Analyst

Preparation of Molecularly Imprinted Polymers for the Recognition of Proteins via the Generation of Peptide-Fragment Binding Sites by Semi-Covalent Imprinting and Enzymatic Digestion

Journal:	*Analyst*
Manuscript ID:	AN-COM-12-2014-002299.R1
Article Type:	Communication
Date Submitted by the Author:	14-Jan-2015
Complete List of Authors:	Taguchi, Hironori; Kobe University, Graduate School of Engineering Sunayama, Hirobumi; Kobe University, Graduate School of Engineering Takano, Eri; Kobe University, Graduate School of Engineering Kitayama, Yukiya; Kobe University, Graduate School of Engineering Takeuchi, Toshifumi; Kobe University, Graduate School of Engineering

Journal Name

RSCPublishing

COMMUNICATION

Preparation of Molecularly Imprinted Polymers for the Recognition of Proteins *via* the Generation of Peptide-Fragment Binding Sites by Semi-Covalent Imprinting and Enzymatic Digestion

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Hironori Taguchi, Hirobumi Sunayama, Eri Takano, Yukiya Kitayama and Toshifumi Takeuchi*

Molecularly imprinted polymers bearing peptide fragment-based binding sites within the protein-imprinted cavities were prepared by copolymerization of acrylated protein with 6-monoacryloyl-trehalose and 6,6'-diacryloyl-trehalose as hydrophilic comonomer and crosslinker, followed by enzymatic decomposition of the grafted protein into the polymer matrix with pepsin, resulting in the creation of peptide fragment-based protein-binding sites.

The use of biologically based molecular recognition materials such as antibodies, enzymes, and other binding proteins in proteomics and disease diagnostics has been clearly established,[1-3] given the fact that they have molecular recognition properties towards their target molecules. However, these biomaterials have some limitations, including high cost and instability. Therefore, the development of artificial molecular recognition materials has been investigated extensively in order to obtain suitable alternatives for biologically based materials.

The synthesis of molecularly imprinted polymers (MIPs), via a template polymerization technique, has become a useful method involving the preparation of synthetic polymer receptors capable of molecular recognition.[4-8] This technique, in which functional monomers, comonomers and crosslinkers are copolymerized with template molecules, involves a process whereby functional monomers are used to form binding sites within their imprinted cavities. The imprinted cavities are formed following the removal of the template molecules after polymerization, and crosslinkers are used to construct polymer matrices to maintain the shapes and sizes of the imprinted cavities. Various efforts have been made to improve the suitability of MIPs for the recognition of biologically active substances, and recently, in-cavity chemical modification after the construction of molecularly imprinted cavity, *i.e.* post-imprinting modification, has been developed for posteriori introduction of on/off switching of molecular recognition ability, signal transduction activity for binding events, photo-responsive activity, and, catalytic activity. [9-16]

The focus of the use of MIPs has shifted from small molecules to the recognition of biomacromolecules, in order to further expand their use to clinical and pharmaceutical applications, given that there are many reports describing the potential of MIPs to have selective binding activities towards target proteins such as natural antibodies. [17-29] In this study we synthesized cytochrome-c (Cyt) specific MIPs, where the MIPs were prepared by semi-covalent molecular imprinting,[30] using covalently conjugated template molecules, followed by the removal of the template molecules; resulting in the formation of binding sites within the imprinted cavities that were capable of non-covalent interactions with the target molecules. In this process, acrylic acid was conjugated covalently with the lysine residues of Cyt, yielding acrylated Cyt, which was used as a template molecule. Following copolymerization with our newly synthesized hydrophilic comonomer and crosslinker; 6-monoacryloyl trehalose (MAT) and 6,6′-diacryloyl trehalose (DAT), respectively (Figure 1a), the Cyt moieties were removed by enzymatic digestion using a protease, pepsin, after which oligopeptide fragments remained to function as binding sites for Cyt within the imprinted cavities (Figure 1b). The combined use of DAT and MAT in radical polymerization may lead to MIPs with higher crosslinking density in aqueous solution and the resulting MIPs could be highly water-compatible compared to those prepared by commonly used acrylamide and *N*,*N*'-methylenebisacrylamide. Unlike previous conventional removal processes in molecular imprinting by chemical reactions such as alkaline hydrolysis, our enzymatic biological removal process would be a gentler alternative and result in intact polymer matrices, which are important constituents for the shape and size of the binding cavities of MIPs. Moreover, only the pepsin-accessible regions of MIPs can be transformed into Cyt-binding cavities, which reduce the possibility of non-specific binding sites commonly left by alkaline hydrolysis of the MIPs. For the simultaneous analysis of the proteins tested, we used matrix-assisted laser desorption/ionization time-of- flight mass spectrometry (MALDI TOF MS) as a tool for examining the binding characteristics of the proteins.[31-33] Poly(ethyleneglycol) (PEG) was used as an internal standard to improve the reproducibility of quantification, given that the reproducibility of the ionization efficiency in each spot on a sample plate for MALDI TOF MS was low. Here, effectiveness of the proposed semi-covalent molecular imprinting technique was demonstrated on the selective detection of Cyt in conjunction with MALDI TOF MS as a tool for the detection of proteins.

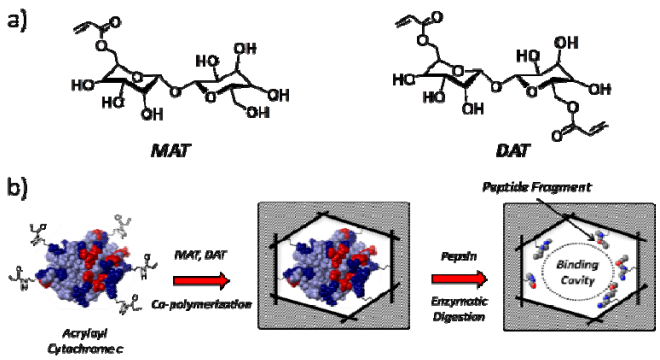

Figure 1. a) Chemical structures of MAT and DAT, b) Schematic illustration of Cyt-imprinted polymers bearing peptide-fragment binding sites prepared by semi-covalent imprinting and enzymatic digestion.

In this study, we prepared thin films of protein-imprinted polymers using a semi-covalent approach, whereby acrylic acid was covalently conjugated with Cyt by an amine coupling reaction between the lysine residues of Cyt and *N*-acryloyl succinimide. Figure 2 shows the MALDI TOF mass spectra of native Cyt and acrylated Cyts, which were prepared with 3, 5, and 10 molar ratio equivalents of *N*-acryloyl succinimide to Cyt (3acrylated Cyt, 5acrylated Cyt, and 10acrylated Cyt). The resulting *m/z* peaks were 12593, 12761, and 13033, respectively. Because the unit molecular weight of an acryloyl group is 54, Cyt was conjugated roughly with *N*-acryloyl succinimide quantitatively.

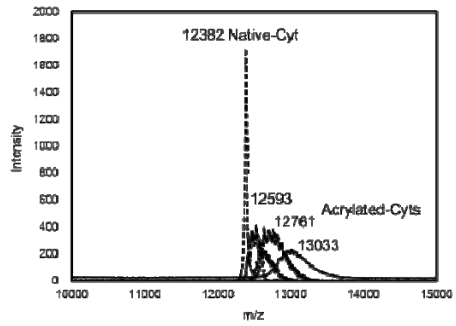

Figure 2. MALDI TOF MS spectra of native Cyt and acrylated Cyts prepared with 3, 5 and 10 molar ratio equivalents of *N*-acryloyl succinimide to Cyt.

Following the conjugation of Cyt with *N*-acryloyl succinimide, we examined the secondary structure of the conjugated Cyt by measuring the circular dichroism (CD) of native Cyt and 10acrylated Cyt (Figure S1 in ESI). We observed little difference between the θ values, derived from the Cyt α-helix, of the native and acrylated Cyt at wavelengths between 215–230 nm, even after the conjugation process. This indicates that the secondary structure of the conjugated 10acrylated Cyt is similar to that of the native Cyt, to the extent that the acrylated Cyts were usable as template molecules for Cyt-imprinted MIPs (Cyt-MIPs).

We used enzymatic digestion with pepsin in order to remove the template Cyt, since the more commonly used alkaline hydrolysis unfavourably cleaves the ester bonds of DAT and MAT. As a preliminary experiment, native Cyt and 10acrylated Cyt were digested by pepsin overnight at 37 °C (Figure S2 in ESI). The results show that the MALDI TOF MS spectra of acrylated Cyt, produced after the pepsin digestion, were similar to that of the native Cyt. This indicates that the enzyme digestion proceeded successfully, even after the conjugation of the acrylated groups to Cyt.

Acrylated groups were expected to be conjugated to ε-amino groups of lysine residues located on Cyt. Following the decomposition of Cyt by pepsin digestion, we hypothesized that the fragment peptides, containing the lysine residues grafted to the polymer backbone, would be retained in the cavities that were formed. The important point to note in this strategy, involving the formation of the binding cavity by biological decomposition, is that only macromolecule-accessible regions can be transformed into imprinted binding cavities, which may be difficult to attain otherwise by conventional chemical hydrolysis or non-covalent molecular imprinting methods. Additionally this strategy is advantageous in reducing the non-specific binding sites located outside the imprinted cavities.

Cyt used in this work is known to have 19 lysine (K) residues (PDB ID: lHRC). Hamuro et al.[34] reported a total of 27 lysine-contained fragments resulting from pepsin digestion. If K residues are located at positions close to the C- and N-terminals, it is likely that pepsin may not be able to gain full access to Cyt in order to digest it fully. Therefore, in our presumption of the peptides in the cavities, we excluded the peptides having K(s) at the first and the last four positions from the list reported by Hamuro. Accordingly, the seven remaining peptide fragments, which contained K residues that may be grafted to the polymer matrices, were as follows: 1) GDVEK^{5}GK^{7}K^{8}IFVQKCAQCHTVE; 2) TYTDANK^{53}NK^{55}GITWK^{60}EETLM; 3) YTDANK^{53}NK^{55}GITWK^{60}EETLM; 4) YTDAK^{53}NK^{55}GITWK^{60}EETL; 5) YTDANK^{53}NK^{55}GITWK^{60}EET; 6) IFAGIK^{86}K^{87}K^{88}TEREDL; and 7) IFAGI K^{86}K^{87}K^{88}TEREDLIA. When attention is aimed at amino acid residues except for Ks that may be conjugated with acrylic acids, more negatively charged D and E are contained than the basic R and H, suggesting that such anionic residues could predominantly interact with Ks in Cyt via electrostatic interaction, although it is still unclear which and how many fragments are available in an imprinted cavity for Cyt binding, following the decomposition of Cyt.

MIPs were prepared on the acrylated glass substrate by photo-initiated radical polymerization of a mixture in 10 mM Tris-HCl buffer (pH 7.4) containing acrylated Cyt, MAT, DAT, and VA-080 water-soluble initiator (see ESI). In order to examine the effect of the crosslinking density of DAT, we synthesized 3acrylated Cyt-based MIPs and NIPs, bearing three different densities of DAT (5 %, 25 %, 50 %). The remaining Cyt within the polymer matrices was detected by ultraviolet-visible measurements of Coomassie Brilliant Blue-stained Cyt, carried out before and after the enzymatic digestion. The absorbance, at approximately 650 nm, in the MIPs composed of 5 % and 25% DAT were clearly lower (Figure 3a and 3b), however, there was no significant change in the absorbance with the 50 % cross-linked MIP (Figure 3c), indicating that the enzyme digestion proceeded successfully with the 5 and 25 % DAT cross-linked polymers, but that pepsin may not have been able to access the 50 % cross-linked polymer enough to decompose and remove Cyt. Moreover, the absorbance of the polymers at around 400 nm before the pepsin digestion, which was derived from the copolymerization of the heme moiety of Cyt in the polymers, was higher with the 25% cross-linked MIP than with the 5 % cross-linked MIP, revealing that more acrylated Cyt was copolymerized in the MIP with 25 % DAT. In this instance, it is possible that the lower percentage of crosslinking agents attenuates the likelihood of crosslinking between the monomer species in solution and the acrylated groups immobilized on the glass substrate. Accordingly, there may be reduced linkage between the glass substrate and the propagated polymers, which could wash out more easily, as a result, from the surface of the substrate, reducing the apparent remaining Cyt. From

these results, the following experiments were performed using Cyt-MIP prepared with 25% DAT.

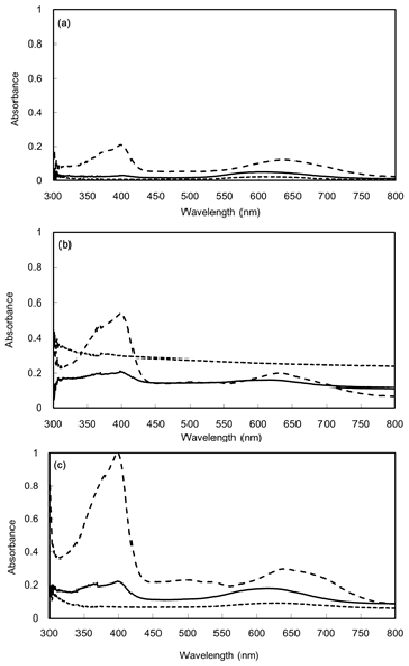

Figure 3. UV-Vis spectra of MIP, before (broken line) and after digestion (solid line) by pepsin, and NIP (dotted line). These polymers were prepared with three different DAT concentrations: (a) 5 %; (b) 25 %; and (c) 50 %.

The binding selectivity of the two acrylated Cyt-MIPs, 3 and 5acrylated Cyts, were examined using Cyt (*Mw*: 12 kDa; p*I*: 10.6) and Lyso (*Mw*: 14 kDa; p*I*: 11.2). In addition, an MIP prepared with free Cyt (free Cyt-MIP), *i.e.*, a non-covalent molecular imprinting approach, was synthesized using acrylic acid as a functional monomer in the presence or absence of Cyt as the template molecule. The binding behaviour of Cyt and Lyso towards free Cyt-MIP, 3acrylated Cyt-MIP, 5acrylated Cyt-MIP, and NIP were evaluated with MALDI TOF MS, in the presence (Figure 4a) or absence (Figure 4b) of PEG, respectively. The standard deviations in Figure 4a were significantly smaller than those given in Figure 4b, and further, by comparison with the data obtained from surface plasmon resonance measurement of 3acrylated Cyt-MIP prepared on a gold coated glass substrate, A ratio of the bound amount of Cyt to that of Lys toward 3acrylated Cyt-MIP in the SPR measurements was ca. 1.6, and the fugure is consistent with the results from MALDI TOF MS (Figure S3 in ESI), indicating that the measurements of the binding behaviour, using PEG as an internal standard, were more reliable.

Figure 4a shows that there was more Cyt bound to 3acrylated Cyt-MIP, 5acrylated Cyt-MIP and free Cyt-MIP than Lyso, while the value of the adsorption for Lyso binding to NIP was greater than that for Cyt binding, and further, the all MIPs showed less affinity toward Lyso having more basic pI than Cyt, implying that simple ion-exchange process is not a predominant driving force and the selective binding property of the MIPs toward Cyt was as a result of the imprinting process (imprinting effect). In addition, the adsorption value for the binding of Lyso to free Cyt-MIP was greater than that for binding to 3acrylated Cyt-MIP and 5acrylated Cyt-MIP, which may be due to non-specific binding of Lyso to the polymer matrix caused by randomly located acryloyl residues outside the imprinted cavities. The results indicate that the semi-covalent imprinting was an effective approach to obtain the selective binding cavities.

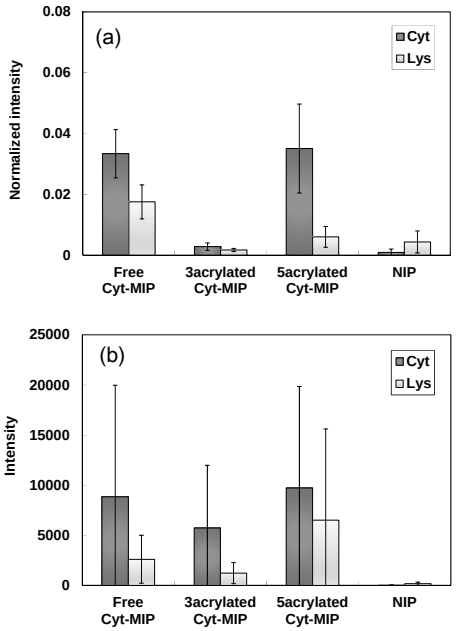

Figure 4. Binding behaviour of Cyt and Lyso to free Cyt-MIP, 3acrylated Cyt-MIP, 5acrylated Cyt-MIP, and NIP measured by MALDI TOF MS with (a) and without (b) PEG as an internal standard.

Moreover, 5acrylated Cyt-MIP adsorbed a larger amount of Cyt compared with 3acrylated Cyt-MIP, suggesting that the number of grafted peptide-fragments involved in the binding of Cyt in the cavities increased with an increase in the amount of the conjugated acrylated group in Cyt, which may underlie the apparent elevated binding ability. The peptide-fragments were only grafted within the binding cavities, therefore, the increase in the interaction sites may not lead to non-specific binding, but affected the specific binding behaviour toward Cyt, resulting in the enhanced-selectivity of 5acrylated Cyt-MIP.

Previously Shimizu et al. pointed out that when methacrylic acid was used as a functional monomer, the self-dimerization (dimerization constant: 1.9 M^{-1} in acetonitrile) influenced the efficiency of non-covalent imprinting process.[35] Presumably this "dimerization issue" happened in hydrogen bonding-based MIPs. In the present work, the polymerization proceeded in aqueous solution and the concentration of acrylic acid used for the preparation of free Cyt-MIPs and NIP was 3 mM (3 nmol/μL). Under the condition employed, the dimerization may not occur, therefore, such dimerization effect was not considered on the evaluation of binding activity.

In conclusions, we successfully demonstrated the preparation of a semi-covalent molecular imprinting-based Cyt-MIPs, using acrylated Cyts and the hydrophilic crosslinker/comonomer (DAT and MAT). The formation of Cyt-binding cavities, by the enzymatic decomposition of Cyt, was confirmed, in which the peptide fragments that were left after the pepsin digestion acted as binding sites for Cyt. The binding experiments were quantitatively evaluated using MALDI TOF MS with PEG as the internal standard, and the

selective binding ability of the MIP was confirmed to be as a result of the molecular imprinting technique, where greater binding ability was observed when more acryloyl moieties were conjugated with Cyt. The MIPs, prepared via the semi-covalent-imprinting process, showed greater selectivity than those prepared by non-covalent binding-based molecular imprinting. We believe that our method provides a novel way to develop protein recognition elements for chemosensing and affinity separation.

Notes and references

Graduate School of Engineering, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan.

† The authors thank Dr. Tooru Ooya (Kobe University, Japan) for his valuable comments on this work. We also appreciate System Instruments Co. Ltd. (Tokyo, Japan) for their technical assistance. This work was supported in part by grants (A-STEP, Seeds Validation type) from Japan Science and Technology Agency (JST).

Electronic Supplementary Information (ESI) available: materials, apparatus, synthesis, MIPs and NIP preparation, binding experiments, MALDI TOF MS spectra of native and 10acrylated Cyt after the digestion, SPR measurements. See DOI: 10.1039/c000000x/

1. C. A. K. Borrebaeck and C. Wingren, *Expert Rev. Mol. Diagn.*, 2007, **7**, 673-686.
2. C. A. K. Borrebaeck and C. Wingren, *J. Proteomics*, 2009, **72**, 928-935.
3. D. J. Brennan, D. P. O'Connor, E. Rexhepaj, F. Ponten and W. M. Gallagher, *Nat. Rev. Cancer*, 2010, **10**, 605-617.
4. B. Sellergren, *Molecularly Imprinted Polymers: Man-Made Mimics of Antibodies and Their Applications in Analytical Chemistry*, Elsevier, Amsterdam, 2001.
5. M. T. Komiyama, T. Mukawa, T. Asanuma H., *Molecularly Imprinting: From Fundamentals to Applications*, Wiler-VCH, Weinheim, 2003.
6. M. Yan and O. Ramstron, *Moleculary Imprinted Materials: Science and Technilogy*, Marcel Dekker, New York, 2005.
7. K. Haupt, *Molecular Imprinting*, Springer, Berlin, 2012.
8. S.-W. Lee and T. Kunitake, *Handbook of Molecular Imprinting: Advanced Sensor Applications*, Pan Stanford Publishing, Singapore, 2013.
9. T. Takeuchi, N. Murase, H. Maki, T. Mukawa and H. Shinmori, *Org。Biomol. Chem.*, 2006, **4**, 565-568.
10. T. Yane, H. Shinmori and T. Takeuchi, *Org。Biomol. Chem.*, 2006, **4**, 4469-4473.
11. S. Murakami, K. Yamamoto, H. Shinmori and T. Takeuchi, *Chem. Lett.*, 2008, **37**, 1028-1029.
12. K. Takeda, A. Kuwahara, K. Ohmori and T. Takeuchi, *J. Am. Chem. Soc.*, 2009, **131**, 8833-8838.
13. T. Takeuchi, T. Mori, A. Kuwahara, T. Ohta, A. Oshita, H. Sunayama, Y. Kitayama and T. Ooya, *Angew. Chem. Int. Ed.*, 2014, **53**, 12765-12770.
14. Z. Y. Tao, E. C. Tehan, R. M. Bukowski, Y. Tang, E. L. Shughart, W. G. Holthoff, A. N. Cartwright, A. H. Titus and F. V. Bright, *Anal. Chim. Acta*, 2006, **564**, 59-65.
15. Y. Suga, H. Sunayama, T. Ooya and T. Takeuchi, *Chem. Commun.*, 2013, **49**, 8450-8452.
16. H. Sunayama, T. Ooya and T. Takeuchi, *Chem. Commun.*, 2014, **50**, 1347-1349.
17. S. Li, S. Cao, M. J. Whitcombe and S. A. Piletsky, *Prog. Polym. Sci.*, 2014, **39**, 145–163.
18. A. Mujahid, N. Iqbal and A. Afzal, *Biotechnol. Adv.*, 2013, **31**, 1435-1447.
19. E. Saridakis and N. E. Chayen, *Trends Biotechnol.*, 2013, **31**, 515-520.
20. Y. Lv, T. Tan and F. Svec, *Biotechnol. Adv.*, 2013, **31**, 1172-1186.
21. K. Yang, L. Zhang, Z. Liang and Y. Zhang, *Anal.Bioanal. Chem.*, 2012, **403**, 2173-2183.
22. D. R. Kryscio and N. A. Peppas, *Acta Biomaterialia*, 2012, **8**, 461-473.
23. M. J. Whitcombe, I. Chianella, L. Larcombe, S. A. Piletsky, J. Noble, R. Porter and A. Horgan, *Chem. Soc. Rev.*, 2011, **40**, 1547-1571.
24. E. Verheyen, J. P. Schillemans, M. van Wijk, M.-A. Demeniex, W. E. Hennink and C. F. van Nostrum, *Biomaterials*, 2011, **32**, 3008-3020.
25. D. Cai, L. Ren, H. Z. Zhao, C. J. Xu, L. Zhang, Y. Yu, H. Z. Wang, Y. C. Lan, M. F. Roberts, J. H. Chuang, M. J. Naughton, Z. F. Ren and T. C. Chiles, *Nat. Nanotech.*, 2010, **5**, 597-601.
26. Y. Ge and A. P. F. Turner, *Trends Biotechnol.*, 2008, **26**, 218-224.
27. N. M. Bergmann and N. A. Peppas, *Prog. Polym. Sci.*, 2008, **33**, 271-288.
28. T. Takeuchi and T. Hishiya, *Org. Bio. Chem.*, 2008, **6**, 2459-2467.
29. D. E. Hansen, *Biomaterials*, 2007, **28**, 4178-4191.
30. M. J. Whitcombe, M. E. Rodriguez, P. Villar and E. N. Vulfson, *J. Am. Chem. Soc.*, 1995, **117**, 7105–7111.
31. T. Kondo, S. Sawa, A. Kinoshita, S. Mizuno, T. Kakimoto, H. Fukuda and Y. Sakagami, *Science*, 2006, **313**, 845-848.
32. S. D. Hanton, *Chem. Rev.*, 2001, **101**, 527-569.
33. J. Schiller, R. Suss, B. Fuchs, M. Muller, O. Zschornig and K. Arnold, *Front. Biosci.*, 2007, **12**, 2568-2579.
34. S. J. Coales, J. C. Tornasso and Y. Hamuro, *Rapid Commun. Mass Spectrom.*, 2008, **22**, 1367-1371.
35. Y. Z. D. Song, L. M. Lanni, K. D. Shimizu, Macromolecules, 2010, 43, 6284-6294.

