present low solubility and exchange between unimers and self-assembled species is extremely slow. Therefore, the vesicle size is often strongly dependent on the fabrication process. For instance, the spontaneous formation of uniform polymer vesicles by the diffusion of water into self-assembled micron-size patches of block copolymer printed on a silicon substrate has been shown by Ryan *et al.* [32]. In this configuration, the final size of the polymersomes was dictated by the area of the patch. In a recent study performed on poly(trimethylene carbonate)-b-poly(L-glutamic acid) (PTMC-b-PGA) diblock copolymers, our team showed another example of the importance of the process used on the size of the vesicle [33]. Vesicles were obtained by two different techniques, either by direct dissolution in water or by the solvent injection method (also called "nanoprecipitation"). Large vesicles presenting relatively high dispersity were obtained by direct dissolution, whereas smaller polydispersity indexes of the vesicles were obtained by the nanoprecipitation process. In this last method, which can be seen as a nucleationgrowth process, parameters such as the order of addition (water to solvent addition or opposite), addition duration, copolymer concentration and solvent affinity to water play a crucial role on the growth of the self-assembled copolymer aggregates and consequently on the final size of the vesicles. By playing with these parameters that control the diffusion kinetics and the local concentration during self-assembly, we were able to obtain vesicles ranging from ~ 50 nm to ~ 500 nm in radius with polydispersity indexes ranging from 0.02 to 0.25.

1.4 What is known about polymersomes' membrane properties?

Vesicles obtained by self-assembly of amphiphilic molecules comprise essentially liposomes, constituted of lipid, and polymersomes constituted of block copolymers. As stated before, liposomes were the first synthetic analogues to cells. Due to their biocompatibility (when natural lipids are used) and ability to encapsulate hydrophilic or hydrophobic molecules, they have been used a lot as models to understand the behavior of cell membranes and for drug delivery studies. However, even if their properties can be modulated (*e.g.*, increase of bending modulus by cholesterol insertion or by partial pegylation of the phosholipid head) a lipid bilayer keeps a relatively fluid character with a low surface shear viscosity, thin membrane thickness (3–5 nm) and low maximum strain before lysis, limiting their stability. Besides, they present a highly permeable membrane to small neutral molecules such as water, alcohols or sugars [34]. Since their inception, polymersomes have been often compared to liposomes [35] because of their similar amphiphilic character of their constituting units. However, comparison to viral capsids seems more appropriated since the molecular weight of block copolymers is in the range of the amphiphilic proteins constituting the viral capsids and because of their similar robustness and low permeability. The properties of polymersomes have been studied essentially on coil-coil diblock or triblock copolymers. Typical values and scaling laws are summarized in table 1 and compared to values commonly observed for liposomes. If the membrane thickness d was measured mostly by TEM for sub-micron size polymersomes, a number of physical properties were obtained on giant vesicles using different techniques such as the suction method inside a micropipette as for liposomes [36–40]. Using the micropipette aspiration technique, it has been shown that the resistance to membrane dilation (stretching) of a polymersome is only governed by the interfacial tension between the blocks and therefore controlled by polymer chemistry and solvent quality. The characteristic values of stretching moduli are below those reported for lipids [36]. However, polymersomes membrane appear to be far more viscous than liposomes as stated by membrane viscosity [40,41] and lateral diffusivity measurements [42]. As a consequence, the energy barrier for pore formation and the lifetime of a pore once formed are considerably higher values than in lipid bilayer membranes [43,44]. Moreover, the fluidity of the membrane decreases as molecular weight increases [42] according to a Rouse scaling. This toughening of the membrane is more pronounced above a critical molecular weight of the

polymer because of chain entanglement of the hydrophobic blocks. The ability of a membrane to be bent appears to scale with d^2, as shown by measurement of bending moduli at different layer thicknesses [38], in agreement with existing theories on bilayer membranes [45].

One of the first approaches to modify membrane physical properties of polymersomes consists in mixing block copolymers with similar chemical structures to obtain vesicles with a homogenous repartition in the membrane and to cross-link one of the components. The extent of cross-linking, X, was therefore directly related to the molecular composition of the blend [39]. Above a critical value of X the membrane lost its fluid character and the mechanical behavior presented strong similarities with red cells in which laterally cross-linked structures of proteins (spectrin skeleton) exist. A complex behavior relative to bending and stretching is thus obtained, modifying the stress response to deformation compared to the one of a fluid membrane. The tension of rupture as well as apparent moduli can reach orders of magnitude higher than for non-cross-linked sample.

One of the great advantages of amphiphilic polymers compared to phospholipids is that membrane thickness can be modulated by playing on the molecular weight of the hydrophobic block. As the thickness of the membrane governs most of their physical properties, its control is relevant and directly related to the molecular weight of the block copolymers. However, only a few systematic studies have been performed, exclusively on coil-coil block copolymers. Poly(ethylene-oxide) as hydrophilic block, poly(butadiene) [36], (PBut) poly(ethylethylene)(PEE) [49] and poly(butylene oxide)(PBuO) [53] as hydrophobic block were considered. Data from studies focusing on other issues can be found in the literature and concerned polystyrene-block-poly(acrylic acid) and poly(2-vinylpyridine)-block-poly(ethylene oxide) block copolymers [54,55]. Membrane thicknesses were measured using TEM and cryoTEM techniques [36,49,55] sometimes completed by SAXS [53] or SANS [54] analysis. The scaling laws obtained are all summarized in fig. 4. The membrane thickness scales with $M^{\sim 0.5}$ for PBut and PEE, and this scaling has been confirmed by simulation (step-wise coarse-grain approach) for membrane thickness higher than 7 nm [49]. The exponent reflecting a non-perturbed state of the chain (Gaussian like) in the membrane could be surprising in a first approach. However, it has been proposed that in the case of PBut-b-PEO the PEO chains collapse at the interface between the PBut blocks and water shielding the hydrophobic membrane even further [56]. It is intriguing to note that in another study, by mixing different PBut-b-PEO to obtain a series of block copolymer presenting variable molecular weight and polydispersity in the range of those investigated in a previous study [36], a scaling law $M^{0.66}$ has been obtained (data not shown in fig. 4) [50]. Nevertheless, this result was not commented related to those obtained in ref. [36].

In case of PBuO-b-PEO, for a roughly similar number of hydrophobic units the scaling law is close to 0.66, in agreement with strong-segregation theory based on
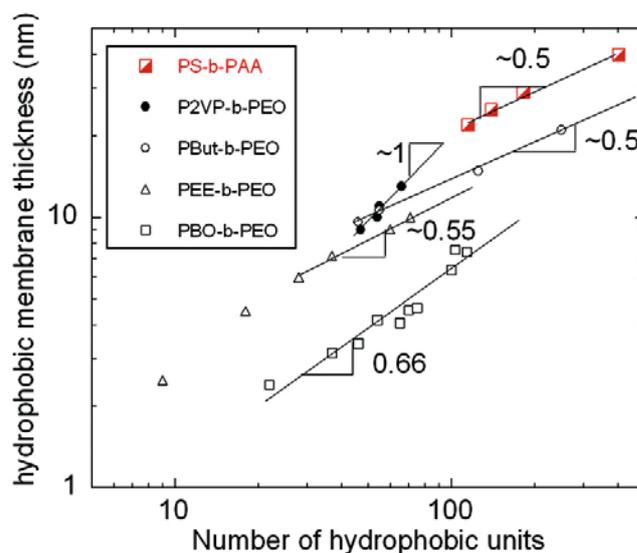
Fig. 4. Master curve of the evolution of the membrane thickness with number of hydrophobic units of the block copolymers, built from data reported in the literature.

the interplay between chain entropy and interfacial tension [57,58]. Interestingly, a scaling law with an exponent close to 0.83 has been found by numerical simulation [49] reflecting a more stretched or ordered configuration of the chains for membrane thickness below 7 nm. A careful look at the data shows that even in the case PEE-b-PEO, the exponent seems to increase for $DP < 20$ (or $d < 7$ nm), (fig. 4). This would confirm that at low bilayer thickness the chains tend to be stretched in the membrane. Another explanation for the 0.5 exponent experimentally observed for PBut-b-PEO and PEE-b-PEO has been suggested by Srinivas *et al.* based on their simulation studies [49]. At very low membrane thickness, (*e.g.* when the molecular weight of the hydrophobic block is around 1000 g/mol, typical of the hydrophobic tail of a lipid) the chains are stretched and the density of the hydrophobic block in the bilayer center is very low. When membrane thickness increases, this hydrophobic density dip is smoothed illustrating interdigitation and melting of the two leaflets, which presumably results from an effective increase in chain flexibility that opposes strong segregation and stretching due to interfacial tension. In this way the classical strong-segregation limit exponent of 2/3 is reduced to an exponent of 1/2 that is more typical of chains in a non-constraint melt state.

Finally from a series of poly(2-vinylpyridine-*b*-ethylene oxide) (P2VP-block-PEO) block copolymers [54] the membrane thickness scales as $M^{\sim 1}$, which is characteristic of a completely stretched chain. Although the range of molecular weights investigated is more restricted compared to other studies, the value of thicknesses has been measured using Cryo TEM and SANS and good agreement has been obtained between the two methods [54], limiting the uncertainty of this scaling law. Other data obtained on a series of Polystyrene-b-Poly(acrylic acid) (PS-b-PAA) [55] show a scaling law $d \sim M^{\sim 0.5}$ in agreement with data obtained on PBut-b-PEO and PEE-b-PEO.

Globally it seems that the scaling law $M^{\sim 0.5}$ is universal for coil-coil block copolymer but is obtained only above a certain critical length or molecular weight of the hydrophobic block. Below this value, which seems to be specific to every system, a more extended conformation is obtained and the exponent varies. To our opinion, systematic studies are clearly needed to clarify these transitions in which entanglement and interdigitation of chains play a subtle role.

The absolute value of membrane thickness for a given hydrophobic block length is another important aspect. In the following we comment results obtained with PEO as hydrophilic block, which is not visible in TEM, the technique essentially used to determine bilayer thicknesses. In the case of PBuO-b-PEO for roughly similar chain length the membrane thickness is below those measured in PBut-b-PEO. This has been ascribed to the fact that PBuO form more flexible chains than PBut ones. Thicknesses reported for vesicular membrane of poly(propylene)oxide-block-poly(ethylene oxide) copolymers are in reasonable agreement with those reported of PBuO-b-PEO for a similar number of hydrophobic units (5 nm for 68 units) illustrating the higher flexibility of a PBuO backbone compared to a polyvinyl chain [59]. However PEE-b-PEO and PBut-b-PEO present similar membrane thicknesses for a similar degree of polymerization, suggesting that their hydrophobic blocks are in comparable coil state.

It has to be noted that a study realized on poly(styrene)-block-poly(acrylic acid) polymersomes reported that the membrane thickness decreases when vesicle size decreases below a critical value of the radius. The authors showed that the relation between size and thickness was directly linked to repulsive interactions between PAA chains in the internal cavity of the vesicle. This evolution is rather particular and is probably suitable only for a limited class of polymersomes with polyelectrolyte coronas [60].

The permeability of polymersomes is far below the value encountered for liposomes (typically 10 to 20 times lower) as illustrated in table 1. The thickness of a membrane seems to be, at least for passive diffusion, the most obvious parameter which can influence permeability to small molecule. Only a few studies give quantitative information about polymersomes' permeability. Most of the data available concern permeability to water. Values of $0.8\,\mu$m/s, $2.5\,\mu$m/s and $7\,\mu$m/s have been reported, respectively, for poly(2-methyloxazoline)-b-poly(dimethylsiloxane)-b-poly(2-methyloxazoline) (10700 g/mol) [61] poly(ethylene oxide)-b-poly(ethylethylene) (3900 g/mol) [37] and for poly(butyl acrylate)-b-poly(acrylic acid) (5500 g/mol) [51] and are well below the order range commonly observed for liposomes (15–150 μm/s).

There is only one study which reports, for a given series of block copolymer poly(butylene oxide)-block-poly(ethylene oxide), on the relationship between permeability and membrane thickness. Such measurements have been performed by quantifying the permeation from outside to inside of a small molecule (5, 5'-dithiobis-2-nitrobenzoic acid), which forms instantaneously a colored complex with a previously encapsulated hydrophilic molecule allowing UV titration. Permeability scales as d^{-1} as predicted by the first Fick's law, d being the thickness of the membrane [52].

2 Tuning of the polymersome membrane permeability

In the following we will present and discuss recent approaches that have been used to modify the permeability or the ability to deliver small solutes previously entrapped in the polymersomes (fig. 1).

2.1 Stimuli-responsive vesicles

Polymersomes present the great advantage to be a nice structure with stability far above their lipid analogues, liposomes. This is due to a higher lysis strain conferred by a higher membrane thickness. The drawback is a low permeability to small molecule, as previously illustrated, that can lead to severe limitation in terms of applications of theses object as drug carriers and nano- or micro-reactors. Therefore different approaches have been elaborated to modify the permeability of polymersomes' membranes. The use of stimuli-responsive block copolymers is one elegant way but most often the stimulus leads to a morphological transition or a simple disruption of the vesicle because of the membrane tension created, leading to a brutal escape of a previously entrapped molecule. In the past ten years many research groups then used different triggers to make polymersome responsive to temperature [62–67], pH [68–74], redox [75–78] or light [79–83] for the most studied and biologically relevant systems. These different approaches have been recently reviewed [5,7].

In the following we will essentially comment the approaches which consist in modulating the polymersomes permeability or the ability to release [33] an entrapped molecule, using stimuli-responsive block copolymers but with maintaining the integrity of the vesicles when the stimulus is applied (*i.e.*, no destruction or disassembly). One method that has been employed is to cross-link the stimuli-responsive blocks after self-assembly into vesicles prior to the application of the stimulus. Quite recently Du and Armes have developed a hydrolytically self-cross-linkable copolymer, poly(ethylene oxide)-*block*-poly[2-(diethylamino)ethyl methacrylate-*stat*-3-(trimethoxysilyl) propyl methacrylate], [PEO-b-P(DEA-*stat*-TMSPMA)] which forms vesicles in THF/water mixtures. The hydrophobic P(DEA-*s*-TMSPMA) block forms the vesicle membrane, which can be subsequently cross-linked by the *in situ* sol-gel reaction of TMSPMA in the membrane in aqueous solution. PDEA is pH responsive and becomes soluble in water at low pH because of its protonated amines. This produces some leaky sites in the membrane in aqueous solution at pH < 7, whose size and number depend on the pH reached. Increasing back pH above 7, PDEA becomes

hydrophobic again and leaky sites disappear. Playing with the ratio DEA/TMSPMA the authors were able upon pH variation to create volume variation of the vesicles between 22 and 123%, indicating variable permeability of the vesicle walls, the integrity of the vesicle being ensured by the *in situ* sol-gel chemical cross-linking [69,84].

Recently, Eisenberg *et al.* have found an elegant way to control the size and permeability of polymersomes, using asymmetric triblock copolymer poly(ethylene oxide)$_{45}$-*block*-polystyrene$_{130}$-*block*-poly(2-diethylaminoethylmethacrylate)$_{120}$ (PEO$_{45}$-b-PS$_{130}$-b-PDEA$_{120}$) self-assembled into vesicles at pH 10.4, where PDEA is hydrophobic [85]. They clearly demonstrated that the membrane was made of 5 layers in the following order: PEO-PS-PDEA/PDEA-PS-PEO. In other words, the PDEA blocks were trapped between two glassy PS layers. This leads to striking features like a controlled increase of the vesicle size accompanied by a controlled increase of the vesicle thickness when pH decreases from 10.4 to 3.4. This was caused by the swelling of PDEA layer by protonation and stretching of the PS chain to limit the interfacial area with PDEA chain which becomes hydrophilic. Interestingly at low pH (< 6) the PS layers crack to release the stress created by the high pressure of PDEA layers but the vesicular morphology remains intact.

From a kinetic analysis of the swelling and de-swelling of the vesicles, the authors were able to estimate the diffusion coefficient of water and of protons through the membrane. Real values of permeability were not evaluated because of the variation of layer thickness during swelling process but it was shown that in the swollen state the vesicles were highly permeable to water and that permeability to proton was strongly dependent on pH.

Very recently, by using polymersomes made from the self-assembly of a temperature-responsive poly(trimethylene carbonate)-block-poly(L-glutamic acid) copolymer, our group demonstrated a temperature-increased permeability and mobility inside the membrane which favor the Fickian diffusion of entrapped doxorubicin molecules through the membrane [86].

Finally, very recent studies have shown that polymersome's membrane properties could be controlled by adding amphiphilic photo sensitizer molecules in membranes of non-stimuli-responsive polymersomes. Depending on the concentration of the photo sensitizer, its chemical nature and the chemical nature of the hydrophobic polymer block, the membrane permeability and mechanical properties could be fine tuned with light irradiation [87]. The polymersome's membrane can be also affected by adding conjugated multiporphyrin chromophore solute in the membrane and Dextran [88] or luminal protein solute ferritin [89] in the hydrophilic cavity of the vesicle. In that case it is suggested that these hydrophilic compounds interact with the inner leaflet of the polymersomes causing a differential response of the two leaflets of the membrane upon illumination of the dye. This leads to subtle behavior of the membranes: stretching, fusing, budding, invagination or simple disruption can be observed under optical
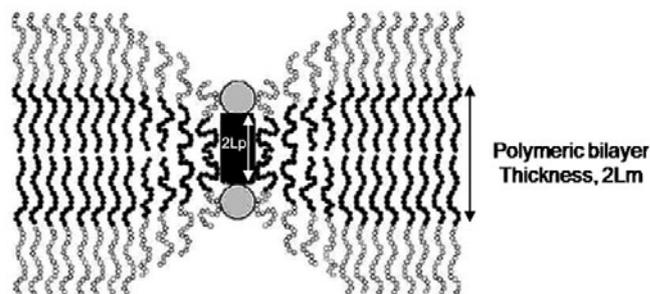
Fig. 5. Conformation of polymer chains near a protein inclusion in a polymeric bilayer: $2Lm$ is the thickness of a flat bilayer, $2Lp$ the inclusion thickness. Adapted from [90].

excitation, depending of the amount and molecular weight of the hydrophilic macromolecules encapsulated.

2.2 Membrane formulation

2.2.1 Incorporation of natural protein

Integral membrane proteins are one of the essential components of cell membranes and play an important role in the transport of nutrients or cell communication. Incorporation of channel proteins in vesicles membrane is a step towards the development of artificial cells and for applications such as micro- or nano-reactor and drug delivery. For polymersomes it implies that the polymer chains adopt the dimension of the protein in its immediate neighboring [90]. This can be done by the establishment of a compression profile of the chains close to the protein in addition to a natural local segregation of the chains due to their polymolecularity, which tends to concentrate short chains around the protein (fig. 5). The first successful incorporation of a membrane protein in polymersomes has been reported in the early 2000's [91].

Up to now all the results concerning protein insertion have been obtained using exclusively poly(2-methyloxazoline)-b-poly(dimethylsiloxane)-b-poly(2-methyloxazoline) (PMOXA-b-PDMS-b-PMOXA) block copolymers. The extreme flexibility of the hydrophobic block may allow the block copolymer membrane to adopt, without considerable loss of energy, the specific geometrical and dynamical requirements of membrane proteins to keep their functionalities. That feature seems to be unique for the polymer membranes since lipid bilayers, the natural component of cell membrane, are nearly incompressible due to the low number of possible configurations of their hydrophobic tails within the membrane.

The incorporation of proteins into polymersomes membrane can fine-tune their permeability to different species, depending on the nature of the protein. Selective transport of calcium ions into the intra-vesicular space has been achieved using in N, N-dicyclohexyl- N', N'-dioctadecyl-3-oxapentane-1,5-diamide (ETH5234), an ionophore which is specific for calcium ions transport. This has been proven by following mineralization of calcium phosphate into a vesicle which was formed in phos-

phate buffer then incubated in CaCl$_2$ solution after addition of a small amount of ionophore [92]. On similar block copolymer (PMOXA-b-PDMS-b-PMOXA) the use of non-specific membrane channel bacterial porin (Ompf) led to the creation of a water-filled channel allowing passive diffusion of small solutes. In that case the authors chose to evaluate the diffusion of ampicillin inside the cavity of the polymer vesicle, rendered possible by the presence of Ompf in the membrane. When entered inside the vesicle ampycillin is hydrolyzed by a previously entrapped enzyme. The product of this hydrolysis (ampicillinoic acid) is ejected from the vesicles through the protein channel and induces the reduction of starch iodine in the outside medium which is followed by UV measurement [91]. In another case, Ompf allows the diffusion of protons and triggers the reaction of a *p*H-sensitive enzyme which then transforms inside the vesicle cavity a soluble non-fluorescent substrate into a non-soluble fluorescent one [93]. The use of a channel protein has been shown also to be very useful for selective and efficient local activation of drug precursors by enzymatic hydrolysis in their cytotoxic base, which can be advantageous for tumor treatment to decrease systemic toxicity [94]. As previously, the polymersome is used as a nano-reactor in which a pro-drug activating enzyme (purin specific nucleoside hydrolase *Trypanosoma vivax* (TvNH)) is encapsulated into its cavity annihilating its immunogenicity. The polymersomes were permeabilized by incorporation of OmpF or Tsx, two bacterial outer membranes channel forming proteins, called porines. OmpF works as a molecular sieve, allowing concentration-driven diffusion of solutes $< 600\,\mathrm{Da}$, whereas Tsx allows specific transport of nucleosides and nucleotides. Small substrates can diffuse into the interior of the polymersomes where they are activated by the enzyme (TvNH). The resulting products can then diffuse outside the vesicle. Interestingly the kinetic of hydrolysis is directly related to the molar amount of OmpF. However, at sufficient high concentration of OmpF or using the specific transporter TsX, the kinetics of hydrolysis is higher than that of the free enzyme. This result is not yet fully understood. Incorporation of a channel membrane protein has been shown to be very useful to develop more complex bio-mimetic structures. It has been reported that bacterial channel forming protein (LamB) can been successfully inserted into artificial membranes of nanometer size polymersomes and still works as a receptor of bacteriophages, allowing the translocation of DNA into polymersomes [95].

Water permeability can be also dramatically changed by incorporating water channel proteins into the membrane. Quantitative data have been obtained on PMOXA-b-PDMS-b-PMOXA vesicles containing various molar amount of Aquaporin Z, a protein naturally found in the eye-lens cells [61], by analyzing the contraction of vesicle suddenly mixed with $1.7\,\mathrm{mol/L}$ of NaCl with a stopped flow apparatus. The authors established that, depending on the molar ratio of channel protein with respect to the block copolymer, the water permeability can be 70 to 800 times higher compared to the permeability without protein. The activation energy of water transport was evaluated with and without protein inside the membrane. The values found for pure block copolymer vesicles ($\sim 9\,\mathrm{kcal/mol}$) and for protein-loaded block copolymer vesicles ($\sim 3\,\mathrm{kcal/mol}$) indicate that water transport is mediated by diffusion through the polymer in the absence of protein whereas a channel-mediated water transport is evidenced in presence of Aquaporin.

If the incorporation of membrane protein channels can modify the permeability of polymersomes, the molecular architecture of the block copolymer can also play a major role on the activity of a certain class of reconstituted proteins. Recently it has been shown that the oxidoreductase activity of the NADH:ubiquinone complex (complex 1), when reconstituted into PMOXA-b-PDMS-b-PMOXA polymersomes membrane, not only keeps its activity as in a phospholipid membrane environment but can also be modulated by the respective sizes of the hydrophobic and hydrophilic blocks [96]. Typically an increase of hydrophobic length increases its activity whereas the opposite is observed by increasing the hydrophilic length. This has been explained by a higher mechanical stress imposed by the protein on the membrane and the conformational flexibility of the copolymers which could favor a more active conformation of complex 1. The activity decrease when hydrophilic chain length increases has been tentatively explained by a perturbation of proton translocation involved in the oxidoreductase catalytic mechanism.

2.2.2 Incorporation of artificial proteins

Even if the incorporation of natural channel proteins in polymersomes' membranes is the most elegant approach to selectively control their permeability, it appeared to be a very difficult task that can only be realized with extremely fluid membranes (*e.g.*, with PDMS as the hydrophobic block). As an alternative to natural channel protein, dendritic dipeptides and dendritic esters have been shown to self-assemble into helical pores in polymersomes' membranes made from the self-assembly of poly(butadiene)-block-poly(ethylene oxide). Their function was evaluated by proton transfer experiments [97]. Proton transport was evidenced using a *p*H-sensitive fluorescent dye (8-hydroxypyrene-1,3,6 trisulfonic acid) (HPTS) loaded in polymersomes and a precise controlled addition in the vesicle's suspension of NaOH or HCl to modulate *p*H in the extra-vesicular medium. A very clear proton transport was observed upon the insertion of dendritic species in the membrane, and increases accordingly to the number of dendritic pore per vesicle, whereas pure polymersomes were impermeable to protons. Polymersomes with pores made from dendritic esters showed a far greater proton transport compared to the one loaded with dendritic dipeptides. The authors proposed an explanation based on the better solubility and stability of dendritic ester in the bilayer compared to the dendritic dipeptides. Another possibility could be proton hopping across the peptide system which could inhibit transport of protons in the dendritic dipeptide pores [97]. It is interesting to note that, unlike all the previous cited works about the incorporation of channel membrane proteins in PMOXA-b-PDMS-b-PMOXA

triblock copolymer vesicles, this study on dendritic helical pore incorporated polymersomes has been realized on PBut-b-PEO diblock copolymer ($M_n = 3800$ g/mol). The incorporation of dendritic ester and dipeptides into PMOXA-b-PDMS-b-PMOXA polymersomes was indeed not successful as ascribed to a significant difference of solubility between PDMS ($\delta = 7.9$) and Pbut ($\delta = 8.5$) and of chemical architecture.

It has to be noted that some block copolymers do not need artificial transport mediators in the membrane to exhibit specific permeability or, in other words, show natural permeability to small molecules while being impermeable to large solutes. This is the particularity of Polystyrene40-b-poly(isocyanoalanine(2-thiophen-3-ylethyl)amide)$_{50}$ (PS-b-PIAT) [98–100].

2.3 Gelification of the internal cavity

Up to now most of the works on gel-filled vesicles has been realized on liposomes. These structures are well known for a long time and a non-negligible activity is devoted to the improvement of their stability as well as their use as a cellular model. However, the lack of equivalents to cytoskelton within their primitive structures limits the relevance of giant lipidic vesicles as model systems for chemical reaction kinetics, migration, diffusion studies in the context of biological cells. Filling a phospholipid vesicle with a gel can be a way to add internal complexity to a structurally simple vesicular system and to modify its membrane properties. The reader could refer to a recent review on the subject which covers the different strategies to incorporate gels and fibrillar networks into liposomes and their effect on their shape, stability and compartmentalization [101]. In the present context we are focusing on the modification of permeability induced by the incorporation of gelified structures into polymersomes. The consequence on mechanical properties of the vesicle membrane will be described in sect. 3.2.

Relatively few results are available on the influence of the presence of gelified structures in the cavity on the membrane permeability. According to the results obtained on gel-filled polymersomes and liposomes by small molecule transport studies and vesicle behavior under osmotic shock [102,103], it appears that the diffusion of the species is essentially governed by the membrane and that the role of the entrapped gel is negligible. In these studies the entrapped gel probably behaves as a porous medium with a characteristic mesh size much larger than water molecules.

Modification of the vesicle permeability by incorporation of a hydrogel needs probably two criteria to be fulfilled. Firstly, the characteristic mesh size of the gel must probably be lower than the membrane mesh size, implying a very high cross-link density of the hydrogel. Secondly, the membrane must be tethered to the network by some interaction in order to prevent a loose structure between the hydrogel and the membrane that would favor "free" permeation of solutes. These two conditions were fulfilled in a recent study concerning poly(N-isopropylacrylamide)

PNIPAM hydrogels inside polymersomes of PDLLA-b-PEG [104], prepared by the solvent injection technique. Compared to empty polymersomes, PNIPAM-filled polymersomes presented a clear slowing down of release kinetics of encapsulated FITC labeled dextran (4000 g/mol) at 37 °C, a temperature at which PNIPAM chains are partially dehydrated and form a gelified network structure. In their study the authors showed that the PNIPAM hydrogel polymerized preferentially in the vicinity of the bilayer, forming an additional barrier for release of encapsulated drug and limiting free membrane area for permeation. It was proposed that the PEG of the vesicle shell may form hydrogen bonds with the amide side-group of the PNIPAM chains, as supported by a previous work on PNIPAM-filled liposomes [105].

3 Tuning of the polymersomes' mechanical properties

In the following we will describe the different approaches used to modify mechanical properties and stability of vesicle membrane. It must be emphasized that most of the physical properties of polymersomes' membrane were measured on giant vesicles, allowing the observation of the vesicle behavior under different conditions (pipette suction, osmotic shock, ...) with a simple optical microscope. However, a non-negligible part of the applications of polymersomes imply a nano-scale size, typically under 200 nm for drug delivery applications. The question arises if the mechanical properties and scaling laws established on giant vesicles can be transposed at nano-scale. Some elements of information have been recently brought by Chen *et al.* [55], using AFM measurements which have been already used to probe mechanical properties of nano-scale liposomes [106]. Using a series of polystyrene-b-polyacrylic acid block copolymers with different molar masses, the authors were able to show that bending moduli of the membrane were two orders of magnitude higher compared to values encountered for lipid bilayers and follow a scaling law with the membrane thickness as for giant vesicles [38], although the exponent was slightly higher (2.5 instead of 2). However an increase in membrane thickness also resulted in a decrease of the apparent Young's modulus, typically from 60 MPa at 22 nm to 43 MPa at 40 nm. According to the authors, this would coincide with a decrease of stretching of PS chains in the membranes, which then would show a more isotropic elastic behavior.

3.1 Incorporation of artificial proteins

As illustrated before, the incorporation of either dendritic peptide or dendritic ester into vesicles can dramatically modify their permeability towards protons. It is obvious that the incorporation of different components in a membrane can only be advantageous if these components bring specific properties without modifying other functional or physical properties of the membrane. The first relevant

Table 2. Modification of mechanical properties of PBut-b-PEO polymersomes by incorporation of dendritic esters and peptides.

(%mol)	Dentritic ester incorporation				Dentritic peptide incorporation		
	0	2.5	11	20	0	1	5
Stretching modulus Ka (mN m^{-1})	125	84 ± 8.7	88.9 ± 14.5	105.6 ± 29.9	125	93.3 ± 32.6	153.5 ± 34.2
Critical area strain	≥ 15	≥ 15	≥ 15	≥ 20	≥ 15	≥ 22	≥ 11

parameter to consider is the mechanical stability of the "modified" membrane, often evaluated by micropipette aspiration techniques on giant vesicles ($\sim 20\,\mu m$ diameter) [97]. The vesicle areal strain was measured and plotted *versus* the applied membrane tension and compared to the unloaded polymersomes. The renormalized area stretching modulus (Ka) obtained from the slope of the curve, as well as estimates of the lysis area strains are summarized in table 2.

The modification of the membrane properties is strongly dependent on the molar amount of the dendritic species as well as their nature. Globally a softening of the membrane vesicle (*e.g.*, greater deformation for same applied stress) is obtained when dendritic ester is incorporated up to 11 mol%, but the softening effect is less visible when reaching higher dendritic ester contents (*e.g.*, 20%). In contrast, the incorporation of dendritic peptide results in the softening of the membrane at very low level content, whereas a hardening of the vesicles is clearly obtained at moderate level (5%). The authors suggested that aggregation of dendritic peptides occurs if they are incorporated above their solubility threshold, leading to the stiffening of the membrane. In all cases the critical area strain is almost unchanged or only a slight increase is observed, except for the highest incorporation of dendritic peptide, demonstrating the stable incorporation of these dendritic molecules in PBut-b-PEO polymersomes.

3.2 Gelification of internal cavity

In biological cells a network of protein filaments is linked to the inner leaflet of the membrane via integral membrane proteins (so called "cytoskeleton") and confers unique mechanical stability and visco-elastic properties to the cell. Therefore mechanical measurements can be a good way to attest the stability of gel-filled vesicles. Up to now most of the work has been realized on gel-filled liposomes with limited use of polymersomes. In poly(N-isopropylacrylamide) (NIPAM) gel-filled vesicles of DOPC/DPPC mixture, where the hydrogel was linked physically to the membrane [107,108], micropipette aspiration experiments show that the mechanical stability is largely increased, the lysis tension being 30 times larger than the lysis tension of a pure DOPC membrane. Increase in the tension that can support a gel-filled vesicles membrane has been also reported in phospholipid vesicles filled with poly(allylamine) chains (partially protonated with acetic acid) cross-linked with poly(ethylene glycol) diglycidyl ether [109]. However the authors suggested that
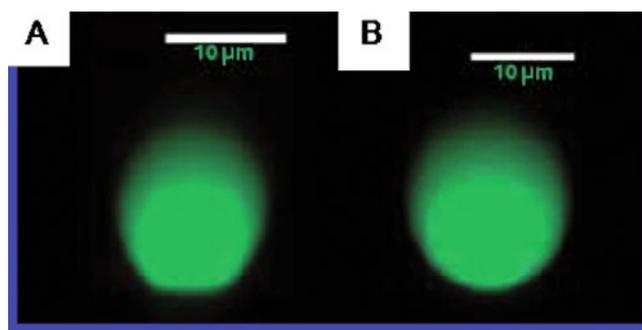
Fig. 6. Dependence of the deformation of Hydrogel Containing Vesicle (HCV) PEO$_{89}$-PBut$_{120}$ with hydrogel cross-link density. The pictures are images of HCVs that have fluorescein acrylate conjugated to the interior hydrogel network. The left image (A) is of a HCV at a 38:1 acrylamide to methylenebisacrylamide ratio and the right image (B) is of a HCV twice more reticulated, which presents a lower contact area with the substrate and a lower deformation compared to the unperturbed spherical shape. Adapted from [111].

these modifications were driven by the interior (*e.g.* the gel) and not really by the bilayer itself.

The stiffness of the vesicles can also be modulated by the incorporation of a gel in the cavity. Micropipettes aspiration experiments carried on in the low deformation regime allow measuring the Young's modulus of the elastic vesicles [110]. DOPC vesicles filled with PNiPAM cross-linked by UV irradiation exhibit moduli values between 500 Pa and 25000 Pa [107] which are similar to that of common cells like *Dicytostelium fungi*, endothelial cells, fibroblasts, cardiocytes and inactivated or activated platelets. This increase in rigidity is also observed in polyacrylamide gel-filled polymersomes made from the self-assembly of PBut-b-PEO, which show a lower deformability when deposited on a glass surface (fig. 6). This deformability is linked to the extent of cross-linking of the gel inside the vesicle [102].

3.3 Molecular design of the amphiphilic block copolymers

3.3.1 Rod-coil block copolymers

As described previously, the essential knowledge of the membranes' properties of polymersomes has been acquired using coil-coil block copolymer for the elaboration of the vesicles. Despite their strong tendencies to form vesicles [73,112] the impact of rod-coil block copolymer on

the membrane properties of vesicle has not been studied, probably because the vesicles formed up to now were most often in the nano-scale range, limiting the utilization of classical techniques adapted on giant vesicles, like micropipette suction experiments.

Quite recently Min-Hui Li and coworkers have studied with this technique the mechanical properties of giant polymersomes made from the self-assembly of block copolymers with PEG as the hydrophilic block and liquid crystalline polymers as hydrophobic blocks [113]. Poly(4″-acryloyloxybutyl) 2,5-bis(4′-butyloxybenzoyloxy) benzoate (PA444) was used as a side-on nematic polymer and PA6ester1 as an end-on smectic polymer, which are both in a glassy liquid crystalline state at room temperature. The authors showed that under an applied suction pressure such polymersomes behave like a visco-elastic solid with a slow deformation step before reaching an equilibrium value, which allowed them to use a simple analogy to Kelvin Voigth's model to quantify their results. The vesicle long-time response led to a measurement of the stretching modulus $Ka = 81 \pm 10 \, \mathrm{mN \, m^{-1}}$ for PEG-b-PA6ester1 and $Ka = 30 \pm 15 \, \mathrm{mN \, m^{-1}}$ for PEG-b-PA444. These relatively low values seem to be contradictory at first glance with the behavior observed (slow deformation, reflecting gel-like or solid phases). Since the stretching modulus is essentially linked to the interfacial tension ($Ka = 4\gamma$) which is in the range of 20–30 $\mathrm{mN \, m^{-1}}$ for an oil/water interface, this attested the coherency of the value obtained for PEG-b-PA6ester1. The authors suggested that Peg-b-PA444 should present a lower Flory interaction parameter (*i.e.*, a lower incompatibility of the blocks), justifying the very Low Ka value.

At short times, the deformation is dominated by the viscous response of the Kelvin Voight's material. Membrane surface viscosities were found to be $\eta_s = 7.9 \pm 0.2 \, \mathrm{mN \, m^{-1} \, s}$ for PEG-b-PA6ester1 and $\eta_s = 30 \pm 15 \, \mathrm{mN \, m^{-1} \, s}$ for PEG-b-PA444. This is 5 to 6 orders of magnitude above surface viscosities of lipid bilayers and 3 orders of magnitude above coil-coil polymersomes [40]. Rescaling surface viscosities by membrane thickness to convert values in "bulk" viscosities gives a value of $10^6 \, \mathrm{Pa \, s}$ typical of polymer melts with a very high molecular weight (10^6 g/mol). The origin of this huge surface viscosity is not yet understood and cannot be ascribed to inter-leaflet or lateral chain entanglement, as demonstrated for coil-coil block copolymers [49].

3.3.2 Dendrimersomes

In the field of vesicles obtained by self-assembly, "Janus" dendrimers have been introduced as another type of amphiphilic macromolecules in addition to block polymers [114]. By synthesizing a large library of amphiphillic Janus dendrimers coupling different amphiphilic building blocks in an arborescent manner, Percec *et al.* performed a systematic study of their self-assembly behavior. Using different preparation techniques, they were able in some cases to observe the formation of vesicles that they called dendrimersomes. The size dispersion of those vesi-

cles seems to be lower than commonly observed for liposomes and polymersomes. Preliminary experiments on giant dendrimersomes showed that their stretching moduli Ka can be tuned over a wide range between 42 and 976 $\mathrm{mN \, m^{-1}}$, which is comparable to liposomes containing cholesterol added to their membrane and above the highest values reported for coil-coil block copolymer polymersomes. This very high stiffness contrasts with a quite low critical strain lysis (5%) similar to those of liposomes, which is still 4 to 10 times below values encountered for polymersomes. Interestingly a pore-forming protein (melittin) could be inserted in the dendrimersomes' membranes as easily as in a phospholipid membrane, thus reinforcing their structural and functional similarities with liposomes.

4 Emerging approaches

Membrane properties of polymersomes can be modulated by numerous methods among which, the molecular design of the amphiphilic constituting unit is up to now the most frequently employed (stimuli-responsive block, rod-coil or even dendrimeric block, etc.). However this chemical approach can be sometimes tedious. Formulation of the membrane by addition of different components is another approach that can be advantageously used to tune their properties as illustrated in sect. 2.2 and 3.2. In the following, we will comment other formulation approaches that seem very promising to modulate the membrane properties of polymersomes and to strengthen their biomimetic character.

4.1 Hybrid membranes (incorporation of nanoparticles)

The embedding of nanoparticles into vesicles is an important issue in medical science, nanotechnology and industry, and can lead to various applications like catalysis, drug delivery and sensing technology, depending on the nature of the particles. If the incorporation of nanoparticles in polymersomes has already been performed, the current knowledge of the keys factors that govern the assembly process of such hybrid structure is limited. Different complex phenomena can occur in block copolymers bilayers stuffed with nanoparticles such as morphological transition (bilayers to vesicles) as was observed for PS-b-PAA bilayers loaded with iron oxide magnetic nanoparticles [115]. The size and shape of particles and the interactions developed with the block copolymer constituting the membrane in which they have to be inserted are all factors that will determine the formation (or not) of a hybrid membrane and its final morphology. For instance, gold nanoparticles (Au-Np) have been inserted in polymersomes either by *in situ* formation of the particles after the membrane formation [69,116] or by direct incorporation of preformed nanoparticles during the self-assembly process [117]. With the last approach, stable hybrid polymersomes could not be obtained above a critical amount of nanoparticles ($\sim$ 65 polymer/particle ratio) and the

Au-Np were not distributed evenly in the polymersomes made of PBut-b-PEO block copolymers: only a part of them contained a large amount of nanoparticles whereas the majority was empty. This was attributed to a favorable interfacial stabilization of the polymersome membrane due to the presence of the nanoparticles.

It has been shown recently, using TEM, that nanoparticles could be inserted only into the central portion of the polymersome walls, *i.e.* approximately halfway between the external and internal interfaces. This was realized by coating the particles surface with diblock copolymers presenting the same chemical composition that the block copolymer used for the preparation of the polymersomes (PS-b-PEO or PS-b-PAA) [118].

When hydrophobic particles are inserted into polymersomes membrane, the question arises about the nanoparticle size limit allowing their insertion and on the consequences on the membrane stability. Recently Mueller *et al.* [119] have proven that quantum dots coated with oleic acid/oleyl amine as ligands could be inserted in PBut-b-PEO polymersomes' membrane using different preparation modes (co-solvent or hydration method). Cryo TEM images reveal that the quantum dots were inserted in the middle of the vesicle shell right between the two poly(butadiene) layers creating a curvature into the assembled shell. This was rendered possible by the fact that only hydrophobic interactions between the quantum dot and block copolymer could be established and because of the relative small size of the particles considered (~ 5.5 nm diameter) compared to the hydrophobic layer of the membrane (~ 16 nm) as in previous studies on hybrid magnetic polymersomes [120–122]. There is probably an upper limit in the size of the nanoparticle relatively to the bilayer thickness above which the insertion is not possible but this has not been established yet. A systematic study with different nanoparticles' sizes and membrane thickness is necessary to reveal how far the double layer can curve to embed nanoparticles.

A study was conducted in that purpose by Ghoroghchian *et al.* [123] who studied the capacity of PBut$_{46}$-b-PEO$_{30}$ polymersomes to incorporate large hydrophobic molecules using multi-porphyrin-based fluorophores (PBF) of different characteristic lengths from 1.4 to 5.4 nm. They demonstrated that the polymersomes' membrane can be loaded up to 10 mol% of PBF relative to polymer even for PBF that are larger than half the thickness of the bilayer polymersome's membrane (9.6 nm). However, the repartition and precise localization of PBF inside the membrane was not commented. Interestingly the loading did not affect significantly the mechanical toughness and the critical areal strain (lysis strain) of the membrane attesting the stability of the resulting "hybrid" polymersomes.

Among the different particles that can be inserted into polymersomes membranes, magnetic nanoparticles are very interesting candidates because they offer in the context of therapeutic applications different possibilities in addition to their contrast enhancing character in Magnetic Resonance Imaging. Nano-carriers can be magnetically moved to the specific cells or organs by applying external magnetic-field gradients. Using ultra-small super-paramagnetic iron oxide (USPIO) as inorganic load, tumors can be thermo-ablated by hyperthermia thanks to the USPIO heating effect in radio-frequency magnetic fields (RF). This effect can also be exploited to release an encapsulated drug provided that the nano-carrier undergoes a transition (*e.g.* the main chain transition T_{m} from gel to a fluid state for a lipid membrane or a LCST for a thermo-sensitive polymer such as PNiPAM) under application of RF magnetic field. Such an approach, named magneto-chemotherapy, is original in the therapeutic research.

The added value brought by magnetic nanoparticles on vesicles regarding drug delivery applications has been very recently proven on nano-scale liposomes [124]. The incorporation of super-paramagnetic iron oxide nanoparticles (SPIO) within lipid bilayers at different lipid-to-nanoparticles ratios allows a controlled release triggered by RF magnetic field of carboxyfluorescein used as a model drug and shows that spontaneous leakage (without application of magnetic field) decreases with increasing nanoparticle loading. This was attributed to an increased bilayer stability brought by the magnetic nanoparticles and a reduction of the lipid surface area available for leakage through the bilayer for decorated magnetoliposomes compared to unloaded liposomes. Recently in an analogous study [125] the anti-cancer drug Doxorubicin was encapsulated into magnetic vesicles with a lipid membrane (DPPC) initially in the gel state and becoming fluid at $T_{\mathrm{m}} = 42\,^{\circ}\mathrm{C}$, a temperature reachable by magnetic hyperthermia. A massive release of encapsulated DOX was indeed observed under the application of a RF magnetic field even though the macroscopic temperature of the sample remained below T_{m}.

Despite these promising results, the incorporation of magnetic nanoparticles (MNP) into liposomes can lead to morphological modification such as rigidification and faceting depending on lipid and MNP concentration [124, 126]. Therefore polymersomes could be an alternative to liposomes for such applications.

Pioneering work concerning the incorporation of MNPs in polymer vesicles was performed in our team [120–122]. We have loaded iron oxide MNPs presenting a hydrophobic coating and with a diameter close to 8 nm into vesicles formed by poly(butadiene)-*b*-poly(L-glutamic acid) (PBut-b-PGA) block copolymers with a hydrophobic membrane thickness of 14 nm. Interestingly we could control the deformation of the resulting hybrid vesicles under magnetic field, attested by anisotropic Small Angle Neutrons Scattering (SANS) measurements. The deformation of vesicles under a magnetic field seemed to be an interesting way to trigger the release of entrapped molecules, as expected from the membrane tension created during the deformation that possibly leads to the formation of pores. However there was no experimental data available to prove that this phenomenon really occurs. More recently other teams have incorporated magnetic nanoparticles into polymersomes made from the self-assembly of poly(isoprene)-block-poly(ethylene oxide) block copolymers [127]. In that case probably because

of the higher dimension of the nanoparticles ($\sim 14\,$nm) compared to the bilayer thickness ($12\,$nm) and incomplete miscibility with PI domains, the nanoparticles were situated at the interface of PI/PEO. This induced binding of adjacent bilayers bridged by nanoparticles, so that exclusively multi-lamellar vesicles (onions typed) were obtained, showing a strong magnetophoretic mobility.

In our group, we are working currently on the incorporation of hydrophobically modified MNPs of maghemite (γ-Fe2O3) in the membrane of poly(trimethylene carbonate)-b-poly(L-glutamic acid) (PTMC-b-PGA) block copolymer vesicles using a nanoprecipitation process [128]. The resulting hybrid vesicles are highly magnetic (up to $70\%\,$w/w of magnetic nanoparticles) and present a deformation under magnetic field of low intensity ($0.1\,$T). These magnetic vesicles exhibited a long-term colloidal stability and showed suitable properties for biomedical applications: being guided by an external magnetic field gradient obtained with a small permanent magnet they also showed an important contrast enhancement in Magnetic Resonance Imaging with a particularly low (sub-nanomolar) detection limit. Moreover, the drug release rate of encapsulated doxorubicin could be enhanced twice under the application of a RF oscillating magnetic field at $500\,$kHz, presumably by the melting of the semi-crystalline PTMC blocks induced by a local hyperthermia effect only at the scale of the membranes (but non macroscopically).

4.2 Blend approaches

Another way has been envisaged very recently to modulate and enrich the functionality and physical properties of polymersomes based on a simple mixing approach. The first experiments performed aimed at modulating the release kinetics of vesicles by hydrolytic degradation of one component of the bilayer. This was realized with "hybrid" vesicles prepared using hydrolysable diblock copolymers of poly(ethylene glycol)-block-poly(-L-lactic acid) (PEG-b-PLA) or poly(ethylene glycol)-block-polycaprolactone (PEG-b-PCL) blended at different molar ratios with a non-degradable, PEG-based block copolymer (PBut-b-PEO) [129].

In our opinion blending different block copolymers into one vesicle is a simple and versatile way to enrich the functionalities of these systems and the previous example is only a brief insight into the possibilities that can be reached. One can imagine to modulate precisely the surface functionality of the membrane (*e.g.* for biological targeting), to associate the advantages of robustness and great controlled permeability by mixing large-molecular-weight neutral block copolymers with stimuli-responsive block copolymers or inserting channel proteins. Besides, the blending approach can be a step towards more sophisticated membrane structures by the creation of "domains" analogous to the lipid "rafts" or to the points of focal adhesion enriched in integrins within cell membranes.

Very recently Discher *et al.* used a mixture of block copolymers with either a neutral or a negatively charged hydrophilic block (PBut-b-PEO and PBut–b-PAA) to prove that mesoscale domains can be induced by addition of divalent cations in a relatively narrow window of *p*H and cation concentrations, centered near the characteristic binding constants for polyacid interactions [130]. This was attested by confocal microscopy and micromanipulation techniques. It was shown that the effective rigidity E_{app} of the membrane can be modulated by *p*H or divalent cation concentration according to a power law form $E_{\mathrm{app}} = A([\mathrm{ion}]/K)^a$ where [ion] is either [H$^+$] or [Ca^{2+}]. The exponent a is negative considering protons, whereas it is positive for calcium ions, illustrating a decrease of rigidity when *p*H decreases and an increase of rigidity when calcium concentration increases. This is linked to the fact that deprotonation causes calcium bridging of polyacrylate leading to lateral segregation into stable mesoscopic gels. Interestingly, the shape of the domains can be controlled via the nature of the cations (calcium or copper) and their number via the hydration time offering numerous possibilities of structural organization and functionalization of these self-assembled synthetic structures.

One may point out that a drawback of polymersomes is their low permeability. This can be turned into an advantage if the hydrophobic block constituting the bilayer of the membrane can adapt a channel protein or if the block copolymer is specially designed to respond to an environmental stimulus. Another approach consists in blending block copolymer with phospholipids well known to form permeable vesicles. Meier *et al.* have shown [131] that such hybrid structures could be obtained by mixing PMOXA-b-PDMS-b-PMOXA ($M_w \sim 9000\,$g/mol) with natural egg phosphatidyl-ethanolamine (Egg-PE) and dipalmitoylphosphatidylcholine (DPPC) at the giant scale ($> 5\,\mu$m) or at nanometric scale ($\sim 100\,$nm). The authors did not focus their effort on the resulting properties of the vesicles but rather on the proof of concept for such a hybrid structure as well as on the repartition of the lipid in the vesicle membrane, which was found to be homogeneous whatever the molar amount of lipid relatively to copolymer. However, the intermediary properties of these hybrid vesicles were shown by a freeze-thawing process where the lipid part allows encapsulation of a model protein, above a certain molar lipid-copolymer ratio, whereas no encapsulation was possible by this process for pure polymersomes.

Blending of block copolymer and lipid has also been used to modulate permeation properties of polymersomes using lipid as a "sacrificial" component of the vesicle membrane. In that case PBut-b-PEO was mixed with 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). After vesicle formation the PBut-b-PEO chains were cross-linked, allowing the extraction of the phospholipid part by Triton X-100 leading to porous polymersomes [132]. This was assessed by the measurement of the longitudinal relaxivity r_1 of the previously encapsulated gadolinium chelates. This parameter r_1 defined as the slope of the T_1 decay time of nuclear spins of water protons *vs.* complexed Gd concentration is known to strongly depend on the exchange rate between bulk water and water

bound to the chelated Gd ions. This relaxivity is strongly increased when the phospholipid part was removed to form a porous polymersome, very permeable to water.

Conclusions and outlooks

Since their inception in the late nineties, polymersomes have seen their properties modulated *via* numerous approaches either based on molecular design of the block copolymers (molar mass and its dispersity for each of the blocks, stimuli-responsive character, cross-linkable block, rod-like or coil character, . . .) or on the incorporation of different components into the membrane or inside the aqueous cavity (membrane formulation).

Most of the physical properties have been studied on coil-coil block copolymers and systematic studies performed in the beginning of the 21st century gave a good overview of the relation between block copolymers' molecular structure and the essential membrane physical characteristics (thickness, viscosity, bending and stretching elasticity, . . .) although still some points remain to be clarified concerning the scaling law established for thickness. Only a few studies have given quantitative information on vesicles made from rod-coil block copolymers and it seems that the major modification compared to "classical" copolymer vesicles deals with membrane viscosity. The origin of such modification is not explained yet. Very recently amphiphilic block dendrimers have been used to generate vesicle called dendrimersomes that finally present all the characteristics of liposomes in terms of mechanical properties and functionality (protein insertion possibility). Another point concerns the knowledge of the physical properties of polymersomes at nanoscale. Up to now, information has been acquired essentially on giant vesicles. However numerous applications imply the use of nanoscale vesicles, for example in parenteral administration of drugs. Therefore the knowledge of the mechanical stability is important, as, for example, during the circulation in the blood vessels the vesicles will be submitted to variable mechanical stress. Some preliminary results have been given by AFM measurements. A structural analysis of nano-scale vesicles (by cryo-TEM or scattering techniques) at different osmotic pressures could be useful to get information about their permeability and elastic properties.

In addition to molecular design, membrane formulation allows the change of physical properties (by incorporation of channel proteins, nanoparticles or simply different amphiphilic species to elaborate the membrane) and can improve the structural analogies with natural cell (*e.g.*, incorporation of gelified structure to mimic cytoskeleton). This approach seems in our opinion particularly promising for various aspects especially in the elaboration of systems with pronounced architectural and functional biomimetic character. Indeed an elegant and subtle approach would consist in designing an artificial cell model by combining lipids and copolymers into a hierarchical structure mimicking not only the plasma membrane but also the organelles within it, realizing basic functions of biological cells such as ion transports triggered by an external stimulus such as light or an electric field.

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