Macromol. Symp. **2005**, *225*, 1-8

Polymer Protein Hybrids

Jurgen M. Smeenk,[1] *Lee Ayres,*[1] *Henk G. Stunnenberg,*[2] *Jan C.M. van Hest**[1]

[1] Department of Organic Chemistry, NSRIM institute, University of Nijmegen
Toernooiveld 1, 6525 ED Nijmegen, The Netherlands
Fax (+31) 24 365 3393, E-mail: vanhest@sci.kun.nl
[2]Department of Molecular Biology, NCMLS, University of Nijmegen, The Netherlands

Summary: Structural proteins form an important source of inspiration for materials scientists, because of the well-defined relationship between their three-dimensional folding pattern and their physical properties. Incorporation of structural peptide elements in synthetic polymers allows the combination of natural structural control with synthetic versatility. In this report, two approaches to introduce silk-inspired β-sheet elements in hybrid block copolymers are described. In our first approach we have successfully applied the controlled radical polymerization technique Atom Transfer Radical Polymerization (ATRP) to construct ABA triblock copolymers, of which the A blocks consist of PMMA and the B block consists of Ala-Gly-Ala-Gly-methacrylate moieties. This method leads to triblock copolymers with the structural peptide elements in the side chain of the middle block.
Our second approach uses protein engineering for the construction of the β-sheet element. The latter is converted into a triblock copolymer architecture by modification of both the N and C ends with poly(ethyleneglycol) chains. In this case the B block consists solely of the β-sheet peptide element. It was demonstrated that it is possible to introduce β-sheet characteristics in the hybrid material by means of both approaches.

Keywords: ATRP; protein engineering; β-sheets; silks

Introduction

Structural proteins have been a source of inspiration for materials scientists for a considerable amount of time. Collagens, keratins, elastins and silk proteins all exhibit mechanical properties that are tailor-made for the specific material functions they have to fulfill in nature[1,2]. These properties are a result of the well-defined three-dimensional architecture of these proteins, which is governed by the primary amino acid sequence. The possibility of proteins to translate information from the monomer level into materials with a predetermined organization is nowadays recognized as an important tool for the construction of a new generation of materials that can fulfill the demands of fields such as nanotechnology, where such a level of control becomes increasingly important. Recent

DOI: 10.1002/masy.200550701

advances in new polymerization techniques, such as protein engineering and controlled radical polymerization, have now provided us with the tools to actively investigate the possibilities of incorporation of protein elements in polymer architectures. This allows us to combine the control over structure and functionality of proteins with the versatility of synthetic polymers.

Silks are an especially interesting class of structural proteins. Silks from both silkworm silk and (dragline) spider silk have been studied extensively[3-8]. Their three-dimensional structure has recently been unraveled and the relation between mechanical properties and the folding patterns is now reasonably well understood. Dragline spider silk has a unique combination of strength and toughness, comparable to the highest grades of high tenacity nylon. In a simplified way it can be described as an all-protein nanocomposite, consisting of alanine-rich β-sheet crystalline domains dispersed in a glycine-rich unordered matrix. The β-sheets introduce the desired stiffness to the material, whereas the matrix is responsible for the toughness. In recent literature different approaches have been followed to obtain substantial quantities of silk. Via protein engineering (parts of) the natural spider silk protein can be produced in other organisms, yielding larger quantities of the desired material [9-11]. Difficulties in this respect are related to the fact that the protein itself does not have the desired properties, and has to be correctly processed to obtain the functional folded structure. A recently developed aqueous spinning procedure has made this approach more promising[12].

Another protein engineering approach focuses on the best defined elements of silk, namely the β-sheet domains. In this case processing problems can be circumvented. Artificial genes of β-sheet mimetic proteins have been constructed and produced as homopolymer, or in combination with other structural protein elements such as elastins (SELPS)[13-15]

A third approach combines peptide chemistry and polymer synthesis to introduce oligoalanine elements into polyethylene glycol[16]. This method is more straightforward but also less controlled than the other synthetic procedures mentioned.

In this report we describe two successful methods to introduce β-sheet elements into well-defined hybrid triblock copolymers. Our first approach makes use of controlled radical polymerization, specifically Atom Transfer Radical Polymerization (ATRP) to prepare block copolymer architectures with β-sheet oligopeptides in the side chains. In a second approach we modify monodisperse β-sheet polypeptides, prepared via protein engineering, on both the N and C termini with polyethylene glycol chains (Figure 1). The results of both synthetic procedures are the basis of this report.

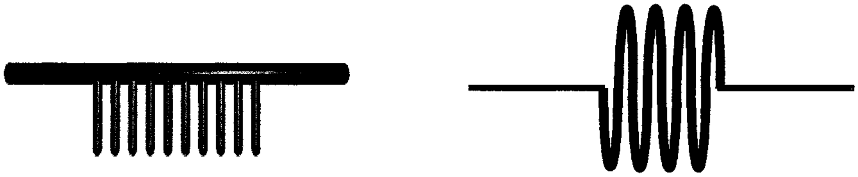
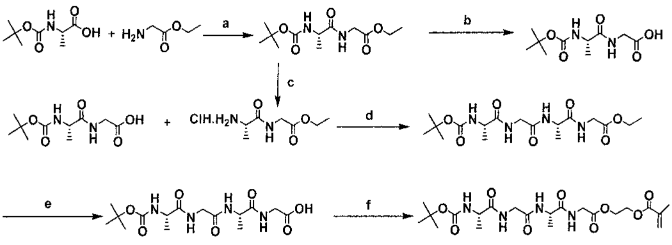


Figure 1. a) Triblock copolymers with β-sheet elements in the side chain; b) Triblock copolymers with the β-sheet element in the main chain.

Triblock Copolymers with Side Chain β-Sheet Elements

In order to incorporate β-sheet elements in the side chain of a triblock copolymer, silk-mimetic peptides that could be polymerized via the controlled radical polymerization technique of ATRP had to be prepared. As peptide sequence, the Alaniel Glycine repeat Ala-Gly-Ala-Gly (AGAG) was chosen. This is the amino acid sequence commonly observed in β-sheets of silkworm silk. It was preferred to the oligoalanine structures found in spider silk because of its better solubility. The peptide was constructed using standard solution phase chemistry. The monomer handle was introduced by a DCC-mediated coupling of hydroxy ethyl methacrylate with the C minus (end) of the tetrapeptide. The overall synthetic procedure is depicted in Scheme 1.

In order to build up the triblock copolymer as depicted in Figure 1a, the polymerization of peptide functional monomer **1** was performed with a bifunctional initiator, 1,4-(2'-bromo-2'-methylpropionato)benzene. The polymerization was conducted at 40°C under normal ATRP conditions, using CuCl/BiPy as catalyst and DMSO-d_6 as solvent. This solvent was used to maintain solubility of the monomer and polymer species, and to be able to follow the reaction kinetics by ^{1}H-NMR spectroscopy. Furthermore, the usefulness of this method was already demonstrated in our labs for the polymerization of elastin based monomers[17].



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Scheme 1. Synthesis of Boc-Ala-Gly-Ala-Gly-ethylmethacrylate, a) DCC, HOBt, DCM, 91% b) 4M NaOH, MeOH, dioxane, quantitative c) EtOAc/2M HCl, quantitative d) DCC, HOBt, DCM, 40% e) 4M NaOH, MeOH, dioxane, quantitative f) DCC, DMAP, HOBt, DMF, 42%, HEMA.

The polymerization of monomer **1** via ATRP reached 92% conversion after 2 hours. Analysis of the kinetics (Figure 2) showed a first-order reaction rate in monomer conversion, which is indicative of a controlled radical polymerization process. This was furthermore confirmed by GPC, which demonstrated that a PDI of 1.17 was obtained.

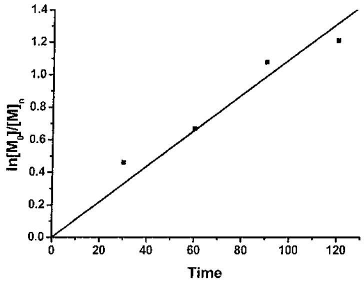


Figure 2. Plot of ln conversion vs. time for the polymerization of Boc-Ala-Gly-Ala-Gly-ethylmethacrylate.

For the A blocks of the triblock copolymer pMMA was chosen. MMA was polymerized via ATRP using the obtained AGAG-containing side chain polymer as macroinitiator. Best results were obtained when this polymerization was conducted *in situ*, without an intermediate work-up procedure of the B block. For the ATRP of MMA, linear kinetics were again observed, and the final triblock copolymer had a well defined architecture, as was demonstrated by the low PDI ($M_{n,\ product}$ = 6.1 kg/mol; PDI = 1.19).

The N termini of the tetrapeptide side chains were subsequently deprotected by removal of the Boc group via treatment with trifluoro acetic acid. After purification this final block copolymer was analyzed for its β-sheet characteristics by IR-spectroscopy. With this technique it was clearly demonstrated that amide I vibrations, indicative of the antiparallel β-sheet conformation, were present (Table 1). This could only be observed with the block copolymer, as the AGAG monomer clearly displayed random coil behavior.

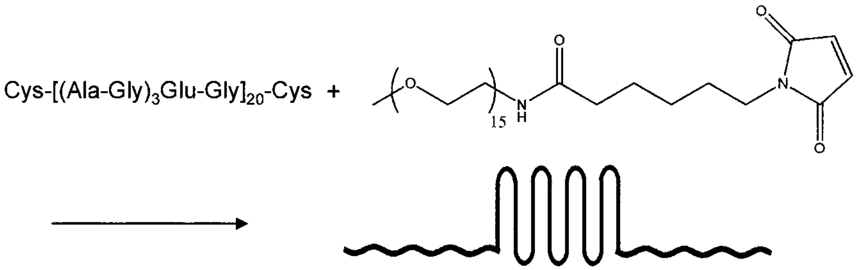
Table 1. Table of standard IR values for the amide I and II bands[18,19] compared to the values obtained for monomer **1** and the respective block copolymer.

Compound	**Amide I**	**Amide II**
Random coil	1656 cm^{-1}	1535 cm^{-1}
Antiparallel β-sheet	1632(s)/1685(w) cm^{-1}	1530 cm^{-1}
BocAGAG-EMA (**1**)	1655 cm^{-1}	1526 cm^{-1}
pMMA-b-pAGAG-EMA-b-pMMA	1624(s)/1683cm^{-1}	1537 cm^{-1}

Triblock Copolymers with a β-Sheet Element in the Main Chain

In a second approach our level of control of the triblock copolymer architecture was further extended by using protein engineering for the preparation of the β-sheet part. Both N and C termini were afterwards modified with a poly(ethyleneglycol) chain to obtain the silk mimetic architecture. The synthetic strategy used to prepare these block copolymers is depicted in Scheme 2.

The synthetic gene encoding for the Cys-[(Ala-Gly)$_3$-Glu-Gly]$_{20}$-Cys β–sheet was based on earlier work by Krejchi et al.[13] and was constructed *via* multimerization techniques. For ease of purification a DNA fragment encoding for a hexahistidine tag was introduced at the beginning of the sequence. It was then transferred to the pET-3b expression vector for production of the desired polypeptide in *E. coli* BL21(DE3)pLysS cells. Since the protein was part of the soluble fraction, Ni-NTA affinity chromatography was carried out under native conditions.



Scheme 2. The antiparallel β-sheet [(Ala-Gly)$_3$-Glu-Gly]$_{20}$ contains cysteine moieties at both N and C terminis. The thiol groups are reacted with maleimide functional poly(ethyleneglycol) to obtain the desired triblock copolymer structure.

Due to low production yields, the purification method had to be extended to gel filtration chromatography to obtain the pure desired product (Figure 3). The protein was identified by MALDI-TOF mass spectrometry and ^{1}H-NMR spectroscopy.

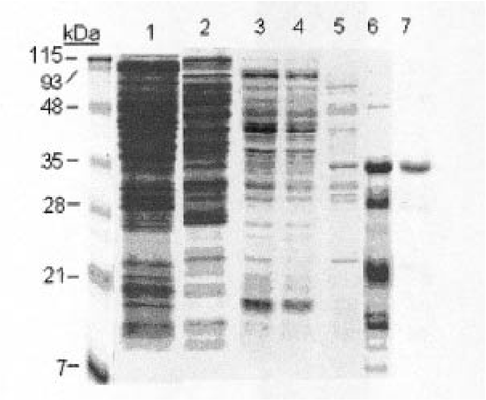


Figure 3. Expression and purification of cysteine-flanked [(AG)$_3$EG]$_{20}$ proteins as analyzed by 15% SDS-polyacrylamide gels stained with Coomassie Blue R-250. Lanes **1** and **2**: whole cell lysate of *E. coli* BL21(DE3)pLysS uninduced and 3 hours after induction with IPTG; lane **3**: clarified soluble fraction of lysate; lane **4**: flow-through of Ni-NTA column; lane **5** elution fraction of Ni-NTA column; lane **6**: soluble fraction after heating for 10 minutes at 70°C; lane 7: protein after purification with Superdex-75 column.

In order to be able to attach the poly(ethyleneglycol chains), the thiol groups first had to be reduced. This could be performed by treatment with an excess of dithiothreitol. After precipitation of the reduced protein with trichloroaceticacid, and subsequent solubilization in phosphate buffer, a 20-fold excess of poly(ethyleneglycol), functionalized with a maleimide moiety, was added. The coupling reaction could be followed by polyacrylamide at room temperature. Using the hexahistidine tag, which was still present, the functionalized protein could be separated from the excess poly(ethyleneglycol).

After the correct structure was established *via* MALDI-TOF spectrometry and ^{1}H-NMR spectroscopy, the effect of the presence of synthetic polymers on the ability of the Ala-Gly peptide to fold in the desired β-sheet structure was investigated. The crystallization process of the protein was performed in a 70% formic acid solution. Methanol was then vapor diffused into the protein solution. Crystallization occurred after 2 days and the correct folded β-sheet structure was obtained (Table 2).

Table 2. Table of standard IR values for the Amide I and II bands[18,19] compared to the values obtained for the triblock copolymer, containing the main chain β-sheet structure.

Compound	**Amide I**	**Amide II**
Random coil	1656 cm^{-1}	1535 cm^{-1}
Antiparallel β-sheet	1632(s)/1685(w) cm^{-1}	1530 cm^{-1}
PEG-Cys-$[(Ala\text{-}Gly)_3]_{20}$-Cys-PEG	1621 cm^{-1}	1521 cm^{-1}

Conclusions

We have successfully developed two new methods for the introduction of β-sheet peptide elements in hybrid block copolymers. Well-defined architectures could be built up by either ATRP or protein engineering. The obtained polymers are now the topic of further investigation with respect to their physical properties and assembly behavior.

Acknowledgments

The authors gratefully acknowledge the Netherlands Technology Foundation for financial support.

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