



Assessing internal structure of polymer assemblies from 2D to 3D CryoTEM: Bicontinuous micelles

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ABSTRACT

The self-assembly behaviour of block copolymers in solution has been of significant interest over the past two decades for a number of applications – for example, as delivery vectors and micro-reactors. More recently, attention has turned to the formation of aggregates with complex internal structure, such as multi-compartment micelles and the so-called “Janus” particles (biphasic aggregates) for their promising application as vectors for the simultaneous inclusion of chemically-different encapsulates and their possible catalytic and templating properties. The challenge has been to observe these complex aggregates in such a way as to be able to characterise their internal morphology whilst preserving their intricate structure. To this end, cryogenic transmission electron microscopy (cryoTEM) has become a powerful and indeed a necessary tool for the elucidation and observation of self-assembled polymer systems. Through its use, a new class of complex micelles has been discovered: amphiphilic block copolymer nanospheres with internal bicontinuous structure. These structures have been observed from a variety of block copolymer amphiphiles, although rarely. Intriguingly, there is no seemingly obvious unifying blueprint for their formation. This review will present the importance of cryoTEM in the elucidation and characterisation of internally-structured polymeric aggregates in recent years and highlight its significance in the definition of bicontinuous dispersions.

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1. Introduction

It is well established that in concentrated aqueous solution, block copolymer amphiphiles can organise into a variety of morphologies; for example, spherical and cylindrical micelles, vesicles [1,2] and more exotic morphologies such as toroids, multi-lamellar vesicles, disk-like micelles and internally-structured nanospheres [3]. Microscopy is an essential part of their characterisation. Traditional TEM techniques such as negative-staining TEM require the removal of water, which prevents an in situ view of the particles and can cause the collapse of soft matter, significantly affecting the structure [4,5]. The heavy metal stain is used to provide and enhance contrast for aggregates constructed from elements with a low atomic number (such as carbon, hydrogen and oxygen). The addition of staining can often obscure any complex internal detail and may even interact with the polymeric structures, causing further deformations. CryoTEM supersedes dry TEM in that the aqueous environment remains undisturbed and the aggregates can be observed in their hydrated state. For cryoTEM analysis, the aqueous dispersion is deposited upon a “holey” carbon coated TEM grid. The

excess water is removed by blotting with filter paper and subsequently, the grid is vitrified by plunging it into liquid ethane; thus forming a thin film of solid amorphous water. This captures and preserves the structures so that they can be observed in their hydrated, near-native state. The vitrified amorphous water in the holes then provides a transparent window through which the aggregates can be observed at cryogenic temperatures (between -160 and -170 °C) without the need for staining or dehydration (Fig. 1).

To avoid damage of the organic structures, or the crystallisation of the amorphous film due to exposure to the electron beam, a low electron dose is used (<100 $e^-/\text{Å}^2$). The inherently low contrast is generally enhanced by observing the objects at negative defocus values, making use of phase contrast imaging.

The contribution of cryoTEM to the progress in the field of polymeric self-assembly has been vital. Through manipulation of architecture and composition, amphiphilic block copolymers can aggregate into a variety of complex morphologies. Dry TEM alone for the characterisation of polymeric aggregates may lead to misinterpretation of the obtained images, and any internal structure may be overlooked. For example, Fig. 2 shows the negative staining micrograph and cryoTEM image of micelles with complex internal structure. The internal detail is significantly obscured by the uranyl acetate staining of the hydrated hydrophilic surface, but is revealed in the vitrified state in the cryoTEM image. This

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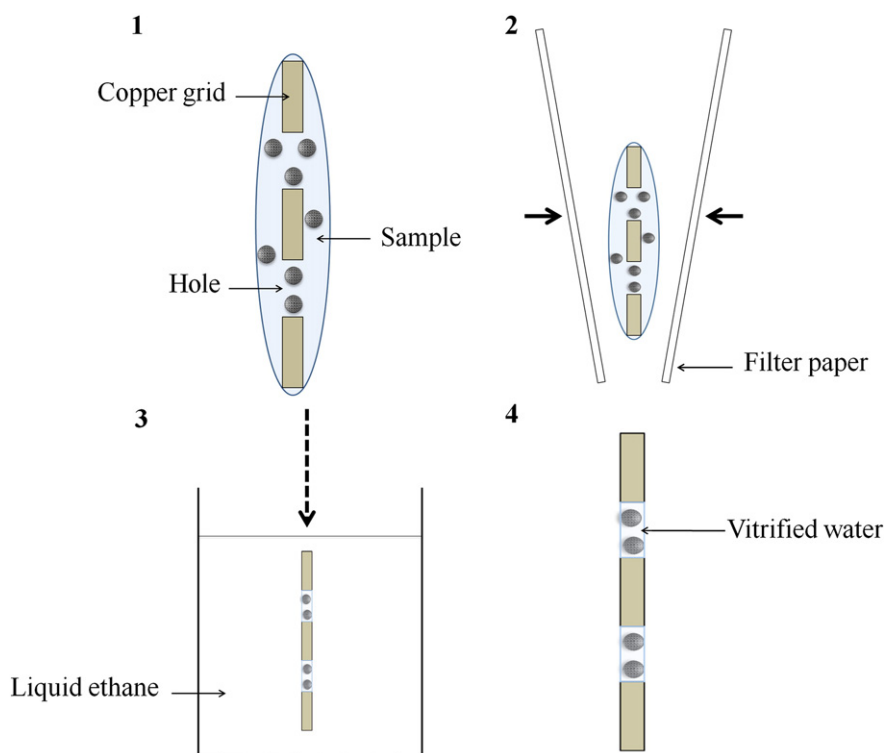


Fig. 1. Representation of the cryoTEM sample preparation method: 30 μL of the aqueous dispersion is deposited onto the grid (1) and the excess blotted away with filter paper to form a thin film (2). The grid is then plunged into liquid ethane (3) to form a vitrified aqueous layer (4).

underlines the importance of cryoTEM as an essential tool for studying polymeric self-assembly.

2. CryoTEM in the study of complex polymer self-assemblies

Aggregates with complex internal structure from amphiphilic di-(AB) and triblock (ABC or ABA) copolymers have received much attention and more recently, are being specifically designed for use as catalytic nano-compartments, delivery vectors for the simultaneous inclusion of chemically different materials and as templates for the organisation of inorganic materials [6–8]. Examples include concentric vesicles [9], spheres with internal hexagonal and lamellar structure [10] and hexagonally packed hollow hoops [11]; however, traditional TEM techniques were employed to visualise these morphologies. Another

example is the design and formation of multi-compartment micelles [12]. Multi-compartment micelles are typically formed from block copolymers containing more than one solvophobic block (ABC triblock copolymers). The blocks phase-separate to form segregated regions within the aggregate core. Further complexity arises when the block copolymer architecture is altered from linear to graft, star-block or miktoarm structure. By directing the relative block compositions, the internal morphology of such aggregates can also be tuned to form “hamburgers”, segmented cylinders and the so-called “raspberry” structures [13••] which further highlights the complexity in the self-assembly of such block copolymers. In addition to this, Laschewsky et. al. [8] discovered an entire range of new morphologies from linear ABC, BAC and ACB triblock copolymers, including core-shell-corona micelles, “soccer ball” micelles and, for the first time, bispherical micelles.

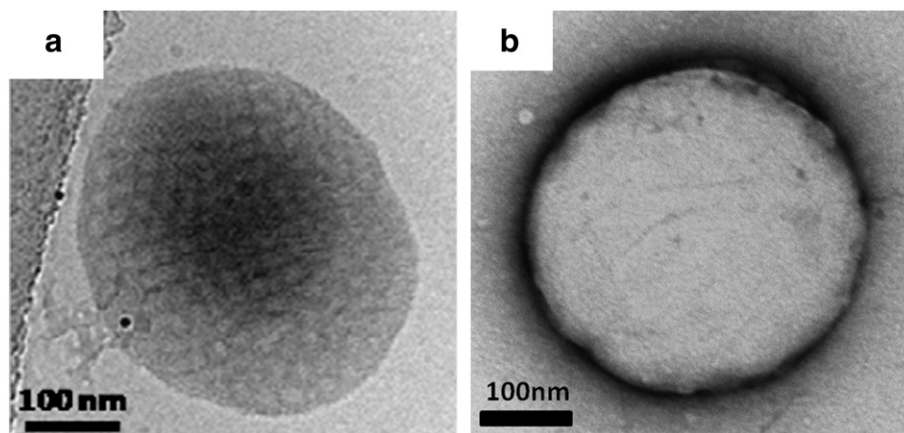


Fig. 2. a) CryoTEM and b) negative-staining cryoTEM 2D images of poly(ethylene oxide)-block-poly(octadecyl acrylate) nanospheres with internal bicontinuous morphology. In a) the sample is vitrified at 4 °C. In b) the sample is observed at room temperature and stained with a solution of 5% uranyl acetate in 1% acetic acid. The internal intricacy is clear in the cryoTEM micrograph; however, the structure is not apparent in the stained particles.

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Of course, the discovery and classification of these novel morphologies is in no small part due to the use of cryoTEM. The effect of variables such as block composition and the preparation conditions upon the internal structure of complex micelles can conveniently be monitored using cryoTEM [13•, 14•, 15,16]. Müller et. al. [17] reported the observation of multi-lamellar and multi-compartment micelles from block copolymers of poly (ethylene oxide)-*b*-poly (*n*-butyl acrylate) (PEO-*b*-P(*n*BA)) through cryoTEM, in which the morphology of the aggregates was strongly dependent upon the length of the P*n*BA block, the initial polymer concentration and the initial cosolvent composition.

2.1. Polymer assemblies with bicontinuous internal structure

In water, the bicontinuous phase [18] forms through the twisting of the block copolymer bilayer into a three dimensional structure with high periodicity, composed of interconnected aqueous channels containing the solvated hydrophilic moieties within a continuous network of the hydrophobic core. Previously, the bicontinuous phase for block copolymers was only observed in bulk melts and in the presence of surfactants [19–21]; however, more recently the bicontinuous morphology has been observed in discrete nanoparticles of aqueous block copolymer dispersions and from a variety of block copolymers [14•, 22•, 23•, 24•, 25•]. It is noteworthy that the majority of these reports have arisen within the last five years. Their formation from block copolymers with dissimilar structural and chemical properties presents future prospects for their use in different applications.

The first evidence for discrete block copolymer particles with bicontinuous internal structure was presented by Eisenberg et. al. [26•] already in 1996. The particles were formed from a highly asymmetric PS-PAA block copolymer (PS₁₉₀-*b*-PAA₂₀) in the presence of NaCl and in a DMSO-water solvent mix. Their structure was inferred

from TEM projection images and proposed to be a three-dimensional network of interconnected rods (Fig. 3a). Wooley and coworkers [22•] also presented the formation of internally-structured nanoparticles from ABC triblock copolymers of poly (acrylic acid)-*block*-poly (methyl acrylate)-*block*-polystyrene (PAA-PMA-PS) with tuneable morphology through alteration of the preparation conditions and relative block composition. Bicontinuous internal structure was proposed for PAA₉₄-PMA₁₀₁-PS₁₈₈ at a THF:water ratio of 1:0.2 (Fig. 3b). Also in this case, the assessment of the internal structure was based on 2D images and no further investigation of the internal arrangement was undertaken.

A further approach was taken by Hamley et. al. [24•] where the addition of tannic acid to a Pluronic ABA triblock copolymer (poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide), PEO₂₀-PPO₇₂-PEO₂₀) led to the formation of nanospheres with an apparent bicontinuous internal structure (termed “emulsomes”) (Fig. 3c). Most recently, particles with a similar appearance were observed from a diblock copolymer of poly (tert-butylacrylate)-*b*-poly (2-[(perfluorononyl) oxy] ethyl methacrylate) (PtBA-*b*-PFNEMA) in a THF-water mix using scanning electron microscopy (SEM) and TEM [25•]. By altering both the preparation temperature and the THF:water ratio, both the internal and surface structures could be tuned. At a water content of 67 vol.%, the particles were proposed to have a cylindrical phase upon the surface which coexisted with an internal bicontinuous phase (Fig. 3d); but no additional definitive characterisation of the internal structure was presented.

2.2. Cryo electron tomography: assessing internal structure

A limitation of cryoTEM – as well as of conventional TEM – is that the image produced is a two-dimensional projection of a three-dimensional

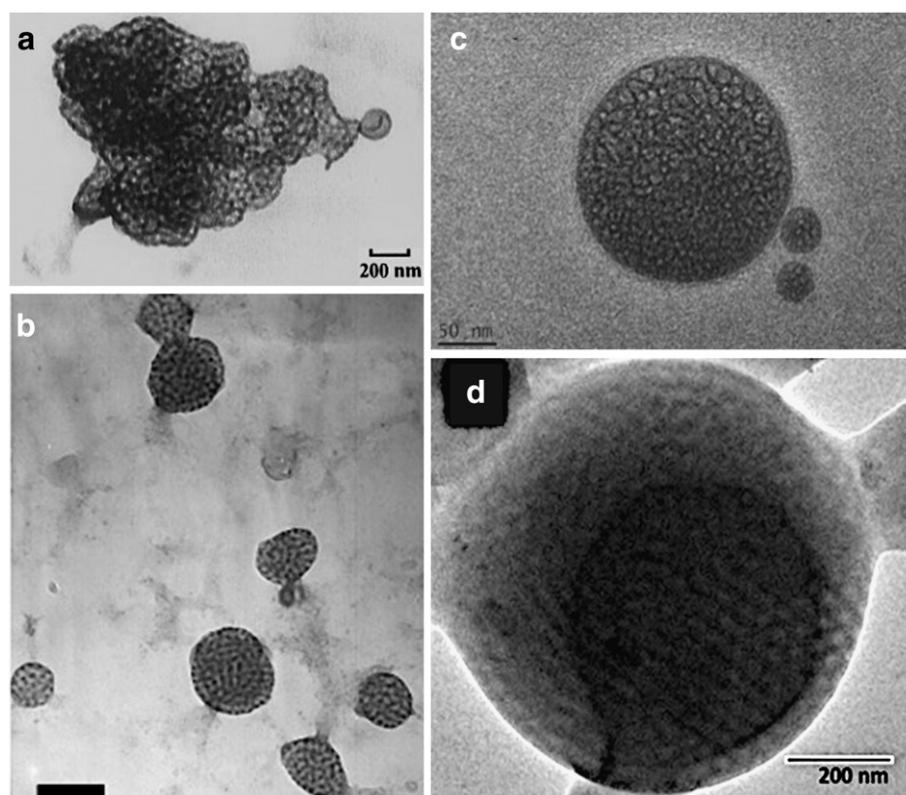


Fig. 3. TEM images of block copolymer aggregates with bicontinuous internal structure: a) aggregate with bicontinuous structure of interconnected rods from a PS-PAA block copolymer in a DMSO-water solvent mix with NaCl. Adapted with permission from Langmuir 1996;12:5980–4. Copyright 2012 American Chemical Society; b) PAA-PMA-PS aggregates in a THF-H₂O mix (ratio 1:0.2). Reprinted with permission from Nano Lett 2008;8:2023–6. Copyright 2012 American Chemical Society. c) So-called “emulsomes” from a Pluronic in water with tannic acid. Adapted with permission from Soft Matter 2011;7:10116–21. Copyright 2012 Royal Society of Chemistry. d) A nanoparticle with apparent bicontinuous internal structure from a PtBA-*b*-PFNEMA block copolymer in a THF-water solvent mix. Adapted with permission from Soft Matter. 2012;8:2471–6. Copyright 2012 Royal Society of Chemistry.

object. Often, internal features may overlap in projection and so their individual contribution cannot be discerned, which can result in inaccurate assessment of morphology and domain sizes [27]. Also, 2D-TEM images do not yield volume information and so the difference between features inside or simply resting on top of the observed object may not be easily discernible [28]. 2D-TEM images therefore cannot conclusively characterise the internal structure of such polymeric assemblies. However, through the use of cryo-electron tomography (cryoET) a 3D reconstruction of the 2D object can be obtained, yielding more complete structural information on the inspected volume [29]. In this technique, images of the vitrified object are acquired at different tilt angles, then aligned at a common point (usually a fiducial marker such as gold nanoparticles) to account for the drift of the object that occurs as it is mechanically tilted (Fig. 4) [30]; and finally, the images are collated and reconstructed using a mathematical algorithm such as weighted back projection and simultaneous iterative reconstruction technique (SIRT) [5]. Although not necessary, computational generation of the rendered 3D volume can be employed to aid visualisation of the tomograms and different colours can be used to highlight separate domains [4].

Dry ET has been utilised to characterise microphase-separated polymeric nanostructures from as early as 1997 [31]; but in partnership with cryoTEM, cryoET has proved a powerful tool for the elucidation and definition of the complex intricacies in internally-structured polymeric assemblies on the nanometer scale. Although ET has found wide-spread use in the analysis of biological structures [30], its use as an analytical technique for the characterisation of synthetic assemblies is still in its infancy.

The first example of the use of cryoET for the investigation of internally-structured polymer nanospheres was illustrated by Parry et al. [23•] in 2008. They investigated dispersions formed from norbornene-based double comb-block copolymers with hydrophobic leucyl-valinyl-leucine methyl ester (LVL) and hydrophilic oligo ethylene glycol (OEG) side chains. CryoET demonstrated clearly that the internal structure consisted of a bicontinuous network of branched peptide-containing worm-like micelles intertwined with aqueous channels decorated with OEG groups (Fig. 5). CryoET allowed the accurate assessment of the diameters of both the hydrophobic domains and the aqueous channels, as well as to establish that the internal porous system was connected to the outside solution (Fig. 5e).

Following this, Laschewsky et al. [32•] presented the internal organisation of complex multi-compartment micelles from a linear ABC triblock copolymer of 2-ethylhexyl acrylate, oligo(ethylene glycol) monomethyl ether acrylate and 1H, 1H, 2H, 2H-perfluorodecyl acrylate (EHA₁₂₀-OEGA₅₀-FDA₄₀). The separate domains arise from the mutual incompatibility of each block causing segregation into the hydrophilic, hydrophobic and fluorophilic domains. CryoET definitively revealed the internal nanostructure: the fluorinated blocks formed small tube-like domains with walls denser than the internal volume, which were dispersed

in a large sphere consisting of the hydrocarbon matrix. The reconstructed volume also showed that the fluorinated domains (with sizes of 5–10 nm) had a tendency to reside close to the core/corona – something that could not be ascertained from 2D images alone.

In a more extensive study, M^cKenzie et al. [14••] presented bicontinuous nanospheres from a highly asymmetric amphiphilic block copolymer of poly(ethylene oxide) and poly(octadecyl methacrylate) (PEO-PODMA). The hydrophilic PEO block is linear, whereas the long and bulky alkyl side-chains of the PODMA segment give rise to a comb or brush-like structure in the hydrophobic block. CryoET revealed that the micellar structure consisted of twisting PODMA-rich channels intertwined with hydrated PEO channels, which were accessible from the surrounding aqueous media (Fig. 6a–d). Significantly, the semi-crystalline order in the brush-like hydrophobic segments of the PEO-PODMA block copolymers proved crucial for the bicontinuous structure of the aggregates. Upon heating the aggregate solution and vitrifying the sample above the PODMA melting point (vitrifying at 45 °C), the complex internal structure was lost and oblate-shaped aggregates were formed with internal morphology consisting of spherical domains of the hydrophilic segments enclosed in a hydrophobic PODMA matrix (Fig. 6e–g). Here, the effect of the temperature on the internal morphology could be clearly observed in the variable-temperature cryoTEM experiments. A similar change in micelle shape upon heating has also been observed for a poly(acrylic acid)-*block*-poly(n-octadecyl acrylate) (PAA-*b*-PODA), though the internal morphology was not investigated [33].

Some of these bicontinuous dispersions have structure similar to that observed in *lipid cubosomes* and *hexosomes*: dispersions of lipid nanoparticles with confined bicontinuous cubic and hexagonal structure respectively. Cubosomes [34] have found potential application in the fields of food science and health care products, stemming from the biodegradability and non-toxicity related to the use of biocompatible lipids such as monooleins and phytanyl-based lipids, their ability to simultaneously solubilise hydrophilic, hydrophobic and amphiphilic molecules, and the high surface area available for reaction or encapsulation [35,36]. Protein-encapsulating cubosomes (or *proteocubosomes*) have already been developed for the protection and controlled delivery of proteins and enzymes [37,38]. Their internal structure is most often probed by cryoTEM (in conjunction with SAXS), which clearly reveals the beauty, intricacy and periodicity of their internal order. Although Wooley et al. have termed their bicontinuous polymer nanospheres “polymer cubosomes”, in none of the systems observed so far has the high degree of order observed in lipid cubosomes or hexosomes been approached.

2.3. Bicontinuous micelles: when and why do they form?

The bicontinuous micelles described above are formed from very structurally and chemically different block copolymers. At present,

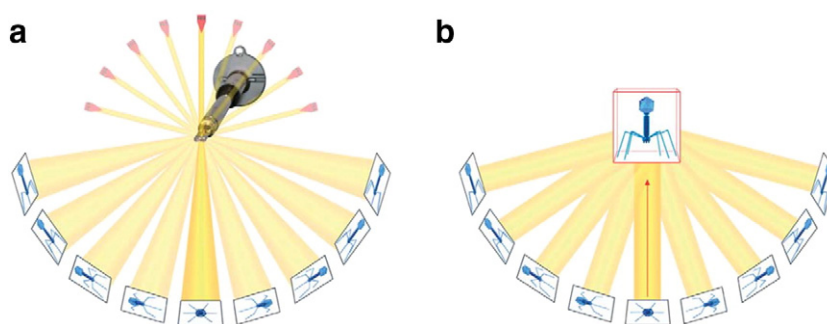


Fig. 4. Schematics representing the principals of electron tomography: In a) a tilt series is shown in which the 3D object within the EM holder (in this case, a bacteriophage) is tilted about an axis perpendicular to the electron beam and a 2D image is acquired at each tilt angle. b) represents the formation of the 3D volume using the back projection method. For each weighted projection, a back projection is calculated and the sum of all of the projected bodies represents the density distribution of the original object. Reproduced with permission from Trends Cell Biol 2005;15:43–51. Copyright 2012 Elsevier.

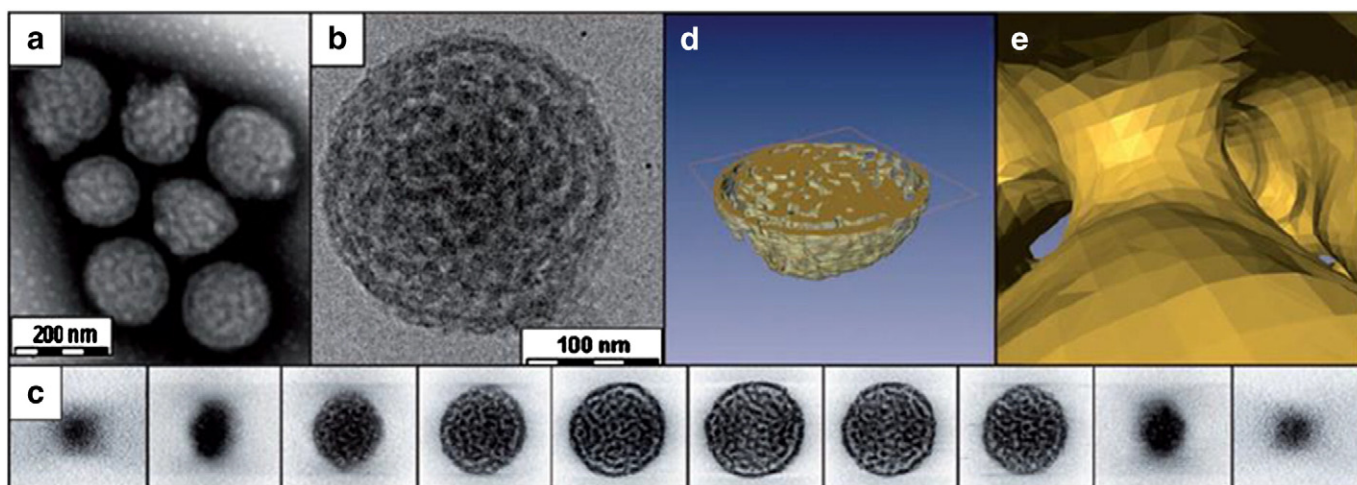


Fig. 5. TEM analysis of aggregates of PNOEG–PNGLF: a) Conventional TEM using negative staining; b) cryoTEM image of a vitrified film; c) gallery of z slices showing different cross sections of a 3D SIRT (simultaneous iterative reconstruction technique) reconstruction of a tomographic series recorded from the vitrified film in (b); d, and e) visualisation of the segmented volume showing d) a cross section of the aggregate and e) a view from within the hydrated channels. Reprinted with permission from Angew Chem Int Ed 2008;47:8859–62. Copyright 2012 John Wiley & Sons.

there is no apparent unifying blue print for the chemical structure that leads to their formation. Moreover, the apparent rarity of their occurrence suggests that they form in only a small region of the phase diagram. The self-assembly process of block copolymers is generally complex, with factors such as block copolymer architecture, relative block volume and the presence of additives having significant bearing on the observed morphology. Hence, the challenge is to understand their contribution on the current process, enabling the tailored synthesis of bicontinuous nanospheres from any block copolymer to target new and exciting applications.

For the self-assembly of block copolymers, the polymer composition, architecture and relative block volumes are fundamental parameters in the aggregation process. The morphology formed is dependent upon the stretching of the core-forming block, the interfacial tension between the solvent and core-forming block and inter-coronal chain repulsion [36]. In analogy to the packing parameter (ρ) model developed by Israelachvili [39,40] used to predict the aggregate morphology of lipids from the ratio between the molecular volume of the hydrophobic chain region, Eisenberg and Discher [41] identified a general relationship between the hydrophilic volume fraction ($f_{\text{hydrophilic}}$) of block copolymers and their preferred aggregate morphology. They found that below a hydrophilic volume fraction of 25% complex micelles are observed. Nevertheless, small differences in the polymer structure that do not affect the hydrophobic/hydrophilic balance or molecular weight, can lead to distinct differences in the aggregation behaviour. This was demonstrated for the norbornene-based amphiphilic double-graft block copolymers discussed above [23••]. CryoET showed that the internal bicontinuous structure for micelles of the polymer containing LVL side chains was lost when GLF (glycyl-leucyl-phenylalanine methyl ester) was present. Instead, worm-like micelles containing loops and branches were formed.

Aside from the block copolymer structure, the conditions under which polymer aggregates are prepared can have a significant effect upon the ultimate morphology [42]. Organic solvents [43] and ions [44] are often used to alter aggregate morphologies through manipulation of the morphology-defining factors, such as inter-coronal repulsion and core-chain stretching [45]. By modifying the preparation parameters, a variety of morphologies can be developed from a single block copolymer, by-passing the need to modify the polymer composition each time.

In contrast to the high energy processes used for lipid cubosomal dispersion preparation, aggregate dispersions of block copolymers are commonly formed by first dissolving the block copolymer in a

water-miscible organic cosolvent followed by the slow addition of water (a poor solvent of the hydrophobic block). The aggregate solution is subsequently dialysed against water to remove the cosolvent. Commonly used solvents are dimethylformamide, dimethylsiloxane and tetrahydrofuran (DMF, DMSO and THF respectively). The type and concentration of the cosolvent as well as the rate of addition of water can strongly influence the micellar kinetics and the subsequent micellar morphology [46,47]. From conventional negative staining TEM Wooley et. al. [22•] inferred that the internal morphology of their PAA-*b*-PMA-*b*-PS aggregates was tuneable by varying the solvent ratios in THF/water mixes. It was proposed that PAA₉₄-*b*-PMA₁₀₁-*b*-PS₁₈₈ produced porous bicontinuous nanoparticles at a ratio of 1:0.8, but that by increasing the volume of water, the structure altered from porous, to a lamellar channel-like structure. They suggested that the bicontinuous phase is a result of swelling of the polymer matrix by the intertwined aqueous channels containing the solvated hydrophilic block. Riegel et. al. [48•] surmised that the formation of porous nanospheres (such as bicontinuous micelles) is due to trapped solvent bubbles that create local inconsistencies in the solvent composition within the premature aggregates.

This idea was consolidated in work by Denkova et. al. [49••], which revealed that fluctuations in regional solvent composition existed in DMF/water mixes, and gave some clues as to the formation of micelles with bicontinuous interior. CryoTEM indicated that bicontinuous micelles were formed from Pluronic P123 (with structure PEO₂₀-*b*-PPO₇₀-*b*-PEO₂₀) at water contents between – 27 and 35 wt.% and were found to coexist with cylindrical worm-like micelles. Coexistence was attributed to a segregation of a polymer-rich phase containing a larger amount of DMF within the micelles, and a polymer-poor phase in the DMF-water solution where the cylindrical micelles resided. Unfortunately, these analyses were based on 2D imaging only as the extreme sensitivity of the aggregates in the DMF/water mixture to the electron beam prevented the recording of multiple images with morphological integrity of the aggregates within a tilt series.

Bicontinuous nanoparticles from amphiphilic block copolymers are rarely observed. One reason might be that the bicontinuous phase is often considered to be a complex metastable morphology (depending upon the strength of segregation between the blocks and the polymeric asymmetry) [19,50], accessed on the way to forming thermodynamically stable morphologies. From the examples given above, it is clear that several parameters may contribute to its formation and may be varied for different block copolymer systems. In any case, definitive probing of the internal organisation for

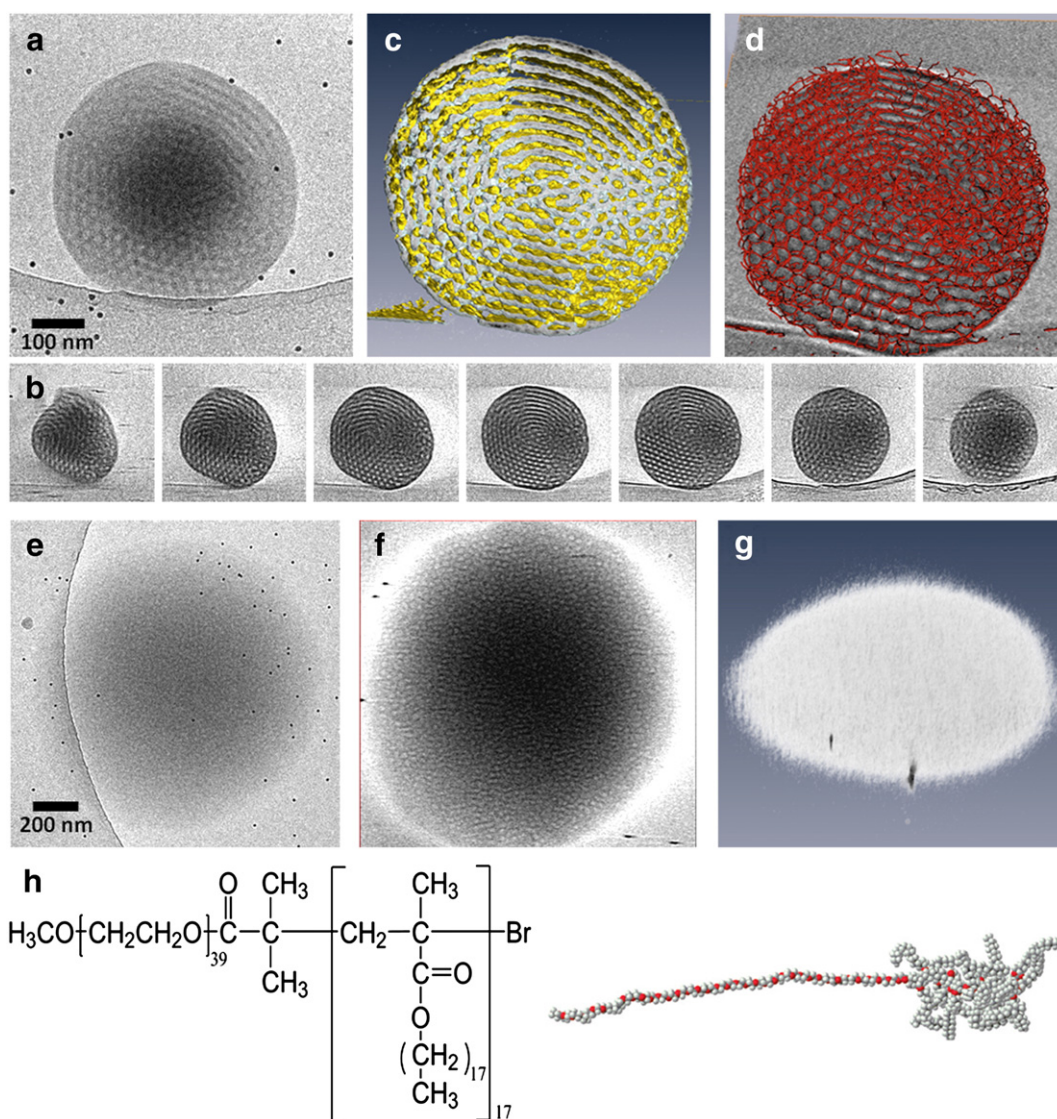


Fig. 6. CryoTEM analysis of PEO₃₉-*b*-PODMA₁₇ bicontinuous micelles: a) cryoTEM image of a vitrified film at 4 °C; b) gallery of z slices showing different cross-sections of a 3D SIRT reconstruction of a tomographic series recorded from the vitrified film in (a); c) computer-generated 3D visualisation showing only an inner section of the whole structure, where all the channels and compartments are visible (the yellow surface is outside of the polymer, the surface in contact with the water); d) skeletonisation of (c), showing only a small section emphasizing that the structure is interconnected and therefore bicontinuous; e) cryoTEM image of a vitrified film at 45 °C, showing the loss of internal order and poorer contrast against the vitrified film; f) slice from a 3D SIRT reconstruction of a tomographic series recorded from the vitrified film in e); g) volume rendering of the structure shown in e), showing the overall morphology; h) chemical structure (left) and 3D representation (right) of PEO₃₉-*b*-PODMA₁₇, highlighting the block copolymer's asymmetry. Adapted with permission from J Am Chem Soc 2010;132:10256–9. Copyright 2012 American Chemical Society.

bicontinuous nanospheres and other complex micellar morphologies is necessary to improve understanding of the self-assembly processes.

3. Conclusions and outlook

CryoTEM techniques have played an integral part in the recent advances in the formation and design of polymeric assemblies with internal morphology. By observing the particles in the hydrated state, the origin of their complexity can be safely attributed to their chemical behaviour and not to sample preparation artefacts. Through its use, new classes of exotic block copolymer aggregate morphologies have been observed and fully characterised, of which bicontinuous nanospheres are the newest and possibly the least investigated. In comparison with their lipid counterparts, “polymer cubosomes” have the added advantage of having tuneable internal morphology through changes in block composition, preparation parameters and external stimuli. Currently, it is still very challenging to specify the

contributions leading to the formation of the bicontinuous phase in discrete aggregates, and the exact combination necessary for their formation is difficult to define. Nevertheless, the complexity, high order and possible applications of bicontinuous micelles certainly qualify them for further investigation. Furthermore, their formation from block copolymers with varying chemical structures and properties – such as stimuli responsive behaviour – presents the possibility for a range of applications. To this end, an understanding of the domain compositions, arrangements and sizes is of great importance. Where 2D cryoTEM cannot, cryoET (3D cryoTEM) yields volume information and a clearer understanding of the internal organisation. Although widely used for the analysis of biological systems, the use of cryoET for synthetic polymeric assemblies is still relatively unexplored. We hope that the work presented illustrates the importance of cryoET for the investigation of polymer self-assembly and that it will inspire more scientists to use this technique for the definitive characterisation of internally-structured polymeric aggregates.

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* Of special interest.

** Of outstanding interest.