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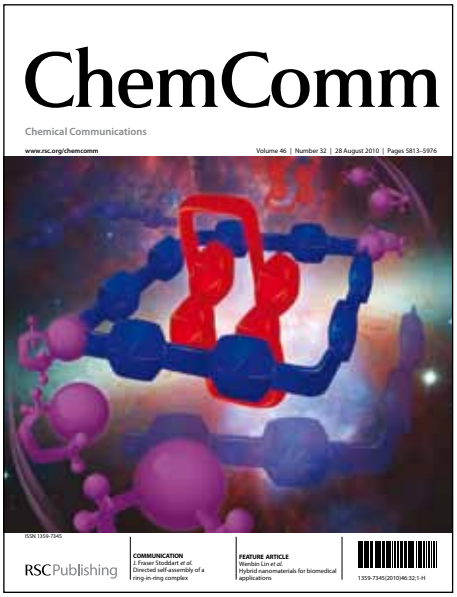

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ARTICLE TYPE

Complex Single-chain Polymer Topologies locked by Positionable Twin Disulfide Cyclic Bridges.

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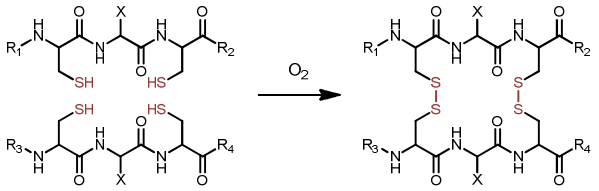
Oligomers containing the peptide sequence cysteine-*any*-cysteine (CXC) were attached, at specific locations, to a linear chain of polystyrene. The polymer-bound peptide motifs were then oxidized in dilute conditions to afford a complex bio-hybrid bi-cyclic topology via intramolecular twin disulfide bridge formation.

It is well known that stereoregular biopolymers such as proteins and RNA fold into compact, ordered conformations exhibiting precise functions.[1] The simplest features of biopolymer folding have been recreated in laboratory conditions using foldamers that are, in most cases, monodisperse stereoregular oligomers.[2] In contrast, atactic polymer chains typically adopt random coil conformations in dilute solution. However, it has been shown in recent years that amorphous random coils can nevertheless be constrained into more defined structures by means of intramolecular chemistry.[3] For example, single-chain globular nanoparticles can be obtained by random intramolecular covalent crosslinking, as shown by Hawker and others.[4] Single-chain globular compaction can also be obtained using cooperative non-covalent interactions as first introduced by Meijer and co-workers.[5] This approach is versatile and allows isotropic[5] or directional[6] compaction of random coils. Thus, although relying on atactic backbones, interesting secondary or pseudo-tertiary structures have been described.[6-7]

The Lutz group has recently extended the effort to create more precisely defined and folded synthetic macromolecules by placing covalent bridges at specific positions along the polymer chain.[8] This strategy is inspired by natural proteins, which employ precisely positioned covalent bridges, such as disulfide bonds, to reduce their conformational space. In this approach, reactive groups are positioned at specific locations along the polymer backbone using a sequence-controlled polymerization process.[9] For instance, the controlled radical copolymerization of styrene with functional *N*-substituted maleimides was used to synthesize such reactive precursors.[10] This method does not lead to perfectly sequence-defined polymers but allows inclusion of discrete functional patches in atactic polystyrene chains. These local reactive groups are subsequently reacted intramolecularly to form precisely folded polymer topologies. For example, positionable covalent bridges formed by intramolecular azide-alkyne 1,3-dipolar Huisgen cycloaddition, alkyne-alkyne Glaser coupling or amine/activated ester chemistry have been described.[8]

The positionable crosslinks reported to date are permanent covalent bonds which, unlike those found in proteins, are not dynamic and do not respond to environmental conditions. In the present work, a redox-responsive cyclic peptide was exploited to form complex bio-hybrid macromolecular topologies. Gauthier, Leroux and co-workers have recently reported that the short tripeptide cysteine-*any*-cysteine (CXC) is of strong interest for folding peptides into multicyclic species.[11] Indeed, as shown in Scheme 1, CXC motifs can be easily oxidized into a 22-membered cyclic dimer closed by two disulfide bridges. Interestingly, the dimer formation is specific, orthogonal to single-cysteine residues,[11] and highly favored as compared to oligomerization.[12]



Scheme 1. Oxidation of two CXC motifs into a twin disulfide cycle. For clarity, only the parallel adduct is displayed.

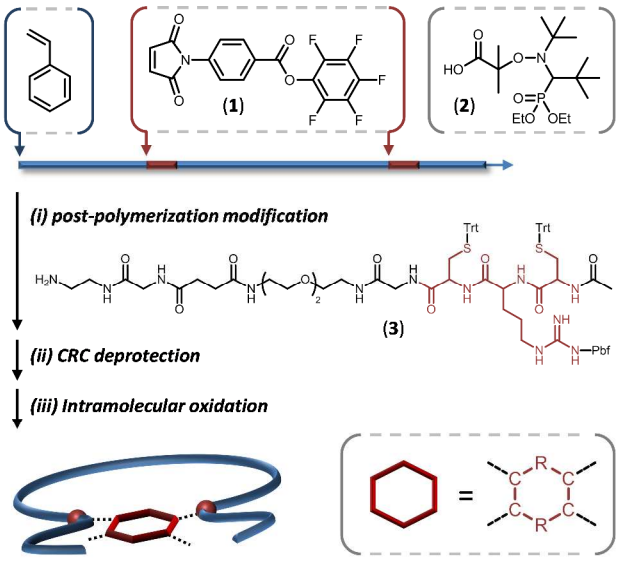


Fig. 1. General strategy studied in the present work for folding well-defined linear polymers, prepared by sequence-controlled nitroxide mediated copolymerization, into defined bicyclic topologies. Experimental conditions: (*i*) RT, NMP, (*ii*) TFA, (*iii*) O_2, RT, NMP.

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Table 1. Kinetic data recorded for the nitroxide mediated copolymerization of styrene with pentafluorophenyl 4-maleimidobenzoate **1**.[a]

	Alk.[b]	$t_{add\,1}$ [min][c]	$t_{add\,2}$ [min][c]	t_1 [min][d]	conv.$_S$ at t_1 [e]	conv.$_S$ at $t_{add\,2}$ [e]	t_2 [min][f]	conv.$_S$ at t_2 [e]	t_{end} [min][g]	conv.$_S$ at t_{end} [e]	M_n[h]	$M_{n\,theo}$[i]	M_w/M_n[h]
P1	**2**	0 (1 Eq.)	46 (1 Eq.)	6	0.12	0.51	52	0.54	100	0.70	9070	8440	1.16
P2	**2**	0 (1 Eq.)	75 (1 Eq.)	8	0.17	0.63	84	0.68	115	0.71	9930	8540	1.14
P3	**2**	0 (1 Eq.)	163 (1 Eq.)	14	0.09	0.63	175	0.64	185	0.69	8640	8330	1.13
P4	**2**	21 (1 Eq.)	71 (1 Eq.)	30	0.32	0.55	80	0.59	124	0.72	9250	8650	1.16
P5	**4**	-	60 (2 Eq.)	-	-	0.48	71	0.55	90	0.60	8520	7670	1.18

[a] Experimental conditions: styrene/anisole 2/1 v:v, 120°C in all cases except for Entry 3 at 115°C; [Styrene]/[Alk] = 100:1; [b] Alk. Means alkoxyamine; [c] Times at which the first and second addition of **1** were performed. The molar equivalents in brackets indicate the added amount of **1**. [d] Time at which full conversion of **1** was observed after the first addition. [e] Styrene conversions calculated from ^{1}H NMR spectra; [f] Time at which full conversion of **1** was observed after the second addition. [g] Final polymerization time; [h] Measured by SEC in THF; [i] $M_{n\,theo} = M_{Alk.} + M_S \cdot conv._S \cdot [S]/[Alk] + M_1 \cdot conv._1 [\mathbf{1}]/[Alk.]$.

It was therefore tempting to investigate CXC motifs for preparing multicyclic synthetic polymers. In the present study, an oligomer containing the cysteine-arginine-cysteine (CRC) sequence was attached at specific locations on atactic polystyrene backbones and used to form intramolecular covalent bridges. A four step strategy was studied to prepare the bio-hybrid bi-cyclic topologies, as depicted in Fig. 1. Well-defined reactive precursors were first synthesized by sequence-controlled copolymerization of styrene with pentafluorophenyl 4-maleimidobenzoate **1**. It was shown in a previous study that the latter monomer can be positioned at chosen locations along polystyrene chains.[13] After polymerization, the activated pentafluorophenyl ester functions of the sequence-controlled copolymer were reacted with the terminal primary amine function of the protected CRC tripeptide derivative **3**. The obtained conjugates were then deprotected and oxidized in dilute solution. The folded polymers were characterized by SEC – one of the most widely used techniques to characterize the change of hydrodynamic volume that occurs when linear polymers are intramolecularly compacted.[14] In general, cyclic polymer topologies exhibit smaller hydrodynamic radius (i.e. higher elution volumes) than linear analogues of comparable molecular weight.[15]

Five sequence-controlled copolymers of styrene and **1** were prepared by nitroxide mediated polymerization (Table 1). In most experiments, the commercial alkoxyamine BlocBuilder MA® **2** was used to initiate and control the radical copolymerization. Two local pentafluorophenyl ester functional sites were included in the chains by adding one molar equivalent of **1** twice during the polymerization of a large excess of styrene. This simple kinetic method allows placement of reactive the groups virtually anywhere along the growing polymer chain.[10e] Alternatively, symmetric microstructures were synthesized using a bifunctional alkoxyamine (**4** in ESI). In this case, the polystyrene chains grow simultaneously in two directions, and therefore two molar equivalents of **1** are added at a given time of the polymerization to afford two individual functional sites.[8a] In all cases, monitoring of the copolymerization kinetics by ^{1}H NMR spectroscopy (Figures S1-S5, ESI) evidenced the precise incorporation of **1** in narrow regions of the formed polystyrene chains. Moreover, after purification and isolation, the synthesized copolymers exhibited narrow molecular weight distributions and controlled molecular weights in agreement with theoretical predictions (Table 1). The presence of pentafluorophenyl ester groups on the purified copolymers was also confirmed by ^{19}F NMR spectroscopy (data not shown) via the characteristic peaks at -153, -159 and -163 ppm.

The sequence-controlled copolymers were then reacted with CRC oligomer **3** by activated ester/amide chemistry.[16] The quantitative conversion of the pentafluorophenyl ester groups of the copolymers into secondary amides was confirmed by FT-IR, ^{1}H NMR and ^{19}F NMR spectroscopy. In all cases, the characteristic signals of the pentafluorophenyl moieties could not be detected anymore in the ^{19}F NMR spectra of the modified polymers, and FT-IR clearly evidenced the formation of amide bonds. In addition, the signal due to the aromatic protons of pentafluorophenyl 4-succinimidobenzoate at 8.2 ppm fully vanished from all ^{1}H NMR spectra and was replaced by a new signal at 7.8 ppm as well as other peaks characteristic of the CRC moieties (Figure S6, ESI).

The formed CRC-polymer conjugates were also characterized by size exclusion chromatography (SEC) in THF, CH_2Cl_2 and DMF; the last two solvents being the most appropriate for solubilizing these chemically heterogeneous macromolecules. For instance, Figure S7 shows typical chromatograms obtained by DMF SEC before and after conjugation with **3**. An apparent molecular weight increase of about 3300 g·mol^{-1} was observed and roughly corresponds to the attachment of 2 units of **3** per copolymer.

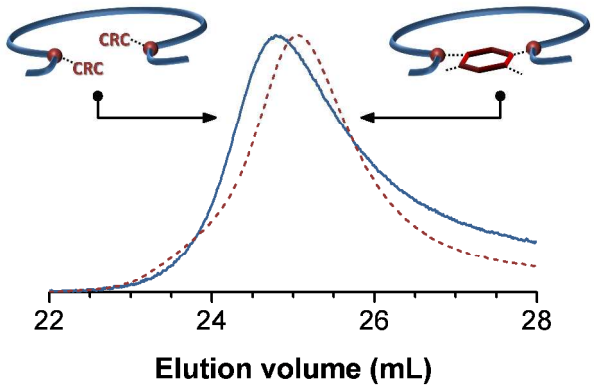

Fig. 2. SEC chromatograms recorded in CH_2Cl_2 before (blue trace) and after (red trace) double cyclization by oxidation. These measurements correspond to polymers derived from precursor **P3** in Table 1.

In a subsequent step, removal of the protecting groups present on the CRC-polymer was achieved with TFA and confirmed by ^{1}H NMR spectroscopy (Figure S8). For instance, signals due to the trityl protecting groups of the cysteine residues fully vanished at 7.41 and 7.26 ppm and a positive Ellman's test confirmed the

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presence of free thiol groups on the obtained polymers.

From their fully deprotected state, the polymers were allowed to fold spontaneously by incubation as a dilute solution in NMP saturated with air (by bubbling). The folding process was monitored by SEC in CH_2Cl_2, ^1H NMR spectroscopy and by Ellman's test. The SEC chromatograms clearly evidenced intramolecular cyclization (Fig. 2). Indeed, after oxidation, the polymers were detected at higher elution volume than the parent CRC-polymer conjugates. As mentioned above, such a shift in elution volume is characteristic of a hydrodynamic volume reduction due to intramolecular cyclization.[14] It should be however noted that a small fraction of the chains may not be folded as shown in Fig. 1. Due to chain-to-chain deviations in composition that are inherent to our sequence-controlled copolymerization method,[10e] some chains can be mono-functionalized and may not fold. In addition, intermolecular reactions may occur during the oxidation step. However, only small high molecular weight shoulders were observed in these experiments, thus suggesting a limited amount of defects due to intermolecular CRC dimerization. Furthermore, Ellman's tests were negative, thus confirming the disappearance of the free thiol functions. All these experimental results account for the formation of complex cyclized topologies. It should however be noted that both parallel and antiparallel CRC dimerization may occur in these systems, but these could not be distinguished experimentally. Nevertheless, the double cyclic topology of these folded macromolecules can be precisely controlled. Indeed, the sequence controlled copolymerization approach, used for the synthesis of the linear precursors, allows control over ring size and external arms sizes. Intramolecular cyclization was clearly observed for precursors with diverse microstructures (Figure S9 in ESI).

Conclusions

In summary, twin disulfide 22-membered rings were studied as positionable bridges for the guided folding of atactic polystyrene chains. Complex bio-hybrid bi-cyclic topologies were obtained by reacting intramolecularly linear precursors containing positionable CRC motifs. Efficient and reagent free folding was observed in all studied cases. In particular, in the studied conditions, intramolecular cyclization was found to be favored as compared to intermolecular reactions. These results confirm that CXC oligomers are highly specific self-associating motifs and emphasize the relevance of these tripeptides for advanced macromolecular engineering. This study also paves the way for designing future water soluble multi-cyclic bio-hybrids,[17] e.g. for enhancing the therapeutic potential of peptide drugs.[18]

Notes and references

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1. a) M. Ouchi, N. Badi, J.-F. Lutz, M. Sawamoto, *Nat. Chem*, 2011, **3**, 917; b) H. G. Börner, *Prog. Polym. Sci.*, 2009, **34**, 811.
2. a) G. Guichard and I. Huc, *Chem. Commun.*, 2011, **47**, 5933; b) E. Yashima, K. Maeda, H. Iida, Y. Furusho, K. Nagai, *Chem. Rev.*, 2009, **109**, 6102.
3. a) J.-F. Lutz, *Kobunshi*, 2013, **62**, 509; b) D. E. Lonsdale and M. J. Monteiro, *Chem. Commun.*, 2010, **46**, 7945; c) N. Sugai, H. Heguri, T. Yamamoto, Y. Tezuka, *J. Am. Chem. Soc.*, 2011, **133**, 19694; d) T. Yamamoto and Y. Tezuka, *Polym. Chem.*, 2011, **2**, 1930.
4. a) E. Harth, B. Van Horn, V. Y. Lee, D. S. Germack, C. P. Gonzales, R. D. Miller, C. J. Hawker, *J. Am. Chem. Soc.*, 2002, **124**, 8653; b) B. S. Murray and D. A. Fulton, *Macromolecules*, 2011, **44**, 7242; c) O. Altintas and C. Barner-Kowollik, *Macromol. Rapid Commun.*, 2012, **33**, 958; d) B. T. Tuten, D. M. Chao, C. K. Lyon, E. B. Berda, *Polym. Chem.*, 2012, **3**, 3068; e) P. T. Dirlam, H. J. Kim, K. J. Arrington, W. J. Chung, R. Sahoo, L. J. Hill, P. J. Costanzo, P. Theato, K. Char, J. Pyun, *Polym. Chem.*, 2013, **4**, 3765.
5. E. B. Berda, E. J. Foster, E. W. Meijer, *Macromolecules*, 2010, **43**, 1430.
6. T. Mes, R. van der Weegen, A. R. A. Palmans, E. W. Meijer, *Angew. Chem., Int. Ed.*, 2011, **50**, 5085.
7. a) T. Terashima, T. Mes, T. F. A. De Greef, M. A. J. Gillissen, P. Besenius, A. R. A. Palmans, E. W. Meijer, *J. Am. Chem. Soc.*, 2011, **133**, 4742; b) N. Giuseppone and J.-F. Lutz, *Nature*, 2011, **473**, 40; c) N. Hosono, M. A. J. Gillissen, Y. Li, S. S. Sheiko, A. R. A. Palmans, E. W. Meijer, *J. Am. Chem. Soc.*, 2013, **135**, 501; d) J. Romulus and M. Weck, *Macromol. Rapid Commun.*, 2013, n/a.
8. a) B. V. K. J. Schmidt, N. Fechler, J. Falkenhagen, J.-F. Lutz, *Nat. Chem.*, 2011, **3**, 234; b) M. Zamfir, P. Theato, J.-F. Lutz, *Polym. Chem.*, 2012, **3**, 1796.
9. a) J.-F. Lutz, M. Ouchi, D. R. Liu, M. Sawamoto, *Science*, 2013, **341**, 628; b) N. Badi and J.-F. Lutz, *Chem. Soc. Rev.*, 2009, **38**, 3383; c) J.-F. Lutz, *Nature Chem.*, 2010, **2**, 84; d) J.-F. Lutz, *Polym. Chem.*, 2010, **1**, 55.
10. a) S. Pfeifer and J.-F. Lutz, *J. Am. Chem. Soc.*, 2007, **129**, 9542; b) S. Pfeifer and J.-F. Lutz, *Chem. Eur. J.*, 2008, **14**, 10949; c) J.-F. Lutz, B. V. K. J. Schmidt, S. Pfeifer, *Macromol. Rapid Commun.*, 2011, **32**, 127; d) M. Zamfir and J.-F. Lutz, *Nat Commun*, 2012, 1138; e) J.-F. Lutz, *Acc. Chem. Res.*, 2013.
11. C. Wu, J.-C. Leroux, M. A. Gauthier, *Nat Chem*, 2012, **4**, 1044.
12. M. Rauschenberg, S. Bomke, U. Karst, B. J. Ravoo, *Angew. Chem., Int. Ed.*, 2010, **49**, 7340.
13. a) R. Kakuchi, M. Zamfir, J.-F. Lutz, P. Theato, *Macromol. Rapid Commun.*, 2012, **33**, 54; b) M. Zamfir and J. F. Lutz, in *Progress in Controlled Radical Polymerization: Materials and Applications*, American Chemical Society, 2012, vol. 1101, pp. 1.

View Article Online
DOI: 10.1039/C3CC47807F

14. a) B. A. Laurent and S. M. Grayson, *Chem. Soc. Rev.*, 2009, **38**, 2202; b) H. R. Kricheldorf, *J. Polym. Sci. Part A: Polym. Chem.*, 2010, **48**, 251; c) D. E. Lonsdale, C. A. Bell, M. J. Monteiro, *Macromolecules*, 2010, **43**, 3331.
15. a) R. Borsali, S. Lecommandoux, R. Pecora, H. Benoit, *Macromolecules*, 2001, **34**, 4229; b) S. Lecommandoux, R. Borsali, M. Schappacher, A. Deffieux, T. Narayanan, C. Rochas, *Macromolecules*, 2004, **37**, 1843.
16. P. Theato, *J. Polym. Sci. Part A: Polym. Chem.*, 2008, **46**, 6677.
17. a) S. Srichan, L. Oswald, M. Zamfir, J.-F. Lutz, *Chem. Commun.*, 2012, **48**, 1517; b) S. Srichan, D. Chan-Seng, J.-F. Lutz, *Acs Macro Lett.*, 2012, **1**, 589.
18. J.-F. Lutz and H. G. Borner, *Prog. Polym. Sci.*, 2008, **33**, 1.

Table of contents entry

Double cyclic polymer topologies were prepared by intramolecular folding of sequence-controlled linear precursors containing precisely positioned self-associating cysteine-arginine-cysteine peptide motifs.