WORM-SHAPED POLYMERSOMES
AND THEIR EFFECTS ON TARGETING EFFICIENCY

BY

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THESIS
Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Chemical Engineering
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2013

Urbana, Illinois

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ABSTRACT

In order to improve the targeting efficiency of polymersomes with a given number of targeting molecules conjugated, efforts are being made to control particle shape. I hypothesized that worm-shaped polymersomes formed from self-assembly between poly(amino acid)s would have more targeting capability than spherical polymersomes. Morphology of the resulting polymersome was observed afterwards. The polymersome was further modified to present Arg-Gly-Asp (RGD) motif, which is known to bind with cells that overexpress integrins. The binding affinities of the polymersomes onto target substrates were assessed using surface plasmon resonance (SPR) spectroscopy and adherence of the polymersomes to a target cell layer was also evaluated using flow chamber simulating blood flow.
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CHAPTER 1: INTRODUCTION

Polymersomes formed from self-assembly between amphiphilic polymer chains have proven their utility in fields ranging from drug delivery to diagnostics due to their capabilities to load hydrophobic and hydrophilic drugs.\textsuperscript{1} Recent studies have focused on development of multi-functional polymersomes that can target specific malfunctional tissues/organs and also treat them.\textsuperscript{1} The targeting capability of polymersomes is typically tuned by varying the number of peptides or antibodies conjugated to the surfaces. However, their function is often limited by steric interference among molecules on the particle surface. In addition, a large number of targeting molecules on particle surfaces can possibly induce non-specific interactions between particles and non-target cells, thus reducing targeting efficiency.\textsuperscript{2}

In order to improve the targeting efficiency of polymersomes with a given number of targeting molecules conjugated, efforts are being made to control particle shape. There have been already some studies on non-spherical particles. Specifically, certain studies demonstrated that long worm-shaped filomicelles increase the circulation time in rodents\textsuperscript{3} and kill tumor stroma more efficiently.\textsuperscript{4} Also, despite the large drag from the flow, they can target the endothelial surface with proper antibodies.\textsuperscript{5} In these ways, particle shape plays a role on targeting efficiency of particles.

I therefore hypothesized that worm-shaped polymersomes formed from self-assembly between poly(amino acid)s would have more targeting capability than spherical polymersomes. In order to examine this hypothesis, poly(L-valine), which has β-sheet structure\textsuperscript{6,7}, was substituted to two systems, a vesicle-forming poly(2-hydroxyethyl aspartamide) and methoxypolyethylene glycol amine; the β-sheet can curve round on itself to form beta barrel, in
which two last strands form a closed cylinder. Morphology of the resulting polymersome was observed afterwards. The PHEA polymersome was further modified to present Arg-Gly-Asp (RGD) motif, which is known to bind with cells that overexpress integrins. The binding affinities of the PHEA polymersomes onto target substrates were assessed using surface plasmon resonance (SPR) spectroscopy and adherence of the polymersomes to a target cell layer was also evaluated using flow chamber simulating blood flow.
CHAPTER 2: MATERIALS AND EXPERIMENTS

2.1: Synthesis of PEG-b-valine

2.1.1: Synthesis of L-valine NCA

Synthesis of N-carboxyanhydride of L-valine (val-NCA) was carried out by the Fuchs-Farthing method using triphosgene. L-valine (Aldrich) and triphosgene (Aldrich) were dissolved in anhydrous THF and heated to 50°C in a strong N₂ atmosphere in order to remove HCl. After 3 hours, the reaction mixture was poured into hexane (Fisher Scientific), and the resulting suspension was stored overnight at -20°C to ensure crystallization.

2.1.2: Synthesis of PEG-b-valine

PEG-NH₂ (2,000 extent of labelling: ≥0.4 mmol/g NH₂ loading; Sigma) was dissolved in N,N-dimethylformamide (Fisher Scientific), and appropriate amount of L-valine NCA was added. The reaction mixture was stirred for 22 hours at 60°C under N₂ atmosphere and poured into diethyl ether (Fisher Scientific). The resulting suspension was stored overnight to ensure crystallization, dialyzed (MWCO 3,500; Fisher Scientific) in deionized water for two days, and lyophilized afterwards.

2.2: Synthesis of PHEA-g-valine

2.2.1: Synthesis of PHEA-NH₂
Polysuccinimide (PSI) was synthesized by polycondensation of L-aspartic acid (Aldrich) using phosphoric acid (Fisher Scientific) in the mixed solvent of sulfolane (Aldrich) and mesitylene (Aldrich) at 160°C under N₂ atmosphere for 18 hours. After cooling to room temperature, the reacted solution was washed with methanol (Fisher Scientific) and then with water several times until it was neutral. The precipitate was lyophilized afterwards.

The purified PSI was dissolved in N,N-dimethylformamide (Fisher Scientific), and appropriate amount of ethanolamine (Aldrich) was added. The mixture was stirred at room temperature for 20 hours. Then it was added to the solution of ethylenediamine and N,N-dimethylformamide (Fisher Scientific) drop-wise. The following mixture was stirred at room temperature for 22 hours. It was dialyzed (MWCO 12,000-14,000; Fisher Scientific) in deionized water for two days and lyophilized afterwards.

2.2.2: Synthesis of L-valine NCA

Synthesis of N-carboxyanhydride of L-valine (val-NCA) was carried out by the Fuchs-Farthing method using triphosgene. L-valine (Aldrich) and triphosgene (Aldrich) were dissolved in anhydrous THF and heated to 50°C in a strong N₂ atmosphere in order to remove HCl. After 3 hours, the reaction mixture was poured into hexane (Fisher Scientific), and the resulting suspension was stored overnight at -20°C to ensure crystallization.

2.2.3: Synthesis of PHEA-g-valine
The purified PHEA-NH$_2$ was dissolved in N,N-dimethylformamide (Fisher Scientific), and appropriate amount of $L$-valine NCA was added. The reaction mixture was stirred for 24 hours at 60°C under N$_2$ atmosphere and poured into diethyl ether (Fisher Scientific). The resulting suspension was stored overnight to ensure crystallization, dialyzed (MWCO 12,000-14,000; Fisher Scientific) in deionized water for two days, and lyophilized afterwards.

2.3: Synthesis of PHEA-g-valine-RGD

2.3.1: Synthesis of PHEA-g-NH$_2$-RGD

The purified PSI was dissolved in N,N-dimethylformamide (Fisher Scientific), and appropriate amount of ethanolamine (Aldrich) was added. The mixture was stirred at room temperature for 20 hours, and appropriate amount of H-GGGG-RGDSP-OH (Mimotopes) was added. The mixture was stirred at room temperature for 18 hours. Then it was added to the solution of ethylenediamine and N,N-dimethylformamide (Fisher Scientific) drop-wise. The following mixture was stirred at room temperature for 22 hours. It was dialyzed (MWCO 12,000-14,000; Fisher Scientific) in deionized water for two days and lyophilized afterwards.

2.3.2: Synthesis of PHEA-g-valine-RGD

The purified PHEA-g-NH$_2$-RGD was dissolved in N,N-dimethylformamide (Fisher Scientific), and appropriate amount of $L$-valine NCA was added. The reaction mixture was stirred for 24 hours at 60°C under N$_2$ atmosphere and poured into diethyl ether (Fisher Scientific).
The resulting suspension was stored overnight to ensure crystallization, dialyzed (MWCO 12,000-14,000; Fisher Scientific) in deionized water for two days, and lyophilized afterwards.

2.4: Characterization of PHEA-NH₂, PHEA-g-NH₂-RGD, L-valine NCA, and PHEA-g-valine structures

\(^1\)H NMR spectra of PHEA-NH₂ and PHEA-g-NH₂-RGD dissolved in deuterium oxide, of L-valine NCA in deuterium chloroform, and of PHEA-g-valine in trifluoroacetic acid-d were collected using a Varian Unity 500 MHz spectrometer, in order to quantify the degree of substitution (DS) and degree of polymerization (DP) of valine chains. Assuming that valine chains are distributed equally on all of the PHEA amine groups, the DS of valine chains equals DS of ethylamine chains.

\[
\text{DS of ethylamine or valine chains} (\%) = \frac{\text{The integral of the peak in 2.85~3.00 ppm}}{\text{The integral of the peak in 2.40~2.90 ppm}} \times 100
\]

\[
\text{DP of valine chains} = \left( \frac{\text{The integral of the peak in 0.9~1.1 ppm}}{\text{The integral of the peak in 3.0~3.2 ppm}} \right) / \text{DS}
\]

2.5: Polymersome formation by thin-film hydration

The polymer was dissolved in dimethyl sulfoxide (Fisher Scientific) with a typical concentration of 1 mg/mL. Then, the organic solvent was removed by rotary evaporation
yielding an evenly distributed film, and the film was hydrated with deionized water or mixture of FITC-BSA and PBS at 60°C for 1 hour; specifically, hydration with deionized water was performed to capture TEM and SEM images of self-assembled nanostructure, whereas, for confocal microscope images, hydration with mixture of FITC-BSA and PBS was performed. Following hydration, polymersome suspension was placed on an ice bath and sonicated for 15 minutes.

2.6: Imaging of polymersomes with transmission electron microscopy (TEM)

The morphology of self-assembled PHEA-NH$_2$ aggregates by direct dissolution in deionized water and of PHEA-g-valine and PEG-b-valine polymersomes by thin-film hydration was observed using JEOL 2100 cryo-TEM. A drop of polymersome suspension containing 8% phosphotungstic acid (Acros) was placed on a 200-mesh Cu grid supported by holey-carbon film (SPI Supplies, West Chester, PA). After drying in air, the sample was imaged at 200 kV.

2.7: Imaging polymersomes with scanning electron microscopy (SEM)

The morphology of self-assembled PHEA-g-valine and PEG-b-valine polymersomes was observed using a Hitachi S-4700 high resolution SEM. Polymersome suspension was added drop-wise to liquid nitrogen, and the frozen drops were lyophilized afterwards. The lyophilized powder was mounted on a SEM stub using conductive carbon tape.
2.8: Imaging polymersomes with confocal laser scanning microscope (LSM)

The PHEA-NH₂, PHEA-g-valine, and PEG-b-valine polymersomes suspension encapsulated with fluorescein isothiocyanate labeled bovine serum albumin (FITC-BSA) was imaged using a 63×/1.40 Oil DIC M27 objective with Zeiss LSM 700 laser scanning confocal microscope (Carl Zeiss AG, Germany). Multiple two-dimensional (2D) image stacks (512-512 pixels) were acquired along the Z axis using a 488 Ar (10 mW) laser line for FITC.

2.9: Measurements of the association/dissociation rates of the polymersomes using surface plasmon resonance (SPR)

A gold sensor chip (GE Healthcare, USA) was modified to present a 11-mercaptoundecanoic acid (MUA, Aldrich) monolayer by immersing the chip in 1.0 mM MUA solution overnight. The carboxylic groups of the MUA monolayer were activated by flowing 0.4 M EDC and 0.1 M N-hydroxysuccinimide (NHS, Aldrich) solutions through the flow cell for five minutes in a Biacore 3000 (GE Healthcare, USA). After the activation, recombinant human integrin α₅β₃ (R&D) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE, Sigma) were chemically linked to the MUA layer by flowing both solutions through the flow cell for ten minutes and five minutes, respectively. The remaining NHS-ester groups on the MUA surface were blocked by injecting 1.0 M ethanolamine hydrochloride into the flow cell for five
minutes. Finally, the PHEA-g-valine and PHEA-g-valine-RGD polymersomes suspended in PBS at a concentration of 1 mg/ml were injected into the flow cell to examine the association and dissociation rates of the polymersomes with the gold sensor chip modified with integrin $\alpha_v\beta_3$ and DPPE, and the media flow rate was kept constant at 5.0 $\mu$L/min. Kinetic data from SPR sensorgrams were collected with the assistance of BIAevaluation version 4.1, which applied a 1:1 Langmuir binding model to quantify the association and dissociation rates.\(^8\)

2.10: Analysis of polymersome adhesion onto a cell layer under flow

Mouse bone marrow stromal cells (BMSCs, ATCC) with a passage number between 28 and 34 were seeded on collagen-coated petri dishes (Falcon, Becton Dickinson) in Dulbecco’s Modified Eagle Medium (DMEM) supplemented by 10% fetal bovine serum and 1% penicillin/streptomycin (GIBCO) at 37°C until reaching sufficient confluency. After formation of a cell layer, the dish was placed into the chamber. The PHEA polymersomes suspension encapsulated with fluorescein isothiocyanate labeled bovine serum albumin (FITC-BSA) and PBS washing were injected via syringe at shear flow rate of 1.74 mL/hr. The cells were then fixed with 10% NBF. The adhesion of polymersomes to the cell layer was examined by measuring fluorescence under the confocal microscope and quantified using Image J software. With at least three samples per condition, four different areas of the cell layer were analyzed.
CHAPTER 3: RESULTS AND DISCUSSIONS

3.1 Preparation and characterization of the PEG-b-valine polymersomes

PEG-b-valine was synthesized from nucleophilic ring-opening chain growth process of val-NCA by amine groups on PEG-NH₂ (Figure 1).

Synthesis of N-carboxyanhydride of L-valine (val-NCA) was carried out by the Fuchs-Farthing method using triphosgene (Figure 2). Its molecular structure was determined by ¹H NMR spectra (Figure 3); a doublet of doublets (dd) was detected on the group (4), which was characteristic of valine.

After preparing polymersomes by the thin-film hydration method, TEM images confirmed worm-like shape of PEG-g-valine polymersomes (Figure 4a). Their polar radius (x) values ranged from 30 to 150 nm, whereas their average equatorial radius (y) was around 10 nm (Figure 4b). The following aspect ratio ranged from 3:1 to 15:1. SEM images, instead, showed a broader range in um scales (Figure 5a & 5b). Their aspect ratios, although they were non-homogeneous in size distribution, matched those of TEM. This result confirmed consistent worm-like shape.

Confocal microscope images of PEG-b-valine polymersomes confirmed encapsulation of FITC-BSA (Figure 6). The sizes of the polymersomes were in um scale, which came from much lower magnification and resolution of the confocal microscope than those of TEM and SEM.

3.2 Preparation and characterization of the PHEA-NH₂, PHEA-g-NH₂-RGD, L-valine NCA, PHEA-g-valine, and PHEA-g-valine-RGD polymersomes
Poly(2-hydroxyethyl aspartamide) substituted with ethylamine chains, termed PHEA-NH₂, was prepared from nucleophilic substitution of ethanolamine and ethylenediamine to poly(succinimide) (PSI; MW 38,000 g/mol) (Figure 7). Its molecular structure was determined by ¹H NMR spectra (Figure 8). The degree of substitution (DS) of ethylamine chains to the PHEA backbone was around 8 mol%, which was quantified from the integrals of two characteristic NMR peaks at 2.40 to 2.85 ppm and 2.85 to 3.00 ppm. Spherical shape was detected with self-assembled PHEA-NH₂ aggregates using TEM (Figure 9).

In addition to PHEA-NH₂, Poly(2-hydroxyethyl aspartamide) substituted with ethylamine chains and RGD, termed PHEA-g-NH₂-RGD, was also prepared from nucleophilic substitution of ethanolamine, H-GGGG-RGDSP-OH, and ethylenediamine to poly(succinimide) (PSI; MW 38,000 g/mol) (Figure 10). Its molecular structure was determined by ¹H NMR spectra (Figure 11). The degree of substitution (DS) of ethylamine chains to the PHEA backbone was around 10 mol%, which was quantified from the integrals of two characteristic NMR peaks at 2.50 to 2.80 ppm and 2.95 to 3.05 ppm.

PHEA-g-valine were synthesized from nucleophilic ring-opening chain growth process of val-NCA by amine groups on PHEA-NH₂ (Figure 12). Their molecular structures determined by ¹H NMR spectra (Figure 13a and 13b). The degrees of polymerization (DP) of val-NCA to the PHEA amine groups were 16 and 38 valine chains per mol% DS, which were quantified from the integrals of two characteristic NMR peaks at 0.9 to 1.1 ppm and 3.0 to 3.2 ppm.

PHEA-g-valine-RGD was synthesized from nucleophilic ring-opening chain growth process of val-NCA by amine groups on PHEA-g-NH₂-RGD (Figure 14) as well.
After preparing polymersomes by the thin-film hydration method, TEM images confirmed worm-like shape of PHEA-g-valine polymersomes (Figure 15a and 15b). Their polar radius (x) values ranged from 50 to 200 nm, whereas their average equatorial radius (y) was around 20 nm (Figure 15c). The following aspect ratio ranged from 2.5:1 to 10:1. SEM images, instead, showed a broader range in nm and um scales (Figure 16a and 16b). Their aspect ratios, although they were non-homogeneous in size distribution, matched those of TEM. This result confirmed consistent worm-like shape.

Confocal microscope images of both PHEA-NH$_2$ polymersomes at DS$_{NH_2}$ of 8 mol% and PHEA-g-valine polymersomes at DS$_{valine}$ of 8 mol% and DP$_{valine}$ of 16 chains per mol% DS confirmed encapsulation of FITC-BSA (Figure 17a and 17b). Based on such confirmation, analysis of polymersome adhesion onto a cell layer under flow was performed. The sizes of the polymersomes were in um scale, which came from much lower magnification and resolution of the confocal microscope than those of TEM and SEM.

3.3 Modification of the PHEA polymersomes with cell adhesion peptides

The PHEA polymersomes were further modified with an Arg-Gly-Asp (RGD) sequence, which is known to bind with cells that overexpress integrins, at DS of 1 mol% (Figure 10 & 14). The binding affinities of the resulting RGD peptide-conjugated polymersomes to a target cell membrane were evaluated using surface plasmon resonance (SPR).

The binding response unit (RU) values were 222 for PHEA-g-valine-RGD and 43 for PHEA-g-valine (Figure 18). Because change in the binding RU value corresponds to change in the mass density of the molecules on the SPR chip$^{2c}$, RGD modification led to an increased
number of polymersomes adhered to the artificial cell membrane, suggesting specific binding between peptides and integrins.

3.4 In vitro evaluation of the targeting capability of the PHEA-NH$_2$, PHA-g-valine, and PHEA-g-valine-RGD polymersomes

The targeting capabilities of the PHEA-NH$_2$, PHEA-g-valine, and PHEA-g-valine-RGD polymersomes encapsulated with FITC-BSA were tested by examining their adhesion to the BMSC cell layer. The model tissue was under shear flow rate of 1.74 mL/hr at which the cell overexpresses $\alpha_v\beta_3$ integrin on its surface. The PHEA-NH$_2$ polymersomes at DS$_{\text{NH}_2}$ of 8 mol% adhered to the tissue minimally (Figure 19a), whereas the PHEA-g-valine polymersomes at DS$_{\text{valine}}$ of 8 mol% and DP$_{\text{valine}}$ of 16 chains per mol% DS adhered more actively (Figure 19b). The result supports the shape effect, in which worm-shaped polymersomes have more targeting capability than spherical polymersomes. Among all, the most significant increase in adherence was with the PHEA-g-valine-RGD at DS$_{\text{RGD}}$ of 1 mol%, DS$_{\text{valine}}$ of 10 mol%, and expected DP$_{\text{valine}}$ of 20 chains per mol% DS (Figure 19c). Therefore, a small amount of RGD substitution of 1 mol% and worm-like shape likely formed by poly($L$-valine) improve targeting capability the most (Figure 20).
3.5 Figures

Figure 1. PEG-b-valine synthesis from nucleophilic ring-opening chain growth process of val-NCA by amine groups on PEG-NH$_2$.

Figure 2. Synthesis of N-carboxyanhydride of L-valine (val-NCA) by the Fuchs-Farthing method using triphosgene.

Figure 3. $^1$H NMR spectrum of N-carboxyanhydride of L-valine (val-NCA).
**Figure 4.** TEM photographs of self-assembled PEG-b-valine polymersomes (a) at expected DP\textsubscript{valine} of 23 chains. The scheme of worm-like polymersome (b) is shown with the polar radius, x, and the equatorial radius, y.

**Figure 5.** SEM photographs of self-assembled PEG-b-valine polymersomes at expected DP\textsubscript{valine} of 23 chains (a) and (b) in um scales.
Figure 6. The LSM photographs of self-assembled PEG-b-valine polymersomes suspension encapsulated with FITC-BSA at expected DP_{valine} of 23 chains.
**Figure 7.** PHEA-NH$_2$ preparation from nucleophilic substitution of ethanolamine and ethylenediamine to PSI.

**Figure 8.** $^1$H NMR spectrum of PHEA-NH$_2$ at DS$_{NH_2}$ of 8 mol%.

**Figure 9.** TEM photograph of self-assembled PHEA-NH$_2$ aggregates at DS$_{NH_2}$ of 8 mol% with negative staining (PTA 8%).
Figure 10. PHEA-g-NH$_2$-RGD preparation from nucleophilic substitution of ethanolamine, H-GGGG-RGDSP-OH, and ethylenediamine to PSI.

Figure 11. $^1$H NMR spectrum of PHEA-g-NH$_2$-RGD at DS$_{NH_2}$ of 10 mol%.
Figure 12. PHEA-g-valine synthesis from nucleophilic ring-opening chain growth process of val-NCA by amine groups on PHEA-NH$_2$.

Figure 13 (cont.)
Figure 13. $^1$H NMR spectrum of PHEA-g-valine at (a) DP of 16 valine chains per mol% DS and (b) DP of 38 valine chains per mol% DS.
Figure 14. PHEA-g-valine-RGD synthesis from nucleophilic ring-opening chain growth process of val-NCA by amine groups on PHEA-g-NH₂-RGD.
**Figure 15.** TEM photographs of self-assembled PHEA-g-valine polymersomes at DS_{valine} of 8 mol\% and (a) DP_{valine} of 38 chains and (b) at DP_{valine} of 16 chains. The scheme of worm-like polymersome (c) is shown with the polar radius, x, and the equatorial radius, y.

**Figure 16.** SEM photographs of self-assembled PHEA-g-valine polymersomes at DS_{valine} of 8 mol\% and DP_{valine} of 16 chains in (a) nm and (b) um scales.
Figure 17. The LSM photographs of self-assembled (a) PHEA-NH$_2$ at DS$_{NH_2}$ of 8 mol% and (b) PHEA-g-valine polymersomes suspension encapsulated with FITC-BSA at DS$_{valine}$ of 8 mol% and DP$_{valine}$ of 16 chains.
Figure 18. Effects of $\text{DS}_{\text{RGD}}$ of PHEA polymersomes on the binding kinetics of polymersomes to $\alpha_\text{v}\beta_3$ integrins.
Figure 19. The LSM photographs of BMSCs (on the right-hand side) after flow chamber test. The adhesion of (a) PHEA-NH$_2$, (b) PHEA-g-valine, and (c) PHEA-g-valine-RGD polymersomes encapsulated with FITC-BSA to the cell layer is examined by measuring fluorescence (on the left-hand side).
Figure 20. Quantitative analysis of fluorescence after examining the adhesion of (a) PHEA-NH$_2$, (b) PHEA-g-valine, and (c) PHEA-g-valine-RGD polymersomes encapsulated with FITC-BSA to the cell layer under the confocal microscope. Each value and error bar in the plot marks the mean and standard deviation for each type.
CHAPTER 4: CONCLUSIONS AND FUTURE WORK

The study demonstrates that beta barrel from $\beta$-sheet of poly($L$-valine) creates self-assembled worm-shaped polymersomes. The worm-like morphology enhanced the targeting capability of polymersomes compared to spherical shape. In addition, there is a significant increase in adhesion to a cell layer when polymersomes are worm-shaped and functionalized with RGD peptides in a circulation mimicking flow.

Because the worm-shaped polymersomes have improved targeting efficiency, future work can focus on turbidity measurements and *in vitro* drug release profiles for drug therapy purposes. Also, unlike other studies of worm-shaped micelles, this study forms polymersomes via thin-film hydration, which can encapsulate hydrophobic and hydrophilic drugs. More studies on application purposes can be looked in as well.
REFERENCES


