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ARTICLE

Enantioselective Assembly of Amphipathic Chiral Polymer and Racemic Chiral Small Molecules during Preparing Micro-scale Polymer Vesicles

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Abstract: Enantioselective assembly is observed during preparing vesicles from a mixture solution of an amphipathic block copolymer bearing pendent chiral L (or D)-phenylalanine groups in hydrophobic segments and racemic chiral small molecules. Primary experimental results reveal that racemic chiral small molecules have been enantioselectively encapsulated into the hydrophobic shells of assembled vesicles with homochiral bilayer membrane structure, showing reverse CD signals compared with the original vesicles. A further study shows that the enantiomeric excess obtained through the enantioselective assembly is directly dependent on the molecular weight of the hydrophobic chiral chain segments, implying both of the hydrophobic interaction and chirality are key points during the assembly of vesicles. Other factors, such as hydrogen-bonding interaction and π-π stack interaction, are found also to be responsible for the enantioselective assembly phenomenon during the formation of the vesicles.

Introduction

Enantioselectivity arises from the phenomenon of the molecular recognition of chiral molecules and depends on synergetic effects, such as hydrophobic/hydrophilic[1], hydrogen bonding[2], electrostatic interactions[3], and steric factors[4]. The materials containing homochiral structure can function as biomimetic materials[5], chiral sensors[6], chiral catalysts[7], and bioactive chiral drugs[8]. Among various applications of homochiral materials, enantioselective separation remains a significant challenge in multidisciplinary scientific research since the biological and chemical activities are highly specific for a particular chiral form[9].

On the other hand, chiral recognition and selection between the enantiomers are also involved in the process of self-assemblies of chiral molecules.[10-13] Smith and Edwards investigated a two-component acid-amine gelation system and found that one enantiomer could be selectively incorporated into assembled gel fibers over the other[2]. In their research on asymmetric non-covalent synthesis, George and Meijer *et al* observed that achiral molecules were assembled according to "Majority Rules" under varying the enantiomeric excess of the mixture of D- and L-chiral molecules[14]. Generally speaking, the chiral molecules can afford the intrinsically chiral centres necessary for the chiral recognition[15]. Even in the spontaneous optical segregation process, homochiral supramolecular polymerization was demonstrated no matter chiral molecules were S-shaped chiral monomers[16] or bowl-shaped chiral macrocycles[17]. Recent work further revealed that the chiral recognition process could be controlled by many factors, for example, the addition of chiral co-anions could selectively suppress the self-assembly process of the enantiomeric macroanions[18]. Obviously, due to so many factors existing in the self-assembly process, there is still a need of more delicate works to explore the detailed mechanism, which will be helpful to explain how homochirality originates and transmits to other biomolecules/assemblies during the evolution of life[18].

It is well known that vesicles are supramolecular assemblies and always used to mimic cells because of their bilayer membrane with the hydrophilic part outside and hydrophobic part inside. Among many kinds of vesicles, homochiral vesicles have many potential applications. For example, Dey's team reported the successful enantiomeric separation using spontaneously formed anionic vesicles as pseudo-stationary phase by electrostatic interaction[3]. In their work, the enantioselective process took place simultaneously with the forming of vesicles in a capillary, the role of many factors that govern the chiral recognition would be connected with the self-assembly of the vesicle-forming surfactants. Furthermore, vesicles are similar to many bio-assemblies, such as cells, in bilayer membrane structures, investigation on chiral recognition during their forming may have contributions to

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clearly understand the homochirality of basic biological system. However, there is few works concerning enantiomeric separation during preparation of micro-scale vesicles through self-assembly, especially for polymer vesicles, which can be studied by direct observation under optical microscopy.

In our previous works, micro-scale polymer vesicles have been successfully fabricated and their characteristic behavior was directly observed under optical microscopy. It has been found that these vesicles have similar properties with the bio-cells, for example, photo-induced fusion[19] and fission[20]. In this work, a kind of polymer vesicles was prepared through self-assembling of an amphipathic block copolymer, which contains amino acid derivatives as the hydrophobic chain segments. Herein, the bilayer membrane of self-assembled vesicle provides not only hydrophobic but also homochiral environment so that it is hoped to make sense for chiral recognition. Enantioselective assembly phenomenon was investigated during self-assembly of the amphipathic copolymer in the solution containing racemic chiral small molecules that were dissolved simultaneously into the solvent with the polymer before self-assembly. It was believed that two enantiomers of phenylalanine derivatives would show a difference in the process of self-assembly.

Experimental

Materials

N, N-isopropylacrylamide was recrystallized twice from hexanes prior to use. Meanwhile α, α'-azoisobutyronitrile (AIBN) was recrystallized from ethanol and stored at low temperature. Tetrahydrofuran (THF) was refluxed over sodium and distilled while pyridine was refluxed with calcium hydride and distilled. N-(tert-Butoxycarbonyl)-DL-phenylalanine (BOC-DL-Phe) was purchased from Aladdin. The other chemicals were of analytical grade and used without further purification.

Synthesis of L- or D-phenylalanine-methyl ester hydrochloride

The two enantiomers of phenylalanine methyl ester hydrochloride were synthesized by L-phenylalanine and D-phenylalanine, respectively. 5.6 ml dimethylsulfoxide ($SOCl_2$) was dropped into 52 ml methanol slowly at -8 ^{o}C, and then the solution was stirred below 0 ^{o}C for 1 h. After that, 5 g phenylalanine was added into the solution with maintaining the low temperature. The thick liquid was stirred for another 3 h at room temperature and then the solution was refluxed at 65 ^{o}C. Finally, the solution was distilled under a vacuum to remove most of the solvent and the residue was recrystallized by mixed solvent of methanol and diethyl ether.

Synthesis of methacryamide-L (or D)-phenylalanine-methyl ester monomer

4.3 g phenylalanine methyl ester hydrochloride was added into pyridine containing 1.85 g methacrylic anhydride and stirred for 24 h then the solution was distilled under a vacuum to remove most of pyridine. After that, the residue was washed by diluted hydrochloric acid sufficiently. The target product was then extracted by moderate dichloromethane (CH_2Cl_2). Finally, the solution was distilled totally and the crude product was achieved as expected. The crude product was subsequently purified by column chromatography with mixed solution of ethyl acetate and petroleum ether as eluent.

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Synthesis of chiral and amphipathic diblock copolymer

The macro chain transition agent (Macro-CTA) of N, N-isopropylacrylamide (NIPAM) was synthesized by reversible addition fragment chain transfer (RAFT) polymerization based on the previously reported work[21]. A distilled THF solution of Macro-CTA (1.4 g), L-monomer or D-monomer (0.6 g) and AIBN (5 mg) in a glass tube was degassed for three freeze-pump-thaw cycles, sealed off under a vacuum and heated at 65 ^{o}C for 48 h. The solution was then precipitated in ethyl ether. Afterwards, PNIPAM capped with chiral and hydrophobic head groups was successfully obtained. The polymer was purified by dissolution in THF and precipitation in ethyl ether for three times. Finally, the collected polymer was dried at 50 ^{o}C under a vacuum for more than 48 h. The number of L-phenylalanine and D-phenylalanine repeating units in the copolymer are characterized to be 57 and 90 while the number of NIPAM is 126 for both of polymer enantiomers.

Preparation of chiral PNIPAM-*b*-P[L (or D)-Phe] vesicles in solution

The PNIPAM-b-P(L-Phe) and PNIPAM-b-P(D-Phe) were respectively dissolved in THF with an initial concentration of 2.0 mg·ml^{-1}. The self-assembly process was performed by adding Milli-Q water at a rate of 5 μl·s^{-1}with stirring into the THF solution of the diblock copolymers. Water was then added until the water content reached 50 vol.-%. Afterwards, the mixture was left to equilibrate for 24 h without stirring. And the temperature was maintained at room temperature.

Self-assembly of mixture solution containing chiral vesicles and racemic chiral molecules

The diblock copolymer (2 mg·ml^{-1}) and racemic BOC-phenylalanine (1 mg·ml^{-1}) were dissolved in THF solution simultaneously. The rest step was just the same with preparation of pure chiral vesicles described as the above.

Filtration operation of mixture solution containing chiral vesicles and racemic chiral molecules

In order to collect the small chiral molecules which are not assembled into the shell of the vesicles in the mixed solutions, a small silica-gel column is used for separating the vesicles and the solution. The diameter of the column is 0.5 mm, and the cubage of the column is 10 ml. A small piece of cotton is put at the bottom of the column firstly, and the 200-300 mesh silica-gel powders with a volume of 1ml are added into the column. After that, moderate mixed solution of THF and water (the volume ratio = 1: 1) is added for saturating the silica-gel powders. Mixture solution containing chiral vesicles and racemic chiral molecules (8 ml) is added into the silica-gel column for filtration. The filtering process is maintaining quite slowly at a rate of 0.5 $\mu l \cdot s^{-1}$. Finally, transparent solution with a volume of 2 ml is collected.

Characterizations and Instruments

All ^{1}H NMR spectra were obtained on a Bruker 300 MHz FT-NMR spectrometer and the samples were analyzed in $CDCl_3$. UV-vis spectra were recorded on a SHIMADZU UV-2550 spectrophotometer. A modified fluorescence microscope (OLYMPUS IX-70) was used to observe the micro-scale vesicles. The molecular weights and molecular weight distributions of the Macro-CTA were determined using gel permeation chromatography (GPC). The molecular weights of copolymers are calculated in terms of data from ^{1}H NMR measurements as shown in supporting information (Figure S1). CD spectra were recorded on a JASCO J-810 circular dichroism (CD) spectrometer. All the operations in this article were carried out at room temperature.

Results and discussion

Structures of PNIPAM-b-P(L-Phe) and PNIPAM-b-P(D-Phe), with chiral amino acid appendages pendent to each repeating unit of the hydrophobic polymer chain, are schematically shown in Figure 1-a. Following the route in Experiment part, vesicles have been successfully prepared through self-assembly and the vesicles composed of the copolymer with chiral segments show sensitive Cotton effects as expected (Figure 1-b). As is well known, the vesicle self-assembled from amphipathic polymers have a shell of bilayer membrane, which is identified as stable condensed state[22]. In addition, the bilayer membrane is hydrophilic outside and hydrophobic inside in solution. In other words, the vesicles self-assembled from the PNIPAM-*b*-P[L (or D)-Phe possess chirality and hydrophobicity in the shell of bilayer membrane. Therefore, such a bilayer membrane of the chiral vesicles owning the chiral and hydrophobic environment may make sense for chiral separation of hydrophobic molecules.

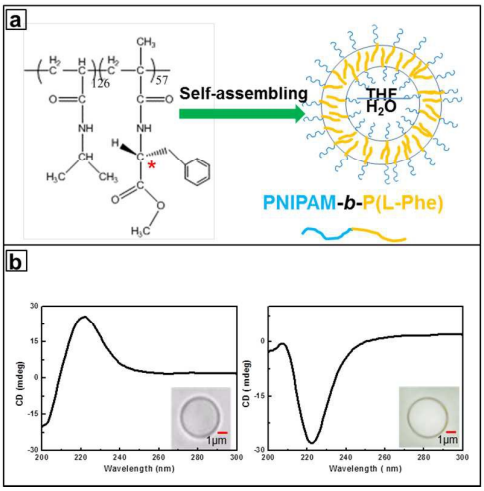

Figure 1 (a): Schematic representation of chemical structure of the copolymer and their self-assembly process; (b): CD spectra and microphotograph (insert) of vesicles composed of PNIPAM-*b*-P(L-Phe) (left) and PNIPAM-*b*-P(D-Phe) (right). The concentration of the two copolymers are both 1 $mg \cdot ml^{-1}$ in mixed solution of THF and water (the volume ratio= 1: 1).

It has been known that there are many methods that can be used in enantioseparations, such as chromatography and electromigration. In one recently published review[23], enantioseparations are systematically categorized into indirect and direct methods. In the indirect approach, the enantiomers are firstly derivatized with a stereoisomerically pure reagent to form diastereomers via covalent bonds. These diastereomers can be subsequently separated, in principle, under achiral conditions. Direct enantioseparation refers to the separation of enantiomers in a chiral environment, which requires the presence of a chiral selector that is either fixed to an immobile support or added to the mobile phase. Dey's group used to work out a giant vesicle (micro size) self-assembled from a novel surfactant (sodium N-[4-dodecyloxybenzoyl]-L-valinate). In this first example of chiral separation by MEKC (micellar electrokinetic chromatography) using a vesicle forming surfactant, it is suggested that the good chiral selectivity and separation could be due to enhanced partitioning of the analytes in the vesicles[24]. After detailed discussing on the factors responsible for chiral recognition of the vesicle forming surfactant system, it was believed that hydrophobic, hydrogen bonding and electrostatic interactions dictated the degree of interaction of the analyte with the chiral selector[3]. In this work, hydrophobic polymer chains with chiral pendents are used as chiral selectors. As shown by CD spectra and photos in Figure 1-b, the selector is located within the shell of micro-scale vesicles, whose diameter is usually several micrometers and can be directly observed under optical microscopy.

In order to verify the chiral separation, a solution of the chiral copolymer with racemic N-(tert-Butoxycarbonyl)-phenylalanine (BOC-DL-Phe) in THF was firstly prepared, and then an assembly of copolymers along with racemic molecules

was performed according to the method described in Experimental part. Because they are totally hydrophobic, D- and L-BOC-phenylalanine were chosen as enantiomers to test the enantioselective self-assembly phenomena. Figure 2-a shows the process of the enantioselective assembly driven by hydrophobicity, chiral steric factors and non-covalent interactions.

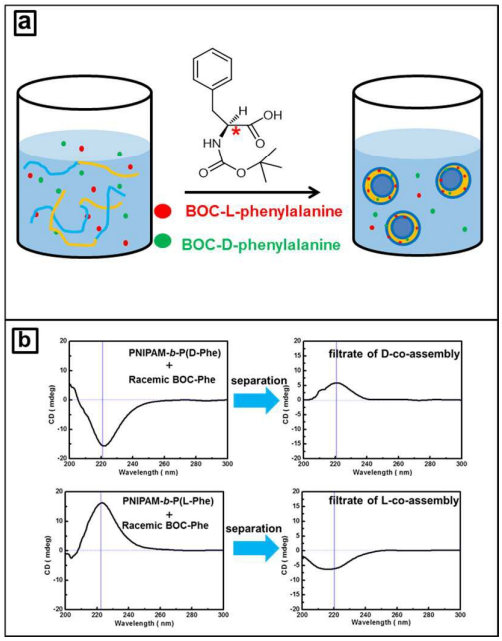

Figure 2 (a): Schematic representation of enantioselective assembly of chiral amphipathic copolymer along with hydrophobic and racemic BOC-phenylalanine; (b): CD spectra of D-vesicles (upper left) and L-vesicles (lower left) along with BOC-phenylalanine before filtration; CD spectra of filtrate of D-vesicles (upper right) and L-vesicles (lower right) along with BOC-phenylalanine after filtration. The concentrations of the polymers are both 1 mg·ml^{-1} in mixed solution of THF and water (the volume ratio= 1: 1). The concentrations of racemic BOC-phenylalanine in the solutions of L(or D)-vesicles are both 0.5 mg·ml^{-1}.

After assembly process, the solution was filtered by a small silica-gel column so that a transparent liquid was obtained, which is hoped to be separated from the solution full of vesicles. The filtering process was maintaining quite slowly at a rate of 0.5μl·s^{-1}. During the operation of filtering, only a quarter of the volume of the assembly solution was collected to guarantee the stability of vesicles during filtering. Detailed description about the filtration can be found in the Experimental part. The CD spectra of the solution of the chiral copolymer and racemic BOC-Phe were measured and shown in Figure 2-b (left), which are similar to the spectra of the pure chiral vesicles as shown in Figure 1-b. The same CD measurements were performed for the filtrate and the results are shown in Figure 2-b (right), from which it can be seen that an opposite Cotton effect is shown comparing with that from the chiral copolymer assembly in Figure 2-b (left). The CD signals of vesicles composed of PNIPAM-b-P(D-Phe) and racemic BOC-Phe, for example, has an intense negative Cotton effect with peak maximum around 220 nm while the CD spectrum of the filtrated solution has a faintish positive Cotton effect at the same wavelength. This phenomenon shows that there is a chiral separation during the assembling of chiral vesicles, although the degree of the separation is low. Furthermore, for two different kind of vesicles, formed by PNIPAM-*b*-P(L-Phe) and PNIPAM-*b*-P(D-Phe), respectively, the same results of opposite Cotton effect can be observed. This result shows that the chiral separation is closely dependent on the intrinsically chiral centres[15] located within the shell of the vesicles.

One of advantages of using micro-scale vesicles to study enantioselective process is that the vesicle can be observed directly under optical microscopy. Figure 3-a shows the vesicles' photos before (left) and after (right) the filtration, from which it can be seen that there is no obvious change of their morphology.

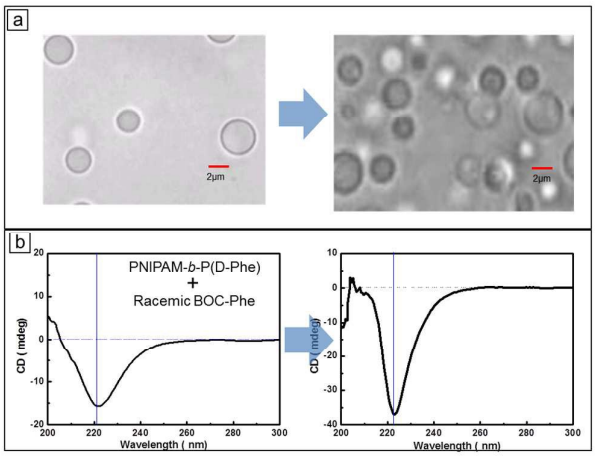

Figure 3 (a): Photos of vesicles under optical microscopy of assembled vesicles containing racemic BOC-phenylalanine before (left) and after (right) filtration; (b): CD curves (left) of D-vesicles along with racemic BOC-phenylalanine before filtration; CD curves (right) of D-vesicular solution along with racemic BOC-phenylalanine after filtration.

In addition, CD spectra of vesicles before and after the filtration also have no obvious change as shown in Figure 3-b (left) and Figure 2-b (left). Moreover, from Figure 3-b (right), it can be found that CD signal around 220 nm is stronger than that before the filtration for the filtrated D-vesicular solution. This phenomenon is caused by the increase of vesicular concentration, which is equivalent to the concentration of small chiral molecules within the shells of the vesicles. To make a quantitatively comparison, the enantiomeric excess (*ee*) of two kinds of assembly systems is calculated according to CD spectra and UV-Vis absorption spectra of two filtrated solutions. From working curves of BOC-L(or D-)-phenylalanine's CD signal and absorption intensity at the wavelength of 220 nm shown in supporting information (Figure S2), *ee* values of the filtrated solutions containing

PNIPAM-*b*-P(L-Phe) and PNIPAM-*b*-P(D-Phe) are +19.8% and -24.2%, respectively. The difference between two values may come from the polymer structure: the higher the chiral monomer content (see data given in Experimental part), the higher the *ee* value. This primary observation is also in accord with the "Majority Rules", implying that more enantiomers have been left with the same chirality as the chiral copolymer

All the observations unambiguously demonstrate that there is indeed enantioselective assembly when chiral copolymer and racemic molecules are assembled together in mixed solution of THF and Milli-Q water. All factors that govern the chiral recognition and separation ability of chiral system, such as hydrophobic/hydrophilic, hydrogen bonding and electrostatic interactions, steric factors, and the length of the chiral chain in the copolymer, may be key factors for the enantioselective assembly phenomenon of the chiral polymer. To demonstrate this hydrophilic/hydrophobic interaction, mixture solutions of chiral copolymer and D- and L-phenylalanine in THF were also used to form the assemblies according to the same method described above. Compared with BOC-D-(or L-) Phenylalanine, D-(or L-) Phenylalanine are totally hydrophilic other than hydrophobic. The experimental results (Figure 4) show that the filtrated solutions are found to be still racemic, namely, there is no Cotton effect for these two assembly systems and the enantioselective assembly does not happen under this occasion. It is suggested that the hydrophobicity crucially affects the enantioselective assembly through hydrophilic/hydrophobic interaction. The chiral and hydrophobic bilayer membrane provides the vital circumstance for homochirality BOC-Phe being preferentially encapsulated.

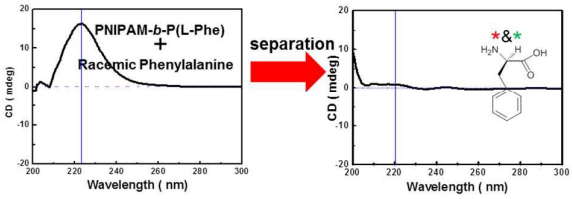

Figure 4 CD spectra before (left) and after (right) filtration of amphipathic copolymer along with hydrophilic and racemic DL-phenylalanine in solution. The concentrations of the PNIPAM-*b*-P(L-Phe) are both 1 mg·ml^{-1} in mixed solution of THF and water (the volume ratio= 1: 1). The concentration of racemic phenylalanine is 0.5 mg·ml^{-1}.

Another interesting problem associated with the enantioselective assembly phenomenon is whether the assembly occurs in the initial formation of the vesicles or dynamic exchange happens between bilayer membrane of vesicle and solution. For most enantioselective processes, it is usually implied that chiral recognition is a dynamic process other than a static one[23]. In another word, the enantioselective assembly may also occur in a way of dynamic exchange and the chiral small molecules of the two enantiomers would be encapsulated into the shell of vesicular structure selectively and dynamically. In order to verify this, a contrast experiment was carried out by changing the adding sequence of the racemic small molecules (BOC-D- and BOC-L-phenylalanine). A solution containing chiral polymer vesicles was prepared without racemic molecules and placed for more than 48 h so that the self-assembly would be stable. Then the BOC-DL-phenylalanine powders were put into the stable vesicle system with slightly stirring. After more than 48 h, the mixture solution was filtered by the same way as mentioned above. The CD spectra of the new filtrated solutions reveals that there is no obviously Cotton effect at around 220nm. In another word, the small molecules in the filtrated solution are still racemic and this reflects that the enantioselective assembly of amphipathic polymers truly occurs during the initial formation of the vesicles.

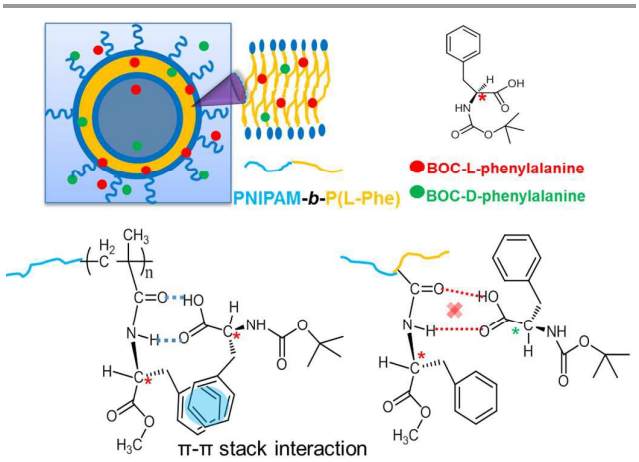

Figure 5 Schematic representation of the structure of the assembled vesicle's shell and the possible hydrogen-bonding interactions between the two enantiomers and the chiral block copolymers. The hydrogen bonds are shown as blue dotted lines.

Another factor may be the non-covalent interactions influenced by steric factors. In other word, the enantiomer of the BOC-D-(or L-) Phe with the homochirality of the chiral copolymer would preferentially enter into the bilayer membrane of the chiral vesicle. The non-covalent interactions between the two enantiomers and chiral segments within the shell of bilayer membrane would be affected significantly by the steric factors, as shown in Figure 5. The hydrogen-bonding and π-π stack interaction of aromatic rings may be influenced by the distance between the amino groups in the hydrophobic segments and carboxyl groups of BOC-phenylalanine. From Figure 5, it is clearly seen that the carboxyl of BOC-phenylalanine with the homochirality as the copolymer will more easily form the hydrogen bonds with amino groups compared to its enantiomer because of steric factors. Many significant works have reported that intermolecular hydrogen bonds and π-π stack interactions will affect the chiral discrimination and separation in various systems[25, 26]. Our experimental observation further demonstrates that the steric

factor is a key factor in enantioselective assembling phenomenon for chiral separation of hydrophobic racemic molecules. Moreover, taking the result shown in Figure 4 into consideration, it is easily deduced that only the steric factor can't fulfill the enantioselective separation of racemic molecules that are hydrophilic. Maybe a further interesting work should be focus on the assemblies of chiral polymers along with chiral molecules with different structures.

Conclusions

In conclusion, enantioselective assembly is observed during preparing micro-scale polymer vesicles through a mixture solution of amphipathic block copolymer and racemic chiral small molecules, in which the hydrophobic chain segments of the copolymer have L- (or D-) phenylalanine groups as the side substitutes. The Results show that the hydrophobic interaction is the key point for this enantioselective assembly process and the enantiomeric excess obtained is directly dependent on the molecular weight of the chiral chain segments. Other factors such as hydrogen-bonding interaction and π-π stack interaction are found also to be responsible for the enantioselective assembly phenomenon during the formation of the vesicles. This primary observation establishes a new method and opens a way to investigate how homochirality originates and transmits to other biomolecules/assemblies during the evolution of life by assembling polymers with different chirality.

Acknowledgements

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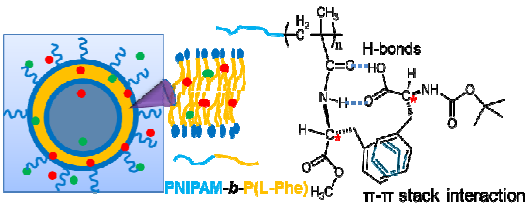

The enantioselective assembly is observed during preparing micro-scale vesicles composed of an amphipathic block copolymer bearing pendent chiral groups.