Preprint Title	Hot Shape Transformation: The Role of PSar Dehydration in Stomatocyte Morphogenesis
Authors	Remi Peters, Levy A. Charleston, Karinan van Eck, Teun van Berlo and Daniela A. Wilson
Publication Date	02 Juli 2024
Article Type	Full Research Paper
Supporting Information File 1	Supporting information.pdf; 10.4 MB
ORCID® iDs	Daniela A. Wilson - https://orcid.org/0000-0002-8796-2274

Hot Shape Transformation: The Role of PSar Dehydration in Stomatocyte Morphogenesis

Remi Peters[‡1], Levy A Charleston[‡1], Karinan van Eck[‡1], Teun van Berlo[1] and Daniela A. Wilson[1]*

[1] Institute of Molecules and Materials, Radboud University, Heyendaalseweg 135, 6525 AJ, Nijmegen, The Netherlands

E-mail: Prof. Daniela A. Wilson - d.wilson@science.ru.nl
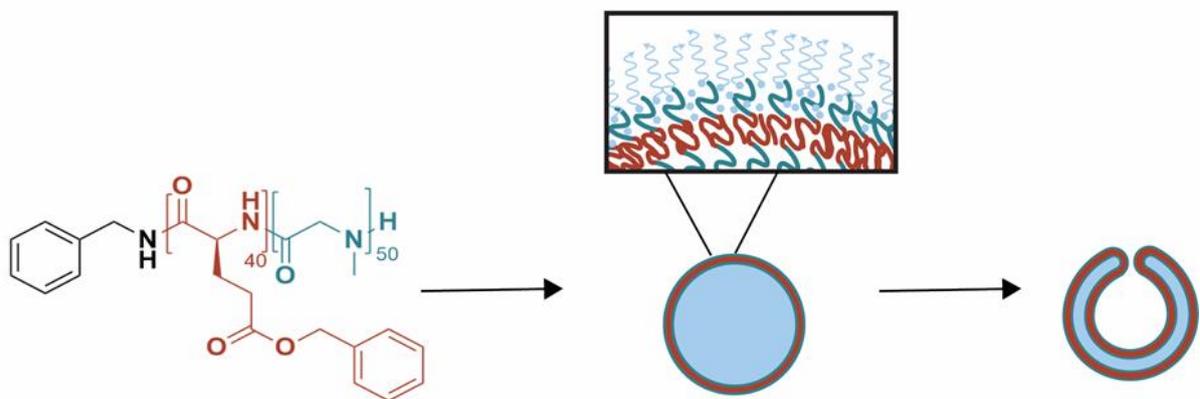
Abstract

Polysarcosine emerges as a promising alternative to polyethylene glycol (PEG) in biomedical applications, boasting advantages in biocompatibility and degradability. While the self-assembly behavior of block copolymers containing polysarcosine containing polymers has been reported, their potential for shape transformation remains largely untapped, limiting their versatility across various applications.

In this study, we present a comprehensive methodology for synthesizing, self-assembling, and transforming polysarcosine-polybenzylglutamate block copolymers, resulting in the formation of bowl-shaped vesicles, disks, and stomatocytes. Under ambient conditions, the shape transformation is restricted to bowl-shaped vesicles due to the membrane's flexibility and permeability. However, dehydration of the polysarcosine broadens the possibilities for shape transformation.

These novel structures exhibit asymmetry and possess the capability to encapsulate smaller structures, thereby broadening their potential applications in drug delivery and nanotechnology. Our findings shed light on the unique capabilities of polysarcosine-based polymers, paving the way for further exploration and harnessing of their distinctive properties in biomedical research.

Keywords

Introduction

Polymeric vesicles represent a promising candidate for usage in drug delivery systems due to their facile assembly and ability to provide a stable soft interface. Among these materials, polyethylene glycol polystyrene block copolymers (PEG-PS) stand out for their versatility and adaptability. These copolymers exhibit a remarkable propensity for self-assembly, allowing the formation of vesicles capable of undergoing diverse shape transformations. Notably, they can adopt the distinctive stomatocyte morphology, characterized by a concave shape with a central cavity [1]. Such structures hold significant potential for drug delivery applications as nanomotors, offering both encapsulation capability and controlled release functionalities [2, 3]. Moreover, researchers have explored novel shapes utilizing PEG-PS copolymers, including the growth of protrusions from the vesicle surface [4]. While these emerging shapes warrant further investigation to elucidate their optimal utilities, their creation underscores the material's versatility in shape manipulation. This ability to engineer a spectrum of shapes from a single material holds profound implications for drug delivery and beyond. By tailoring vesicle morphology to specific requirements, researchers can optimize drug encapsulation, targeting, and release, advancing the efficacy and precision of therapeutic interventions. Continued exploration of these versatile materials promises to unlock new avenues in pharmaceuticals and biomedical engineering. The problem here is that PEG-PS is a non-biodegradable material and this poses significant challenges for its use in drug delivery applications, particularly concerning potential accumulation in the body [5]. Recent advancements

have sought to address this issue by replacing the hydrophobic block with biodegradable alternatives, like polylactic acid, resulting in PEG-PDLLA block copolymers capable of forming vesicles that are able to undergo shape transformation towards stomatocytes [6]. Despite this progress, the persistence of the non-degradable PEG segment is still ongoing as PEG is regarded as the benchmark for hydrophilic polymers used in drug delivery [7].

The non-biodegradability of PEG under most conditions, coupled with recently discovered immunogenic responses to it, has led to increasing concerns [8, 9]. This debate has prompted exploration into novel materials that resemble PEG, retaining its positive attributes, but add biodegradability. Two of such classes of polymers that would form viable alternatives are polyoxazolines and polypeptides [10, 11], both of which possess biodegradable and biocompatible properties. Moreover, these materials offer versatility in their synthesis, allowing for the incorporation of various building blocks to tailor the polymers to desired specifications. Additionally, they lend themselves well to the synthesis of block copolymers, further expanding their potential applications for the use in drug delivery systems [12].

In this regard, synthetic polypeptides emerge as a promising candidate for constructing biodegradable and biocompatible polymersomes. Leveraging the inherent presence of peptide-degrading mechanisms within the human body and the versatile chemical functionalities of naturally occurring amino acids, synthetic polypeptides offer a robust platform for designing drug delivery systems that meet the criteria of biodegradability and biocompatibility [13]. The present study focuses on a polysarcosine and poly-L-benzylglutamate block copolymer

(PSar-PBLG), as it is known to be able to self-assemble in a variety of structures, including micelles, vesicles, and nanoparticles [14]. However, the versatility of these polymers in shaping vesicles into asymmetric structures suitable for nanomotors remains relatively unexplored. While previous research has demonstrated their ability to form round vesicles and some tubular shapes, the pursuit of asymmetric structures, such as the stomatocyte, presents a novel challenge and opportunity in the field [15, 16].

The stomatocyte shape would be an excellent addition because of its demonstrated suitability for nanomotor fabrication [17]. The necessary shape transformation for achieving this morphology is primarily driven by changes in osmotic pressure. Initially achieved through dialysis and later by the addition of PEG solution [1, 17], the process involves deflating the polymeric vesicle, prompting it to bend and adopt the stomatocyte configuration. Several critical factors contribute to this transformation. Firstly, the application of osmotic pressure which must be sufficiently robust. This control is notably easier with the addition of PEG, as it swiftly creates a substantial osmotic gradient [18]. Secondly, the vesicle's properties are crucial, particularly its permeability. Excessive permeability allows water molecules – and potentially larger molecules – to traverse the membrane without exerting adequate force, undermining the transformation process [19, 20]. Lastly, membrane stiffness plays a role; the stiffness of the membrane determines how it responds to the applied force. Besides that stiffness should be sufficient to maintain membrane curvature for a shape transformation to occur [21]. This is particularly important when domains are formed over the membrane with

different stiffness which can lead to an asymmetrical response [22-24]. An excessive stiffness will eventually even impede deformation entirely [25]. This characteristic is harnessed in systems to stabilize shapes by water addition, effectively locking the membrane in a kinetically stable state [26].

In a recent study by Elafify, M. S. Et all it was demonstrated that polysarcosine-based self-assemblies could undergo shape transformation via dehydration induced by heating [15]. This dehydration of the polysarcosine caused in their system a complete shape transformation but it would effectively also reduce membrane permeability and enhance osmotic pressure, as temperature is a factor in the van 't Hoff formula for osmotic pressure [20]. Additionally, dehydration strengthens polysarcosine chains interactions [15], potentially rendering the membrane too rigid for shape transformation. This means that the

heating of the vesicles made with polysarcosine would improve the effectivity of the osmotic pressure applied up to a certain point, after which the membrane would be too rigid for deformation. Within this window it is expected that stomatocytes could be obtained using PSar-PBLG block copolymers.

Results and discussion

Starting with the synthesis of the PSar-PBLG that was used for all the following systems, it was found that a polymer with a length of 50 units sarcosine and 40 units benzylglutamate would be able to form vesicles of around 700 nm [Supp. Figure S16,17]. The synthesis of PSar-PBLG [Scheme 1] involved anionic ring-opening polymerization of N-carboxyanhydrides (NCA's), following a protocol adapted from Tian, Z.Y. et all [27]. High-purity monomers were obtained using this method, ensuring the subsequential polymer
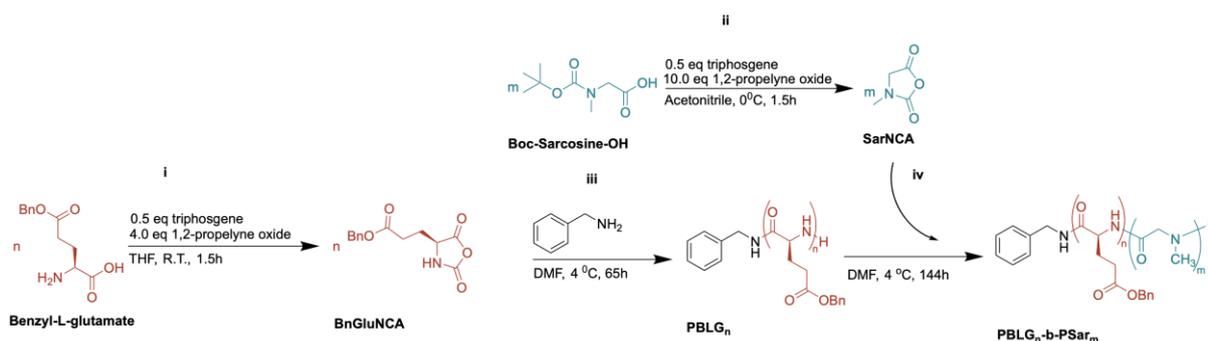
Scheme 1: I Synthesis of benzylglutamate NCA using phosgene and propylene oxide as a scavenger. Ii synthesis of sarcosine NCA starting from boc protected sarcosine. Iii ring opening polymerization of BnGluNCA using benzyl amine as initiator. Iv ring opening polymerization of Sar NCA using the benzyl glutamate homopolymer as macro initiator.p

synthesis proceeded with minimal impurities. Sequential polymerization commenced with polybenzylglutamate (PBLG) as the hydrophobic block, this was done to prevent ending on a primary amine in the end product which could disrupt self-assembly due to their ionizable nature. Subsequently, PBLG served as a macroinitiator for the polymerization of sarcosine NCA, yielding the final polymer with high yield and low polydispersity index (PDI), crucial for effective self-assembly [28].

Previous studies on PEG-PS vesicles demonstrated a solvent exchange method for vesicle formation, followed by a shape transformation induced by osmotic pressure from PEG addition [17]. The structure was then frozen by addition water, this process was facilitated by the glass transition temperature (T_g) of the polymer, marking the transition from a fluid to a glassy state. To replicate this methodology, it is important that the T_g of PSar-PBLG is high enough to freeze the different morphologies by water addition. The T_g was determined using differential scanning calorimetry (DSC) [Supp. Figure S15]. The results revealed a T_g of approximately 100°C for PSar-PBLG, indicating the potential for shape locking through transitions between fluidity and a glassy state. Additionally, examination of the

thermogram unveiled a peak during the initial heating cycle, spanning from 40°C to 90°C [Supp Figure S15]. This peak corresponds with the dehydration temperature of polysarcosine, suggesting its role in facilitating subsequent shape manipulation [15].

The assembly of the PSar-PBLG block copolymers was optimally achieved by dissolving the block copolymers in DMF followed by a solvent exchange method. Here, MilliQ water was gradually introduced to form monodisperse polymersomes [Figure 1 i] [Supp. Figure S16,17]. Hexafluoro isopropanol (HFIP) emerged as another solvent yielding monodisperse assemblies. HFIP promotes formation of alpha helices in peptides and this property yielded vesicles with different morphologies [29]. The resulting vesicles looked darker compared to those formed in DMF, as observed through TEM [Supp. Figure S19].

For the shape transformation experiments only the vesicles assembled in DMF were used. To validate the structure of the self-assembly, cryo-TEM was conducted, revealing that the obtained vesicles exhibited a wrinkled appearance, differing from the typically round vesicles observed in systems like PEG-PS [19] or PEG-PDLLA [30]. This
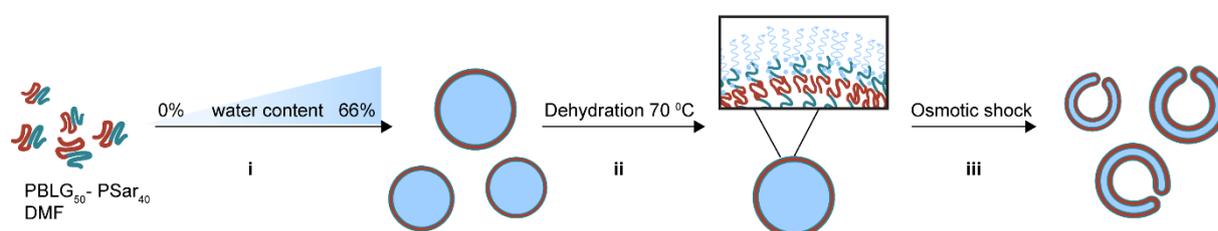

Figure 1: i. The PBLG-PSar block copolymers are dissolved in DMF and then assembled though the solvent exchange method by addition of 66% water. ii. The membrane is dehydrated at 70°C for 1 min before starting the shape transformation. iii. The shape transformation is induced by osmotic shock. This is done by the addition of 5µl PEG_{2000} solution (400 mg/ml). The osmotic shock can also be induced by PSar solution or saccharose solution.

wrinkled morphology suggests that the membrane may possess greater flexibility compared to the aforementioned systems [Supp. Figure S20].

It was attempted to change the shape of the assembled vesicles following a similar method as previously developed for PEG-PS systems. Cryo-TEM analysis revealed only a slight decrease in vesicle volume and a somewhat flattened appearance [Supp. Figure S21]. Based on our observations and those of others working with similar polymers [15], we deduced that the membrane of PBLG-PSar is overly flexible and permeable to water, resulting in a minimal shape transformation. The osmotic pressure exerted on a membrane depends on its permeability to water, with PBLG-PSar experiencing significantly less force on the membrane compared to PEG-PS systems, even with the same amount of added PEG. Simply increasing the

solute concentration proved ineffective; an alternative approach was needed.

Examining the Van 't Hoff equation for osmotic pressure:

$$\Pi = iMRT$$

Four factors play significant roles: solute concentration M (in this case, PEG), the van 't Hoff constant i (which is 1 for non-dissociating molecules), temperature T, and the ideal gas constant R. Increasing the temperature can enhance osmotic pressure, similar to solute concentration. This equation does not directly include membrane permeability as it only relates the osmotic pressure to temperature and concentration. However, membrane permeability indirectly affects osmotic pressure difference over the membrane. Permeability influences the concentration gradient of solute particles across the membrane [20], meaning that in this case membrane permeability affects the osmotic

9

pressure that can be exerted on the membrane. Dehydrating polysarcosine by raising the temperature influences most of these parameters, reducing permeability and increasing membrane stiffness due to enhanced sarcosine chain interactions [15].

Heating the sample to 70°C and then applying osmotic shock through PEG addition, followed by quenching with water, transformed the shape into a stomatocyte as demonstrated in [Figure 1]. The window for shape transformation seems to lie between 60°C and 80°C, with the best result being obtained at 70°C [Figure 3]. When the temperature is too low, the shape transformation cannot happen as the membrane is still too permeable. However, when the membrane gets too stiff, the deformation is prevented regardless of the increased force that is applied.

The progression of shape transformation was monitored from 0 to 120 seconds before quenching. Initially, upon heating and immediate quenching, typical vesicles were observed. This contrasted with earlier observations of wrinkled membranes, suggesting increased membrane rigidity due to heating [Figure 2a]. Within 5 seconds, a slight shape transformation became evident, with most vesicles exhibiting minor dents and some forming stomatocytes [Figure 2b]. By the 60-second mark, all shapes had transitioned into stomatocytes [Figure 2c], with further waiting resulting in a transformation towards disk-like structures [Figure 2d]. This route for the shape transformation appears to deviate slightly from the conventional method, where a disk is first formed and then symmetrically deflated [19]. This deviation might be due to domain formation during dehydration, which results in more rigid domains. The anomalous shapes observed, with

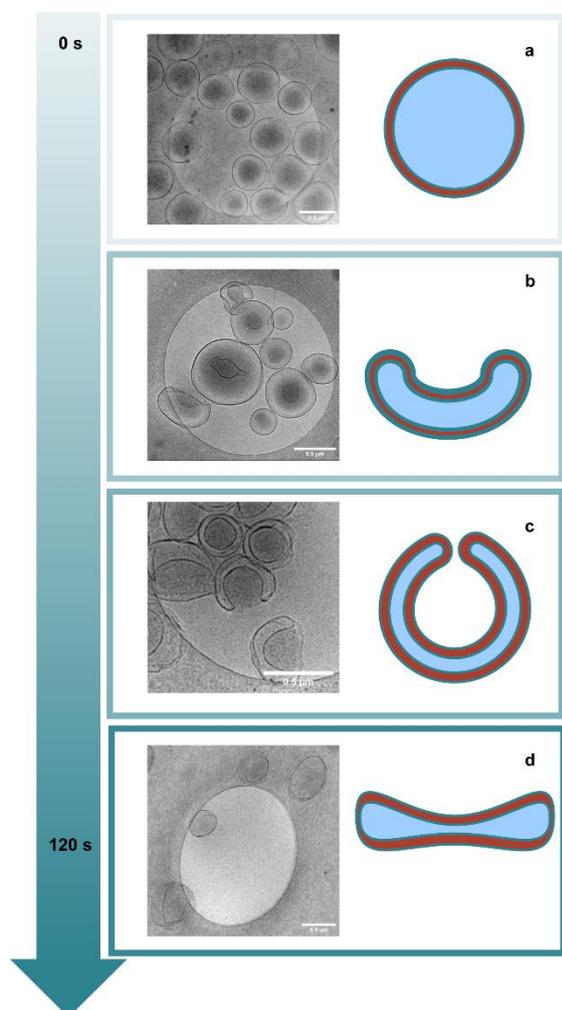bending starting from seemingly random positions, support this

Figure 2: Progression of Shape Transformation of PSar-PBLG vesicles at 70 °C (scalebar 0.5 μm). a. Initiation: Polymersome formation.b. Transition: Formation of a vesicle with a dent and stomatocyte. c. Further development: Transition to a wide-open stomatocyte. d. Completion (angle 35°): Shape evolved into a disk after 120 seconds before quenching.

hypothesis [Supp Figure S32]. These irregular shapes are reminiscent of some anomalous shapes observed in red blood cells [24]. For further affirmation of the mechanism Circular Dichroism spectroscopy (CD) was employed to give more insight on the morphological changes in the polymers secondary structure [Supp. Figure S35]. A slight decrease in signal intensity corresponding to the alpha helix region was observed [31], indicating a minor disordering of the hydrophobic part potentially aiding flexibility for shape transformation. Additionally, the hydrodynamic radius of vesicles decreased slightly during the heating cycle, indicative of membrane dehydration (Figure 3c). Further proof for the changing membrane properties was shown by a fluorescence measurement of pyrene in the presence of the PSar-PBLG vesicles. With the increase in temperature there was a slight change in hydrophobicity of the PSar corona in which the pyrene resided. This change shifted the pyrene signal slightly from 361.4 nm to 361.8

nm, starting around 40°C (Figure 3b). The information obtained from DSC, accompanied by the shape transformation experiments are indicative of a clear correlation between the dehydration of the membrane and its susceptibility to change shape through osmotic shock [Figure 3]. After replacing PEG with PSar in the self-assembled structures we also show that the osmotic pressure can be induced by PSar in similar fashion as with PEG. Hence, solutions of PSar homopolymer and saccharose were employed, initiating the shape transformation as well [Supp Figure S30]. This suggests that various hydrophilic biocompatible additives could be viable for inducing osmotic shock in shape transformation Both PEG and PSar are partially dehydrated at 70°C making them less effective for usage at high temperatures, Another compound was proposes to induce osmotic pressure, namely saccharose.
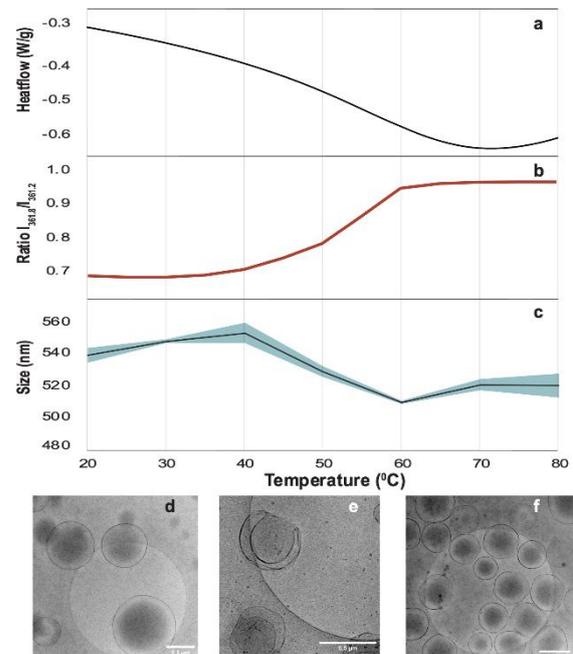

Figure 3: The Temperature-Dependent Behavior of Vesicles and Shape Transformation: a. Thermograph of PBLG-PSar: Showing dehydration with a peak around 70°C. b. The fluorescent intensity of pyrene measured at 361.4 nm divided by the intensity at 361.8 nm tracked over temperatures between 20°C and 80 °C c. DLS Measurement: Demonstrating a slight decrease in hydrodynamic radius of the vesicles at higher temperatures. d-f. Vesicles After Shape Transformation: Illustrated at 60°C, 70°C, and 80°C, respectively.

The dehydration temperature for saccharose is around 100°C, indicating that its hydrophilicity remains high compared to PEG and PSar, which have lower dehydration temperatures [32] [Supp Figure S31]. Due to this higher temperature, sucrose is

expected to induce a greater osmotic shock. This is evident in the results of shape transformation experiments, where more complex structures such as stomatocyte-in-stomatocyte shapes were observed. This suggests that shape transformations triggered by saccharose are more effective at higher temperatures [33].

Conclusion

Self-assembled vesicles crafted from polysarcosine-polybenzylglutamate block copolymers exhibit distinct properties compared to conventional systems like PEG-PS or PEG-PDLLA, notably their inability to undergo shape transformation though osmotic pressure under conventional conditions. This limitation arises from the inherent membrane flexibility and heightened permeability of these vesicles. However, polysarcosine exhibits a dehydration temperature between 40°C and 90°C, triggering the release of water molecules from the hydrophilic layer. Consequently, enhanced interactions between polysarcosine chains ensue, culminating in a more rigid and less permeable membrane structure at an optimum dehydration at 70°C.

This augmented membrane permeability facilitates the buildup of stronger osmotic pressure within the vesicle, driving deformation into a stomatocyte shape. This novel morphology for polysarcosine based polymers opens new avenues for utilizing fully biodegradable polymers as nanomotors, leveraging enzyme encapsulation techniques. Moreover, it was observed that a temperature that is too high does not yield further shape transformation but instead results in the formation of more rigid polymersomes. So, the system only operates accordingly in a narrow window.

Lastly besides the PEG addition method for inducing osmotic pressure most hydrophilic molecules should be able to induce enough osmotic pressure to induce shape transformation. The use of saccharose which has a higher dehydration temperature then PEG or PSar seems to be more effective for use at high temperatures.

In summary, the distinctive properties of polysarcosine-polybenzylglutamate block copolymers pave the way for innovative applications in drug delivery and nanotechnology, marking a significant advancement in the field of biodegradable polymer vesicles. Further exploration into optimizing shape transformation conditions tailored to one's goal and harnessing the unique capabilities of these vesicles holds promise for future biomedical applications.

Acknowledgements

Funding

References

1. Wilson, D.A., R.J. Nolte, and J.C. van Hest, *Autonomous movement of platinum-loaded stomatocytes.* Nat Chem, 2012. **4**(4): p. 268-74.
2. Abdelmohsen, L.K., et al., *Dynamic Loading and Unloading of Proteins in Polymeric Stomatocytes: Formation of an Enzyme-Loaded Supramolecular Nanomotor.* ACS Nano, 2016. **10**(2): p. 2652-60.
3. Tu, Y., et al., *Self-propelled supramolecular nanomotors with temperature-responsive speed regulation.* Nat Chem, 2017. **9**(5): p. 480-486.
4. Sun, J., et al., *Generating biomembrane-like local curvature in polymersomes via dynamic polymer insertion.* Nat Commun, 2021. **12**(1): p. 2235.
5. Mohammad, A.K., et al., *Rapid lymph accumulation of polystyrene nanoparticles following pulmonary administration.* Pharm Res, 2013. **30**(2): p. 424-34.
6. Toebes, B.J., F. Cao, and D.A. Wilson, *Spatial control over catalyst positioning on biodegradable polymeric nanomotors.* Nat Commun, 2019. **10**(1): p. 5308.
7. D'Souza A, A. and R. Shegokar, *Polyethylene glycol (PEG): a versatile polymer for pharmaceutical applications.* Expert Opin Drug Deliv, 2016. **13**(9): p. 1257-75.
8. Knop, K., et al., *Poly(ethylene glycol) in drug delivery: pros and cons as well as potential alternatives.* Angew Chem Int Ed Engl, 2010. **49**(36): p. 6288-308.
9. Hoang Thi, T.T., et al., *The Importance of Poly(ethylene glycol) Alternatives for Overcoming PEG Immunogenicity in Drug Delivery and Bioconjugation.* Polymers (Basel), 2020. **12**(2).
10. Konradi, R., C. Acikgoz, and M. Textor, *Polyoxazolines for nonfouling surface coatings--a direct comparison to the gold standard PEG.* Macromol Rapid Commun, 2012. **33**(19): p. 1663-76.
11. Huesmann, D., et al., *A head-to-head comparison of poly(sarcosine) and poly(ethylene glycol) in peptidic, amphiphilic block copolymers.* Polymer, 2015. **67**: p. 240-248.
12. González-Aramundiz, J.V., et al., *Polypeptides and polyaminoacids in drug delivery.* Expert Opinion on Drug Delivery, 2012. **9**(2): p. 183-201.
13. Skoulas, D., et al., *Amphiphilic Star Polypept(o)ides as Nanomeric Vectors in Mucosal Drug Delivery.* Biomacromolecules, 2020. **21**(6): p. 2455-2462.
14. Birke, A., J. Ling, and M. Barz, *Polysarcosine-containing copolymers: Synthesis, characterization, self-assembly, and applications.* Progress in Polymer Science, 2018. **81**: p. 163-208.

15. Elafify, M.S., et al., *Reversible transformation of peptide assembly between densified-polysarcosine-driven kinetically and helix-orientation-driven thermodynamically stable morphologies.* Biomater Sci, 2023. **11**(18): p. 6280-6286.

16. Watabe, N., C. Joo Kim, and S. Kimura, *Fusion and fission of molecular assemblies of amphiphilic polypeptides generating small vesicles from nanotubes.* Biopolymers, 2017. **108**(2).

17. Sun, J., et al., *Enzyme-Powered Nanomotors with Controlled Size for Biomedical Applications.* ACS Nano, 2019. **13**(9): p. 10191-10200.

18. Li, W., et al., *Shape Transformation of Polymer Vesicles.* Acc Mater Res, 2024. **5**(4): p. 453-466.

19. Rikken, R.S., et al., *Shaping polymersomes into predictable morphologies via out-of-equilibrium self-assembly.* Nat Commun, 2016. **7**: p. 12606.

20. Lewis, G.N., *The Osmotic Pressure of Concentrated Solutions, and the Laws of the Perfect Solution.* Journal of the American Chemical Society, 2002. **30**(5): p. 668-683.

21. Pijpers, I.A.B., et al., *Morphology Under Control: Engineering Biodegradable Stomatocytes.* ACS Macro Lett, 2017. **6**(11): p. 1217-1222.

22. Carlsen, A., et al., *Block copolymer vesicle permeability measured by osmotic swelling and shrinking.* Langmuir, 2011. **27**(8): p. 4884-90.

23. Zong, W., et al., *Deformation of giant unilamellar vesicles under osmotic stress.* Colloids Surf B Biointerfaces, 2018. **172**: p. 459-463.

24. Lim, H.W.G., M. Wortis, and R. Mukhopadhyay, *Stomatocyte-discocyte-echinocyte sequence of the human red blood cell: evidence for the bilayer- couple hypothesis from membrane mechanics.* Proc Natl Acad Sci U S A, 2002. **99**(26): p. 16766-9.

25. Pozrikidis, C., *Effect of membrane bending stiffness on the deformation of capsules in simple shear flow.* Journal of Fluid Mechanics, 2001. **440**: p. 269-291.

26. Mai, Y. and A. Eisenberg, *Self-assembly of block copolymers.* Chem Soc Rev, 2012. **41**(18): p. 5969-85.

27. Tian, Z.Y., et al., *A moisture-tolerant route to unprotected alpha/beta-amino acid N-carboxyanhydrides and facile synthesis of hyperbranched polypeptides.* Nat Commun, 2021. **12**(1): p. 5810.

28. Lynd, N.A., A.J. Meuler, and M.A. Hillmyer, *Polydispersity and block copolymer self-assembly.* Progress in Polymer Science, 2008. **33**(9): p. 875-893.

29. Colomer, I., et al., *Hexafluoroisopropanol as a highly versatile solvent.* Nature Reviews Chemistry, 2017. **1**(11).

30. Yewdall, N.A., A.F. Mason, and J.C. Van Hest, *The hallmarks of living systems: towards creating artificial cells.* Interface Focus, 2018. **8**(5): p. 20180023.

31. Guan, A.J., et al., *G-quadruplex induced chirality of methylazacalix[6]pyridine via unprecedented binding stoichiometry: en route to multiplex controlled molecular switch.* Sci Rep, 2015. **5**: p. 10479.

32. Lu, Y., et al., *Investigating the thermal decomposition differences between beet and cane sucrose sources.* Journal of Food Measurement and Characterization, 2017. **11**(4): p. 1640-1653.

33. Men, Y., et al., *Stomatocyte in Stomatocyte: A New Shape of Polymersome Induced via Chemical-Addition Methodology.* Nano Lett, 2018. **18**(3): p. 2081-2085.