Peptide/protein–polymer conjugates: synthetic strategies and design concepts

Marc A. Gauthier and Harm-Anton Klok*

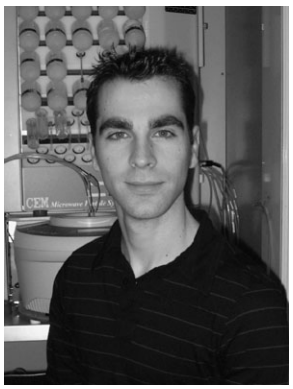
Received (in Cambridge, UK) 21st December 2007, Accepted 6th February 2008
First published as an Advance Article on the web 17th April 2008
DOI: 10.1039/b719689j

This feature article provides a compilation of tools available for preparing well-defined *peptide/protein–polymer conjugates*, which are defined as hybrid constructs combining (i) a defined number of peptide/protein segments with uniform chain lengths and defined monomer sequences (primary structure) with (ii) a defined number of synthetic polymer chains. The first section describes methods for post-translational, or direct, introduction of chemoselective handles onto natural or synthetic peptides/proteins. Addressed topics include the residue- and/or site-specific modification of peptides/proteins at Arg, Asp, Cys, Gln, Glu, Gly, His, Lys, Met, Phe, Ser, Thr, Trp, Tyr and Val residues and methods for producing peptides/proteins containing non-canonical amino acids by peptide synthesis and protein engineering. In the second section, methods for introducing chemoselective groups onto the side-chain or chain-end of synthetic polymers produced by radical, anionic, cationic, metathesis and ring-opening polymerization are described. The final section discusses convergent and divergent strategies for covalently assembling polymers and peptides/proteins. An overview of the use of chemoselective reactions such as Heck, Sonogashira and Suzuki coupling, Diels–Alder cycloaddition, Click chemistry, Staudinger ligation, Michael's addition, reductive alkylation and oxime/hydrazone chemistry for the convergent synthesis of *peptide/protein–polymer conjugates* is given. Divergent approaches for preparing *peptide/protein–polymer* conjugates which are discussed include peptide synthesis from synthetic polymer supports, polymerization from peptide/protein macroinitiators or chain transfer agents and the polymerization of peptide side-chain monomers.

1. Introduction

Peptide/protein–polymer conjugates are hybrid materials, which are either designed to benefit from the synergistic behavior of both components or to overcome shortcomings inherent to the components alone. To date, the most prominent representative of this class of materials are conjugates of peptides/proteins with poly(ethylene glycol) (PEG). Peptide/protein PEGylation is now established as a powerful strategy to improve the *in vivo* properties (circulation half-life, stability, immunogenicity, *etc.*) of therapeutic peptides/proteins.[1] The development of many new and/or improved strategies for the

École Polytechnique Fédérale de Lausanne (EPFL), Institut des Matériaux, Laboratoire des Polymères, Bâtiment MXD, Station 12, CH-1015 Lausanne, Switzerland. E-mail: harm-anton.klok@epfl.ch; Fax: +41 21 693 5650; Tel: +41 21 693 4866



Marc A. Gauthier

Marc A. Gauthier was born in 1979 in Montreal (Canada) and received his BSc in chemistry from the Université de Montréal with distinctions. Marc received fellowships from the Natural Science and Engineering Council of Canada and the Fonds Québecois de la Recherche sur la Nature et les Technologies (FQRNT) and was awarded his PhD in 2007 for his work with T. H. Ellis and X. X. Zhu. He is currently a postdoctoral fellow (FQRNT) with H.-A. Klok (EPFL, Switzerland) in the field of peptide/protein–polymer conjugation.

Harm-Anton Klok

Harm-Anton Klok was born in 1971 and studied chemical technology at the University of Twente (Enschede, The Netherlands) from 1989 to 1993. He received his PhD degree in 1997 from the University of Ulm (Germany) after working with M. Möller. After postdoctoral research with D. N. Reinhoudt (University of Twente) and S. I. Stupp (University of Illinois at Urbana–Champaign, Urbana, IL, USA), he joined the Max Planck Institute for Polymer Research (Mainz, Germany) in early 1999 as a project leader in the group of K. Müllen. In November 2002, he was appointed to the faculty of the Swiss Federal Institute of Technology (Lausanne, Switzerland). There he is currently heading the Polymer Laboratory in the Institute of Materials.

preparation of *peptide/protein–polymer conjugates* over the past years, however, paves the way for numerous novel and elegant applications of this class of materials.

The continuous expansion of the number of synthetic strategies that can be used to prepare *peptide/protein–polymer conjugates* is due to the development of advanced methodologies for preparing synthetic peptides, creative use of controlled polymerization techniques, chemoselective coupling systems and residue-/site-specific protein modification reactions. Currently, the myriad of choices available for preparing *peptide/protein–polymer conjugates* may be overwhelming when attempting to devise an approach for preparing such a material.

The purpose of this article is to provide a useful compilation of the tools available for preparing well-defined *peptide/protein–polymer conjugates*. Herein, well-defined *peptide/protein–polymer conjugates* are defined as hybrid constructs which combine: (i) a defined number of peptide/protein segments with uniform chain lengths and defined monomer sequences (primary structure) with (ii) a defined number of synthetic polymer chains. The constructs are obtained by coupling specific amino acid residues on the peptide/protein with specific functional groups located at defined positions on the synthetic polymers.

This contribution is separated into three major sections, which discuss the elementary steps involved in designing synthetic pathways for the preparation of *peptide/protein–polymer conjugates*. In what follows, we use the terms *polymer-reactive* or *peptide/protein-reactive* as descriptors for functional groups intended for reaction with a complementary functional group on a polymer or peptide/protein. The first section, entitled *preparation of polymer-reactive peptides/proteins* describes methods for post-translational, or direct, introduction of chemoselective handles onto natural or synthetic peptides/proteins. Included topics are residue- and site-specific modification of natural proteins, as well as synthetic and biosynthetic methods for producing peptides/proteins bearing chemoselective groups. The second section, entitled *preparation of peptide/protein-reactive polymers*, describes methods for introducing chemoselective groups onto synthetic polymers either through the use of functional monomers, initiators or terminators, or through quantitative post-polymerization modification. The final section, entitled *preparation of peptide/protein–polymer conjugates*, discusses convergent and divergent methods for covalently assembling polymers and peptides/proteins and includes a discussion of currently available chemoselective coupling reactions as well as the synthesis of polymers from peptide/protein macroinitiators or peptide side-chain monomers.

Given the vastness of the topics addressed herein as well as the overwhelming number of studies published each year in this field, many recent, important, and detailed review articles have been published on individual aspects discussed in this contribution. Reference to reviews of outstanding interest will be made at the beginning of each relevant subsection. This article places particular emphasis on studies which develop new design concepts or synthetic strategies for preparing *peptide/protein–polymer conjugates* and does not specifically discuss applications for such materials.

2. Preparation of *polymer-reactive peptides/proteins*

2.1 Introductory remarks

The synthesis of *polymer-reactive peptides/proteins*, which can be used for the preparation of well-defined *peptide/protein–polymer conjugates*, requires knowledge of the primary structure of the peptide/protein and the presence of specific amino acid residues that can be chemoselectively modified under conditions that do not influence the side chains of the other amino acids. The target amino acid has to be reasonably exposed on the surface of the protein and not buried in the interior of the protein in order for functionalization to occur. Obviously, the residue selected for modification should be chosen so that conjugation with a polymer will not affect protein conformation or function. In practice, information about the protein's three-dimensional structure (single-crystal X-ray and/or solution NMR studies) is necessary, though the primary sequence may be sufficient if only partial knowledge of protein structure is known.[2]

A major challenge in the preparation of *polymer-reactive peptides/proteins* lies in the site-specific introduction of the desired functional groups. While quite a number of residue-specific transformation reactions are known (*vide infra*), proteins, and especially large proteins, are likely to contain multiple copies of some, if not all twenty canonical amino acids and thus directing the modification at a specific site is a daunting task. Consequently, the usefulness of a given residue-specific reaction for preparing well-defined *peptide/protein–polymer conjugates* may be offset by the overall abundance of this residue within the protein's sequence. While clearly the desire for site-specific modification of a protein requires, or assumes, that detailed information on the protein's sequence and three-dimensional structure is available, in certain circumstances such information is not available. In such cases, however, knowledge of natural amino acid abundances, the distribution of amino acids over the primary structure of the peptide/protein as well as information on the average surface accessibility of amino acids may provide rules of thumb that can guide the preparation of site-specifically modified *polymer-reactive peptides/proteins*. Table 1 lists the natural abundances of the 20 canonical amino acids in proteins taken from the SWISS-PROT database (Release 54.5).[3] Selecting one of the less abundant amino acids such as tryptophan or cysteine for the introduction of polymer-reactive groups offers a first strategy to control the degree of modification of the peptide/protein and may facilitate the preparation of well-defined *peptide/protein–polymer conjugates*. A second strategy that may assist in the design of well-defined *peptide/protein–polymer conjugates* is based on the consideration that different amino acids have different propensities to be located in certain sections of the polypeptide or protein. For instance, short peptides/proteins (<50 residues) have been found to over-represent cysteine and tryptophan residues relative to their overall frequency in proteins, particularly in the N-(cysteine) and C-terminal regions (cysteine and tryptophan) (Table 1).[4] Conversely, glutamine is over-represented in the N-terminal region while under-represented in the C-terminal

Table 1 Trends in amino acid (AA) composition[4,5] and average surface accessibility[6] in database proteins

Amino acid	Natural abundance (%)	For short peptides (<50 AA)			Near binding sites	ASA[a]
		Overall	N-terminal region	C-terminal region		
Surface amino acids						
Asp	5.33					0.615
Gln	3.98		+[b]	−[c]		0.573
Glu	6.66	−	−	−	−	0.586
Gly	6.93					0.588
Lys	5.91				−	0.607
Ser	6.90					0.568
Intermediate amino acids						
Arg	5.44				+	0.539
Asn	4.11					0.568
His	2.30				+	0.425
Pro	4.86				−	0.502
Thr	5.39		−			0.480
Core amino acids						
Ala	7.86				−	0.405
Cys	1.50	+	+	+		0.268
Ile	5.86					0.273
Leu	9.67					0.321
Met	2.39		+	+		0.364
Phe	3.94	+		+		0.290
Trp	1.13	+		+	+	0.279
Tyr	3.00				+	0.319
Val	6.72					0.306

[a] Average surface accessibility. [b] +: Over-represented relative to natural abundance. [c] −: Under-represented relative to natural abundance.

region. Furthermore, the binding sites of certain proteins such as enzymes have been shown to over-represent residues such as histidine, arginine, tryptophan and tyrosine while under-representing lysine.[5] A third strategy that can be used to achieve a certain degree of site-specificity in the preparation of *polymer-reactive peptides/proteins* involves considering the relative surface exposure of amino acids. The average surface accessibility (ASA), or propensity for a residue to be present on the surface of a protein, has been calculated for the 20 canonical amino acids based on the known structures of a number of database proteins (Table 1).[6] Knowledge of the ASA of amino acids allows directing of the introduction of polymer-reactive functional groups more towards the interior or towards the surface of the protein.

2.2 Synthetic strategies for rendering peptides/proteins *polymer-reactive*[2,7–10]

This section gives an overview of different strategies that are available to modify existing peptides/proteins with *polymer-reactive* handles and begins by discussing the possibilities, advantages and disadvantages of reactions that are currently available to modify the side groups of specific amino acids. This is followed by a presentation of strategies that are available to selectively modify the N-terminal amino group of peptides/proteins as well as reactions that can be used to selectively modify specific N-terminal amino acids on peptides/proteins. An overview of the reactions that are discussed in this section is presented in Tables 2 and 3.

Lysine. The most popular but one of the least site-specific residue-specific functionalization strategies targets the ε-amino group of lysine residues.[2] The main disadvantage of this strategy is that, generally, proteins possess many surface-exposed lysine residues. Lysine can make up to 6% of the overall amino acid sequence (Table 1). In general, the ε-amino group on lysine is targeted with electrophilic reagents such as activated carboxylic acids (**1**), aldehydes or ketones (**2**) and isocyanates/isothiocyanates (**3**).[8] In the reaction with aldehydes, the reversibly formed imine must be reduced to irreversibly form a secondary amino group. While this reduction can be accomplished by use of harsh, water-sensitive chemicals, which react rapidly and quantitatively with imines,[11] recent developments using iridium catalyzed hydrogenation have been shown to be more mild while maintaining similar efficiency to cyanoborohydride methodologies.[12] Though this modification maintains the overall charge of the protein (even though its isoelectric point may change), the two-step nature of this modification and the more difficult synthesis of aldehyde heterobifunctional spacer compounds (*i.e.* for conjugation with a polymer) *versus* activated carboxylic acids makes this route more challenging.[2] Alternatively, lysine residues may react by amidination with imidoesters (**4**) at elevated pH (~9) or with imidothiolane (Traut's reagent, **5**) near pH 8,[13] the latter being useful for introducing thiol groups. Generally, the latter reaction is selective towards surface accessible lysine residues, though the generated thiol groups may conceivably form intra- or intermolecular disulfide bonds with cysteine residues. Modification of non-surface-accessible lysine residues requires denaturing conditions. Amidination conserves the overall charge of the side group, an aspect which may be advantageous should the charge on this residue be important in the peptide/protein's structure. Another strategy employs the use of thioesters or dithioesters (**6**) for the modification of lysine residues. Aliphatic dithioesters are good electrophiles and have been shown to be interesting, mild reagents for lysine residues in proteins, in the absence of cysteine residues. The reaction of dithioesters is very fast, specific and irreversible.[14] Generally, the water solubility of dithioesters is limited, and their design should therefore incorporate solubility improving groups. Reactions of dithioesters are specific towards primary and secondary amines, though exchange reactions are possible with cysteine groups, depending on the leaving group present on the thioester.[14] Protein thioacylation can be conveniently monitored with UV-Vis spectroscopy due to the difference in the UV-Vis absorbance of the reactants (aliphatic dithioesters, ~310 nm) and products (aliphatic thioamides, ~270 nm).[14] Also, excess dithioester can be quenched by addition of ammonia, which allows for control over reaction time. In the studies discussed in this section, side-reactions involving nucleophilic attack by alcohol groups found on other amino acids were not observed. Side-reactions involving nucleophilic attack of the thiol side chains on cysteine residues would produce thioesters that are sufficiently unstable to prevent their irreversible participation in these reactions.

Cysteine. Cysteine residues are valuable targets for residue-specific modification of peptides/proteins in that these residues

Table 2 Reactions for the post-translational modification of the side groups of specific amino acid residues[a]

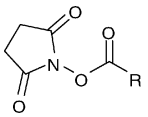
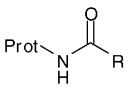
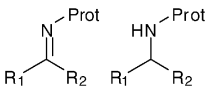
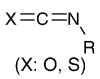
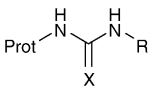
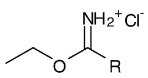
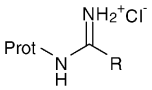
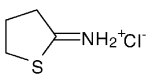
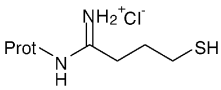
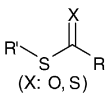
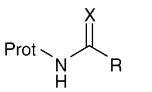
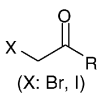
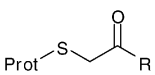
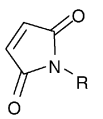
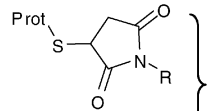
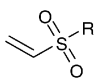
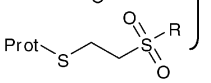
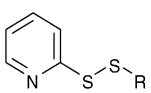
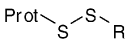
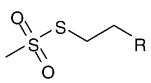
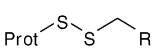
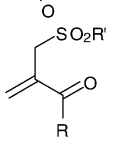
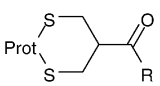
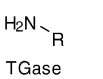
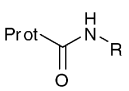
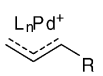
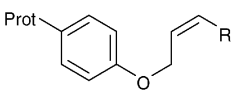
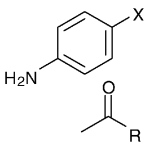
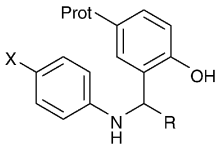
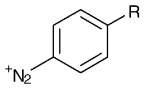
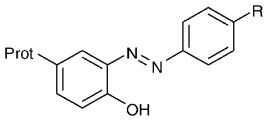
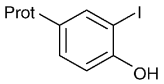
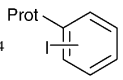
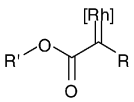
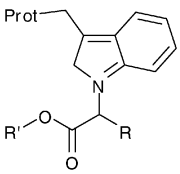
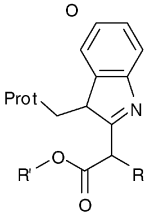
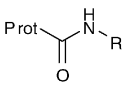
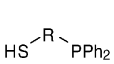
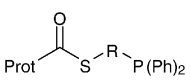
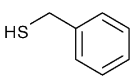
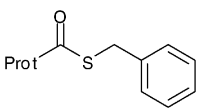
Reagent(s)	Product(s)	Comment
Lysine		
1 		Reagents easy to synthesize or commercially available. Side-reactions are possible.[26]
2 		Reversible imine formation. Reduction to amine can be done in mild conditions (phosphate buffer, pH 7.4).[12]
3 		Formation of stable (thio)urea linkages. Reagents easy to synthesize or commercially available.[8]
4 		Maintains charge of residue.[13]
5 		Useful for generation of a thiol group.[13]
6 		Exchange reactions with free thiol groups can be overcome by adding excess R'SH.[14]
Cysteine		
7 		Formation of stable thioether bonds.
8 		Reaction *via* Michael's addition (section 4.1). Possible side reactions with amines.[27]
9 		
10 		Reversible. Useful for releasing and characterizing R. Prepared by thiol exchange.
11 		Reversible. Useful for releasing and characterizing R. Reagent easy to synthesize.[9]
12 		Reaction with disulfides. Mild reducing conditions. Maintains protein structure.[15,16]
Glutamine		
13 		Possible side reaction with internal Lys residues and N-terminal Gly residues.[28]
Tyrosine		
14 		pH 8.5–9, 45 min at r.t. 50–65% yield. Selective towards solvent accessible Tyr residues.[19]
15 		pH 5.5–6.5, 18 h, 0–80% yield. Yield drops significantly above pH 8.[20]

Table 2 (*continued*)

Reagent(s)	Product(s)	Comment
16 		pH 9, 4 °C, 2 h, ~90% Yield.[21]
17 10% TFA, 1.5 eq. IPy_2BF_4		50–60% yield for short peptides. Does not iodinate Phe residues.[24]
Phenylalanine		
18 10% TFA, 1 eq. IPy_2BF_4 2 eq. HBF_4		85–100% yield for short peptides in 30 min. Cannot be performed in presence of Tyr, Trp or His.[25]
Tryptophan		
19 	 	Rhodium carbenoid generated *in situ*. 60% conversion (combined N- and C-alkylation) after 7 h at r.t. Low pHs required.[29]
Aspartic acid, glutamic acid and C-terminus		
20 		(i) *In situ* activation and coupling with carbodiimide and activating agent (cat.).[30,31] (ii) Separate activation and coupling steps.
21 		For Staudinger ligation. Reaction *via* standard coupling chemistry (*i.e.* for SPPS).[32]
22 		Formation of thioester electrophile for native chemical ligation.[33]

[a] Bold numbers refer to entries, not individual compounds.

are relatively rare, having a natural abundance of 1.5% (Table 1). Cysteine residues are often found partially of fully buried within the protein structure, a property which may limit their accessibility to chemical reagents.[2] Dithiothreitol (DTT) can be used to expose additional cysteine groups participating in intramolecular disulfide bonds, though care should be taken if the three-dimensional structure of the protein is to be maintained following modification, given that disulfide bonds play a significant role in maintaining protein structure. Under appropriate conditions, cysteine can be modified selectively, rapidly, quantitatively and in either a reversible or irreversible fashion. Indeed, the nucleophilic thiol group is reactive towards a variety of reagents, any one of which can be used to introduce a handle for chemoselective coupling. α-Halocarbonyl compounds (**7**), maleimides (**8**) and vinyl sulfones (**9**) react irreversibly with the thiol side-group of cysteine to produce stable thioether bonds.[7] A very specific method for modifying cysteine residues lies in the formation of disulfide linkages, though these may be cleaved with reducing agents such as DTT. This may be an advantage or disadvantage depending on the application for which the conjugate is designed. Formation of disulfide bridges can be accomplished by means of orthopyridyl disulfides (**10**) or methanethiosulfonates (**11**), the latter being synthesized directly from organohalogen compounds.[9] The recent development of bis(thiol) specific reagents (**12**) allows the modification of native disulfide bonds that are crucial to protein structure and biological activity.[15,16] This strategy starts with reduction of the native disulfide bond to release the two cysteine thiols, which then react with a α,β-unsaturated β′-monosulfone reagent (**12**) to form a three-carbon bridge between the two sulfur atoms. The formation of the three-carbon disulfide bridge ensures that the modification reaction does not influence protein structure or function.

Table 3 Reactions for the selective N-terminal modification of peptides/proteins[a]

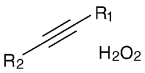
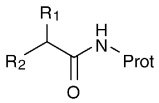
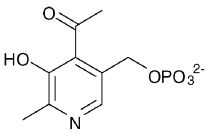
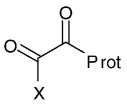
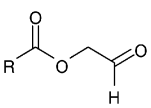
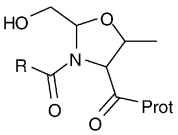
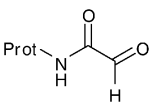
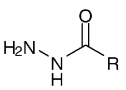
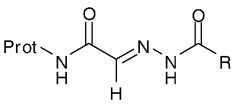
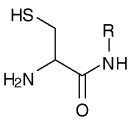
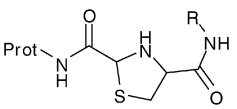
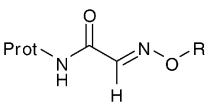
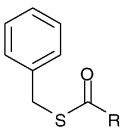
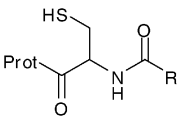
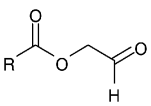
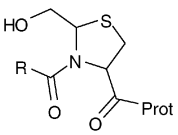
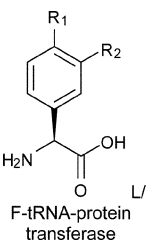
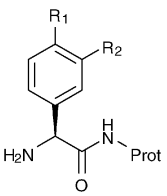
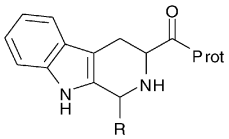
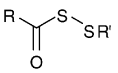
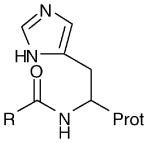
Reagent(s)	Product(s)	Comment
N-Terminus-general		
23 NaI + $NaNO_2$, pH 4		Under basic conditions (pH > 7) side-reactions with Tyr and His observed.[36]
24 		$NaHCO_3$ buffer, H_2O : ACN, 6 h, r.t., 80% Yield. Cysteine and methionine residues were reversibly oxidized.[37]
N-Terminal aspartic acid, glycine, lysine, methionine and valine		
25 		Buffer pH 6.5–8.5, 18–20 h, 37–55 °C, 30–80% Yield. X is the side-chain of the N-terminal group. X = CH_3 for Asp.[38]
N-Terminal serine or threonine		
26 		Pseudoproline ligation. Must be performed in (nearly) non-aqueous media.[39]
27 IO_4^-		Oxidation of the N-terminal residue. H_2O, 10 min, 37 °C, quant.[10]
Reaction of glyoxylyl peptides/proteins produced in entries 25 and 27		
28 		H_2O:ACN (16%) pH 4.5, 70 min, 22 °C, quant. Hydrazone very labile < pH 2.[40]
29 		Buffer pH 5.25, 7–9 h. r.t., >95% yield. Insensitive to internal lysine or arginine residues.[42]
30 		Buffer pH 3–5, 24 h, r.t., 40–100%.[43]
N-Terminal cysteine		
31 		Native Chemical Ligation. 6 M Guanidinium HCl buffer pH 7.5, r.t., quant.[45]
32 		Pseudo-proline ligation
N-Terminal lysine or arginine		
33  F-tRNA-protein transferase		Buffer pH 8, 30 min, 37 °C, 90–100% yield. Works in presence of crude protein mixture.[47] R_1:N_3, $COCH_3$; R_2:H R_1:OH; R_2:NO_2

Table 3 (*continued*)

Reagent(s)	Product(s)	Comment
N-Terminal tryptophan		
34 		Pictet–Spengler reaction. Glacial acetic acid, 24 h, r.t., ~100% yield.[48]
N-Terminal histidine		
35 		Histidine ligation. Buffer pH 5.7, 8 h, 60–75% yield.[39]

[a] Bold numbers refer to entries, not individual compounds.

Glutamine. Glutamine is a moderately abundant target for selective protein/peptide functionalization through transamidation mediated by transglutaminase (TGase).[17] This enzyme catalyzes an acyl transfer reaction between the γ-carboxamide group of protein-bound glutaminyl residues and readily available amino groups on a target compound (**13**).[18] The reaction proceeds at pH 7.5 and leads to the exclusive modification of glutamine residues. Possible side-reactions involve intra- or interprotein reactions with the ε-amino group of lysine.

Tyrosine. Tyrosine residues on proteins have recently been established as targets for residue specific modification. While this amino acid is moderately abundant (~3%, Table 1) its over-representation near binding sites may be disadvantageous if the activity of the protein is to be maintained. Under mildly basic conditions, which deprotonate the phenolic hydroxyl group, tyrosine reacts with π-allylpalladium complexes that can be generated *in situ* from allylic acetates in the presence of $Pd(OAc)_2$ (**14**).[19] This reaction produces the corresponding *O*-alkylated tyrosine residues in 50–65% yield. Site selectivity studies indicated that this modification reaction preferentially targets solvent accessible tyrosine residues. C-alkylation of tyrosine residues can be achieved *via* a three-component Mannich type reaction (**15**).[20] This reaction involves the use of a stoichiometric mixture of an aldehyde and a modified aniline compound, which *in situ* forms an imine that acts as the actual alkylating agent. Reaction times of 18 h resulted in degrees of modification of up to 80% depending on the nature of the aniline compound. This reaction generally proceeds in the pH range of 5.5–6.5 and yields drop significantly above pH 8. Alternatively, electrophilic aromatic substitution of tyrosine side-groups can be achieved by diazonium coupling (**16**) in a highly efficient and selective manner.[21–23] Tyrosine residues can also be selectively mono-iodinated with an equimolar amount of IPy_2BF_4 either in the presence of 10% TFA in CH_2Cl_2 or 1 eq. HBF_4 in acetonitrile or dichloromethane (**17**).[24] Experiments carried out with short peptides containing both tyrosine and phenylalanine residues did not reveal any phenylalanine iodination, which underlines the selectivity of this reagent.

Phenylalanine. In the absence of tyrosine residues, phenylalanine may be monoiodinated in the same fashion as for tyrosine by use of IPy_2BF_4 (1 eq.) in the presence of TFA (10%) in CH_2Cl_2 and HBF_4 (2 eq.) in high yield (**18**). These reaction conditions produce mixtures of the corresponding *ortho*/*para* monoiodinated residues with *o*/*p* ratios varying from 7/1 to 13/1 depending on the length and amino acid sequence of the peptides.[25]

Tryptophan. The residue-specific modification of the indole side-group of tryptophan residues has recently been shown to be possible through the *in situ* generation of rhodium carbenoid reagents (**19**).[29] Given the low natural abundance of tryptophan (1.13%, Table 1), this strategy offers attractive opportunities to produce well-defined *peptide*/*protein–polymer conjugates*. In this strategy, the reactive rhodium carbenoids are generated *in situ* from a vinyl diazo compound and $Rh_2(OAc)_4$. Reaction of the rhodium carbenoid species with tryptophan results in a mixture of the corresponding N-alkylated and 2-alkylated indoles. Experiments carried out with horse heart myoglobin resulted in the modification of ~60% of the tryptophan residues. The chemoselectivity of this reaction is thought to be brought about by the unique ability of tryptophan to outcompete hydrolysis of the rhodium carbenoid.[8] A drawback of the strategy is the relatively low pH (1.5–3.5) that is required for the reaction, which may have undesirable effects on protein structure and function.

Aspartic acid, glutamic acid and C-terminus. The modification of carboxylic acid groups along the peptide/protein backbone or at the C-terminus with amines can be easily accomplished using routine coupling chemistry such as that used in solid-phase peptide synthesis (**20**).[30] Reactions of this type are generally rapid and quantitative, which is why they form the basis for solid-phase peptide synthesis. The activation of the carboxylic acid group, which is typically carried out using a carbodiimide coupling agent and *N*-hydroxybenzotriazole (HOBt) as an additive, can be carried out either in a one-pot, two step process, or in two separate reaction steps (**20**). When modifying complex proteins, care should be taken that excess nucleophile is used to avoid side-reactions with the ε-amino group on lysine residues. Phosphinothiols may be introduced to form thioesters suitable for Staudinger ligation (**21**). A variety of water soluble phosphinothiols have been developed for the purpose of protein modification.[32]

Furthermore, carboxylic acids may be converted into thioesters (**22**)[33] which can be used in native chemical ligation.

N-Terminus-general. The difference in pK_a between the amino group of an N-terminal amino acid residue ($\sim$7.8) and the amino groups in the side-chains of lysine ($\sim$10.5) and arginine ($\sim$12) residues allows the selective N-terminal modification of shorter peptides and proteins by methods discussed for lysine residues.[34] The difference in pK_a comes from the electron withdrawing character of the geminal amide group at the N-terminus.[10] This methodology, however, has not been proven sufficiently selective for general use on proteins.[35] The N-terminal amino group may be selectively iodinated under acidic conditions with sodium iodide and sodium nitrite (**23**). However, under neutral to basic conditions side-reactions with tyrosine and histidine residues are possible.[36] The N-terminus of a protein can be selectively targeted with an alkyne through oxidative amide synthesis using an alkyne and hydrogen peroxide (**24**). This reaction has been shown to promote the exclusive amidation of the N-terminus of unprotected peptides bearing lysine, serine, threonine and tyrosine residues in aqueous acetonitrile at pH 8.3.[37] Another possibility to selectively modify the N-terminus of peptides/proteins uses a transamination reaction that introduces a uniquely reactive ketone or aldehyde group, which allows further modification through oxime or hydrazone formation (**25**).[38] This method has been successfully used to modify the N-terminus of a series of aspartic acid, glycine, methionine, lysine and valine containing peptides and has been applied to selectively functionalize proteins with *p*-bromobenzyloxyamine, alkoxyamine substituted fluorescent dyes or PEG derivatives. This technique, however, is suspected to be incompatible with proteins bearing N-terminal serine, threonine, cysteine, tryptophan, and proline residues.

N-Terminal glycine. N-Terminal amino groups generally do not participate in the TGase mediated reaction with glutamine derivatives. N-Terminal TGase mediated chain end modification, however, is possible with peptides/proteins that contain an N-terminal glycine residue. This N-terminal modification reaction is facilitated if multiple ($>$3) sequential glycine residues are present, or if the second amino acid is basic. In certain circumstances, this reaction is insensitive to the presence of lysine residues, as not all lysine residues are TGase reactive in folded peptides/proteins.[28]

N-Terminal serine and threonine. The nucleophilicity of the N-terminal 1,2-aminoalcohol group of N-terminal serine or threonine residues can be used for pseudoproline ligation (**26**).[39] The reaction proceeds *via* an oxazolidine intermediate, whose formation is not favored under aqueous conditions. Oxaproline ligation, however, can be realized under nearly nonaqueous conditions, such as 90% DMSO or DMF in 10% acetate buffer at pH 5.5.[39] Alternatively, the presence of an N-terminal serine or threonine offers unique possibilities owing to the high susceptibility of 1,2-aminoalcohols to periodate oxidation (**27**). In fact, the periodate oxidation of a 1,2-aminoalcohol proceeds several orders of magnitude faster than for 1,2-diols or 1,2-diamines, making this reaction quite specific.[10] Periodate oxidation of an N-terminal serine or threonine results in the formation of a glyoxylyl group, which can be used to form hydrazone (**28**),[40,41] thiazolidine (**29**)[42] or oxime linkages (**30**).[43] The initial oxidation step proceeds under mild aqueous conditions in the pH range of 6–8 at room temperature in a matter of minutes.[35] The excess periodate used to oxidize the N-terminal residues of proteins has been shown to participate in side reactions, in which methionine is oxidized to methionine sulfoxide.[40] In general, the reaction time must be controlled precisely for large proteins in order to avoid over-reaction during periodate oxidation.[44] Quenching of excess periodate to stop the oxidation reaction can be accomplished by addition of excess of ethylene glycol. While in principle the reactive glyoxylyl group formed by periodate oxidation may participate in intra- or inter-molecular Schiff base formation by reaction with amino groups on lysine residues, these structures are generally sufficiently unstable towards hydrolysis to regenerate the glyoxylyl group, which can then be used for more stable coupling strategies.[35]

N-Terminal cysteine. Peptides/proteins with N-terminal cysteine residues have been exploited for the preparation of amide bonds by native chemical ligation.[45] Native chemical ligation is a two-step process that starts with the reaction of the thiol group of an N-terminal cysteine residue with a thioester compound (**31**). This results in the formation of a thioester intermediate, which in a second step undergoes a rapid and irreversible intramolecular *S*,*N* acyl migration to generate a peptide bond. This reaction proceeds in aqueous solution at neutral pH, is irreversible under the reaction conditions used, and proceeds to high yields.[33] This strategy has not only been successfully used for the total chemical synthesis of proteins, but has also been applied for the preparation of complex *protein–polymer conjugates*.[46] Native chemical ligation can be performed in the presence of additional cysteine groups along the backbone by inclusion of a large excess of competitive thiol molecules to keep cysteine side-chains reduced, to reverse the formation of unproductive thioesters and to potentially convert the easily manipulated benzyl thioester into a more reactive thioester through thiol exchange.[33] In addition to native chemical ligation, N-terminal cysteine residues can also be used for pseudoproline ligation.[39] Pseudoproline ligation starts with imine capture of ester glycoaldehydes *via* the formation of a thiazolidine ester, which occurs in a matter of minutes under either acidic, neutral or basic conditions (**32**). Acidic conditions are favorable since they avoid side-reactions with other nucleophiles. In a second step, the thiazolidine ring undergoes a *O*,*N*-acyl migration to form a proline mimetic 2-hydroxymethyl thiaproline ligation site.

N-Terminal lysine or arginine. Recently, leucyl/phenylalanyl(L/F)-tRNA-protein transferase has been shown to catalyze the transfer of non-natural amino acids bearing azide and ketone functional groups onto the N-terminus of proteins bearing N-terminal lysine or arginine residues (**33**). This transfer has been shown to be quite efficient, even in the presence of other peptides or in crude protein mixtures.[47]

N-Terminal tryptophan. Peptides bearing N-terminal tryptophan residues have been shown to participate in the Pictet–Spengler reaction with aldehydes in glacial acetic acid (**34**). This ligation method produces stable C–C bonds in high yield

after 24 h and is compatible with a variety of amino acid side-groups.[48] The presence of lysine groups on the peptide/protein may, however, interfere with this process through consumption of the aldehyde reagent by imine formation.

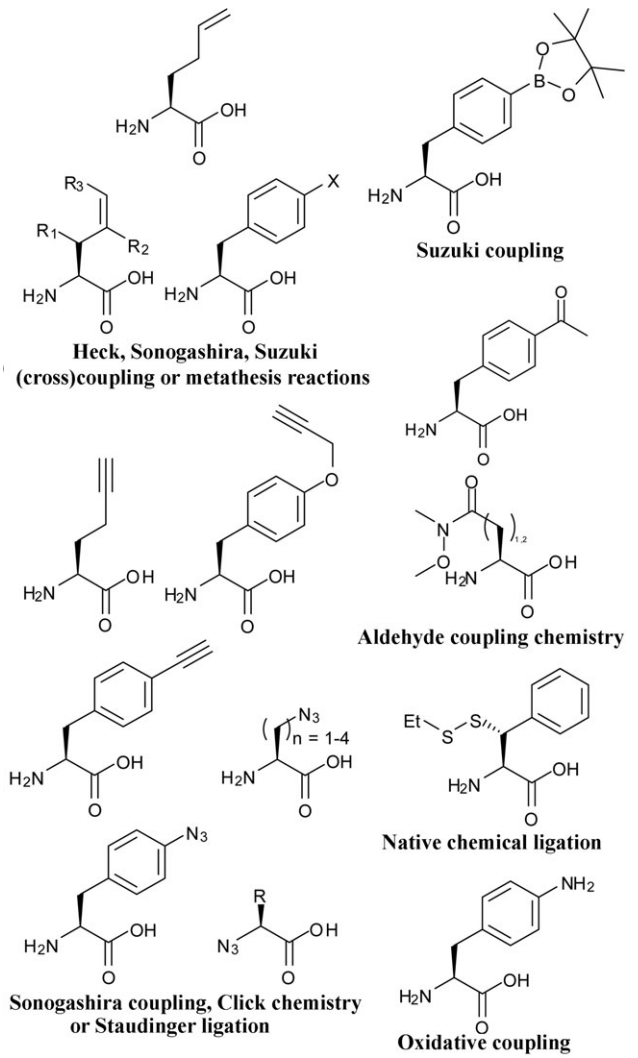
N-Terminal histidine. As for pseudoproline ligation or for native chemical ligation, the nucleophilicity of N-terminal histidine residues may be exploited to form stable amide bonds. In this ligation strategy, acidic conditions are used so that the imidazole moiety is the sole nucleophile on the protein susceptible to react with a dithioperoxy ester reagent (**35**). Following an $N^{\text{imidazole}}$ to N^{α} acyl shift, the imidazole group is regenerated and an N-terminal amide bond is formed.[39]

2.3 Synthetic strategies for directly synthesizing *polymer-reactive peptides/proteins*[7,9,49,50]

Circumstances such as low overall abundance of a natural protein, or difficulties related to its isolation/purification may contribute to the desire to directly synthesize the target peptide/protein. Furthermore, relying on the circumstantial presence of a particular amino acid residue at a particular location on the chain may not always permit the desired site-selective modification of a peptide/protein. In such cases, peptides/proteins containing non-natural residues or site-specific chemoselective handles at defined positions can be directly synthesized *via* various chemical and biological pathways. In this section, we will first present several examples of non-canonical amino acids and fusion tags, which have been used as chemoselective handles. Following this, chemical and biosynthetic methods for introducing such residues in peptides/proteins will be discussed.

Non-canonical amino acids. *Polymer-reactive peptides/proteins* can be synthesized by incorporating either natural amino acids with side chain functionalities that are orthogonal to those of the other amino acids, or by introducing appropriate non-canonical amino acids. Strategies to selectively modify the side chains of natural amino acids have been discussed in the previous section. For the synthesis of *polymer-reactive peptides/proteins* containing non-canonical amino acids, the nature of the non-canonical side group is selected based on its ability to participate in mild and highly selective reactions, which can be used to covalently attach a polymer or polymerization initiator. Some examples of non-canonical amino acids, which have been used for this purpose are summarized in Table 4. Alkenyl or alkynyl side groups can be used to introduce functional groups *via* a number of transition metal catalyzed addition reactions or metathesis reactions.[49,51] *p*-Halo-phenylalanine residues can be introduced in order to take advantage of the Heck reaction with an activated alkene,[52] Sonogashira coupling with a terminal alkyne[53] or Suzuki coupling with boronic esters.[54] Functional groups such as azides or terminal alkynes can be used for Huisgen's copper (I) catalyzed [3 + 2] cycloaddition reaction.[55,56] These coupling strategies will be discussed in greater detail in section 4.1. *p*-Acetyl-phenylalanine[9] or Weinreb amides of aspartic or glutamic acid (masked aldehydes)[57] have been introduced in order to take advantage of their susceptibility towards hydrazone and oxime formation. Weinreb amides may be activated in 60–90% yield in 3.5–24 h using $LiAlH_4$ on a solid support.[57] Amino substituted phenylalanine residues present another non-canonical target for site-selective peptide/protein modification. These residues can be selectively modified under mild oxidative conditions with dialkylphenylene diamine derivatives.[58] Finally, the recent introduction of β-mercaptophenylalanine derivatives has extended native chemical ligation to N-terminal phenylalanine residues.[59]

Table 4 Non-canonical amino acids for preparing polymer–reactive peptides/proteins



Fusion tags. An alternative strategy for introducing site-specific reactivity into peptides/proteins lies in the incorporation of a specific amino acid sequence known as a fusion tag. While in the past fusion tags have generally been quite long (50–80 residues), recent advances have reduced their length to <15 residues. In particular, the C-terminal Cys–Val–Ile–Ala sequence is specifically recognized by the enzyme protein farsenyltransferase (PFTase), which can be used to promote

the selective alkylation of the thiol group within the sequence by means of modified isoprenoid diphosphates bearing functional groups of choice. This strategy has been used to incorporate azide or alkyne groups onto the C-terminus of peptides at 30 °C in neutral aqueous media in 1–3 h.[60,61] This 4-residue fusion tag is quite short and is therefore not suspected to significantly alter protein folding and due to its length can be easily introduced by synthetic means.

Chemical synthesis of polymer–reactive *peptides/proteins*. Peptides containing up to ~50 amino acid residues are most easily prepared using (automated) solid phase peptide synthesis (SPPS), which is a routine technique nowadays.[30] N-Terminal, C-terminal, and site-specific functionality can be introduced in a variety of manners by careful choice of the resin, the linker, cleavage methodology, use of orthogonal protecting groups or appropriate non-canonical amino acids. The site-specific introduction of non-canonical amino acids bearing functional groups can be accomplished by a number of methods. In its simplest form, a non-canonical amino acid bearing the desired side-group can be introduced at a specific step in the peptide synthesis thus allowing for site-specific modification of the peptide. However, this method requires that the side-group of the amino acid being introduced be insensitive to the coupling and deprotection steps. Functional groups such as azides,[62] alkynes,[62] alkenes,[62,63] boronic esters[64] and aryl halides[65] have been shown to be compatible to SPPS reaction conditions. Alternatively, side-group protected amino acids, which are either highly sensitive to the common TFA and piperidine cleavage cocktails or have orthogonal cleavage conditions can be incorporated at specific locations on the peptide and selectively deprotected and modified prior to the global deprotection and cleavage of the peptide from the resin. A variety of such protecting groups exists for side-chain amino, alcohol, carboxylic acid, and thiol groups.[30]

The C-terminus of a peptide can be selectively modified by SPPS by selecting an appropriate linker or cleavage conditions. The cleavage of a peptide from the solid support by means of amines or alcohols *via* nucleophilic attack (in basic conditions) is an effective means of selectively modifying the C-terminus of the peptide with any desired functionality non-susceptible to degradation during side-chain deprotection. The use of a Weinreb amide linker is a convenient method for producing peptides with a C-terminal aldehyde group under reducing conditions and in a highly selective manner.[66] This aldehyde group may be used for coupling to a polymeric moiety or polymerization initiator. C-Terminal peptide thioesters can be produced using sulfonamide linker modified resins.[67] Following SPPS, the sulfonamide linker is cyanomethylated and cleaved with a thiol nucleophile to afford the peptide thioester, which can be further derivatized using native chemical ligation[68] or Staudinger ligation.[69] C-Terminal thioesters have also been produced by SPPS by anchoring the side-chain of trifunctional amino acids such as Lys, Glu, Gln, Asp or Asn onto a resin and introducing the thioester functionality onto the carboxylic acid group of this amino acid following peptide synthesis and its orthogonal deprotection.[70]

N-Terminal modification is straightforward using SPPS given that following deprotection with piperidine, only the N-terminal amino group on the peptide is available for reaction, all others bearing acid-sensitive protecting groups.[71–73] Modification of this amino group with compounds bearing a carboxylic acid group by standard acid/amine coupling methods is a natural extension of SPPS and has been used to produce a number of *peptide–polymer conjugates*.[72–76] Recently, α-azide protected amino acids have been prepared for use in SPPS as a means of producing N-terminal azide bearing *polymer-reactive* peptides, which are able to participate in Staudinger ligation or Huisgen's 1,3 dipolar cycloaddition reaction.[77] N^{α}-(ethanethiol) and N^{α}-(oxyethanethiol) groups may be introduced in three steps onto the N-terminus of resin-bound peptides by first bromoacetylation of the N-terminus, attachment of a thiol-protected amino or alkoxyamine compound by nucleophilic displacement and finally deprotection of the thiol group.[78] These groups are useful for extending the applicability of native chemical ligation. Also, modification of the N-terminus of peptides produced by SPPS with a N^{α}-(1-phenyl-2-mercaptoethyl) auxiliary group has enabled the application of native chemical ligation to generate peptide bonds at amino acid residues other than cysteine. Following thiol exchange and *S*,*N* acyl shift, the auxiliary is removed under acidic conditions to complete amide bond formation.[79]

Biosynthesis of polymer-reactive *peptides/proteins*. While SPPS is the preferred method for producing peptides containing up to ~50 amino acids, longer peptides/proteins are most conveniently prepared using biosynthetic methods. The incorporation of non-canonical amino acids into proteins can be accomplished both in a site-specific and residue-specific manner. Suppressor tRNA methods for introducing non-canonical amino acids in a site-specific manner are relatively new and promising but are highly labor intensive and are currently limited by poor efficiencies.[50,80] The residue-specific multi-site replacement of one amino acid by another, non-canonical, analog is routinely performed by means of auxotrophic bacterial hosts. In this manner, amino acids bearing alkene or alkyne side groups are readily introduced in locations initially occupied by methionine, leucine, isoleucine, phenylalanine or proline.[49] Furthermore, the potential of this method for site-specifically introducing non-canonical amino acids can be harnessed if a site-specific mutation for one of these amino acids can be introduced into the DNA coding for the protein. In this case, expression of this mutant in the presence of a non-canonical amino acid will allow for the incorporation of the latter, specifically at the site of the mutation. Alternatively, a mutation for a canonical amino acid, which has orthogonal reactivity to the other canonical amino acids on the protein, may be introduced. For example, the introduction of a cysteine into a protein deficient in this amino acid would yield a protein with a chemoselective handle suitable for reactions outlined in section 2.2.

3. Preparation of *peptide/protein-reactive polymers*

This section discusses the different methods that are available for producing polymers with chemoselective handles, which can be used to couple peptides or proteins. Peptide/protein conjugation can be accomplished using either side-chain or

end-group reactive polymers. These two strategies will be successively discussed below.

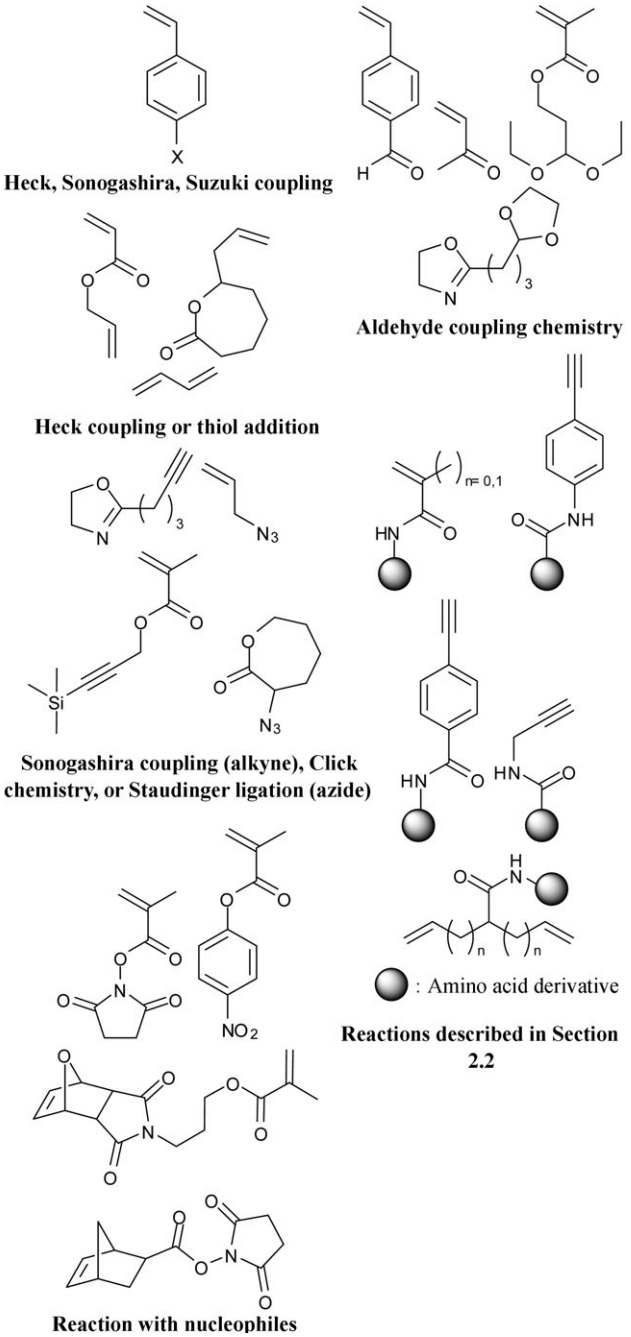
3.1 Strategies for the preparation of side-chain *peptide/protein-reactive polymers*[81–83]

Polymers with side-chain functional groups are of interest for introducing many copies of pendant peptides that may potentially be too long to be used in a "grafting through" strategy (section 4.3), for introducing peptides bearing functional groups incompatible with polymerization conditions and for preparing polymers with higher molecular weight that those accessible with the "grafting through" strategy. This section begins by discussing methods for preparing side-chain *peptide/protein-reactive polymers* by (controlled) radical polymerization and is followed by non-radical methods for producing such polymers.

Side-chain functional polymers may be prepared by a variety of (controlled) radical polymerization methodologies. Within this approach, monomers bearing side-groups susceptible to participate in chemoselective reactions are polymerized either in their protected or unprotected form and subsequently reacted with an appropriately functional peptide/protein. Examples of monomers bearing side-group active esters,[84–86] protected or free ketones and aldehydes,[86–89] protected maleimides,[90] protected alkynes,[91] azides[92,93] and *p*-aryl halides,[94] which have been successfully used in (controlled) radical (co)polymerization can be found in Table 5. The postpolymerization modification of poly(*N*-methacryloxysuccinimide) has been reported to suffer from side reactions, which include ring opening of the succinimide moiety and glutarimide formation by ring-closing attack of amides on presumably neighboring active esters.[26] By carefully adjusting the equivalents of the modification reagents, polymer concentration, reaction time and temperature, however, these side reactions can be overcome. Vinyl monomers containing amino acid derivatives based on Gly, Leu, Tyr, Phe, Pro, His, Ser, Ala, Lys, Glu have also been polymerized and offer the possibility of introducing chemoselectivity through the reactions highlighted in section 2.2.[95–99] The monomers listed in Table 5 are attractive as the resulting polymers can be directly functionalized with the peptide/protein of interest. In addition to direct postpolymerization modification, *peptide/protein–polymer conjugates* can also be generated *via* a two-step protocol in which an additional activation step precedes the actual peptide/protein modification. As an example, *p*-nitrophenyl chloroformate activation of the pendant hydroxyl groups of poly(poly(ethylene glycol) methacrylate) and poly(2-hydroxyethyl methacrylate) has been used to introduce short peptide sequences.[100] In another sudy, side-chain maleimide groups were introduced onto a copolymer of *N*-(2-hydroxypropyl)methacrylamide and *N*-(3-aminopropyl)methacrylamide and subsequently used for grafting long (24–38 amino acid) peptides by Michael addition.[101]

In addition to (controlled) radical polymerization, other polymerization methods have also been used to prepare side-chain functional polymers. For example, amino acid derivative based phenylacetylene (Ala, Val, Ser, *etc.*)[102–104] and propargyl amide (Ala, Ser, Thr, Asp, Glu)[105–108] monomers have been developed for Rh-catalyzed polymerization and offer reactivity as discussed in section 2.2. Living ring-opening metathesis polymerization (ROMP) and acyclic diene metathesis (ADMET) polymerization are also of interest for developing side-chain functional polymers due to their tolerance to many functional groups such as acids, alcohols, aldehydes, ketones, esters, amides as well as to aqueous reaction media.[109,110] Norbornene monomers bearing active esters may be readily polymerized by ROMP for creating side-chain *peptide/protein-reactive* polymers.[111] Azido and alkynyl groups

Table 5 Examples of functional monomers for preparing *peptide/protein-reactive polymers* by methods discussed in section 3.1



may be introduced by post-polymerization nucleophilic substitution of alkyl bromide functionalized poly(oxanorbornenes) produced by ROMP.[112] The direct ROMP of alkyne or azide containing monomers can be problematic. Homopolymerization of alkynyl oxynorbornenes has been reported to result in polymers with a relatively broad molecular weight distribution, probably due to a competing reactivity of the alkynyl moiety with the ROMP catalyst.[113] Homopolymerization of azido functionalized cyclooctenes has also been reported to be difficult, presumably due to irreversible coordination of the functional group to the catalyst.[114] Poly(cyclooctene)s with pendant azide moieties, however, have been successfully prepared by copolymerization of cyclooctene and aryl azide functionalized cyclooctenes.[115] Branched diene monomers containing derivatives of the amino acids Ala, Leu, Lys and Arg have been polymerized by ADMET.[116] Ring-opening polymerization (ROP) of cyclic esters is another useful method for producing *peptide/protein-reactive polymers* bearing side-chain functional groups. Lou *et al.* have recently reviewed methods for preparing polymers bearing ketone, alcohol, carboxylic acid, amine, azide, acrylate, alkene and thiol groups from appropriately protected cyclic ester monomers.[117] Poly(esters) bearing alkynyl[118] and azide[119] groups may also be directly prepared by ROP. Anionic polymerization represents another powerful method for producing polymers with well-defined length and structure. Protecting groups for most functional groups and which are stable under the strongly basic conditions encountered in anionic polymerization exist and have been employed to introduce alkenyl, alkynyl, hydroxyl, amino, thiol, carboxylic acid, ketone and aldehyde groups into polystyrenes.[120] The anionic polymerization of vinyl monomers such as (meth)acrylates is also possible, though side-reactions due to the presence of electrophilic functional groups such as carbonyl groups (ester and amides) and acidic hydrogen atoms (NH of (meth)acrylamides) leads to lesser control over molecular weight and yields greater polydispersity.[121,122] Newer strategies are currently being developed to improve the living character of the anionic polymerization of these monomers. As an example, a solid-supported samarium(III) enolate has been successfully used for the living anionic polymerization of allyl methacrylate.[123] This has led to the production of polymers with pendant alkenyl groups, which may be suitable for reactions such as Heck coupling or thiol addition. Polymers with pendant alkenyl groups are also readily obtained from the anionic polymerization of 1,3-butadiene.[124] The living cationic ring-opening polymerization (CROP) of functional 2-oxazolines has also recently emerged as a method for producing side-chain functional polymers bearing amino,[125] aldehyde,[126] azido[127] and alkynyl[128] groups suitable for different ligation strategies. It must be mentioned, however, that the aldehyde and amino functionalities must be polymerized in a protected form to avoid undesirable side-reactions. Furthermore, aliphatic azides are expected to interfere with the cationic ring-opening process.[128]

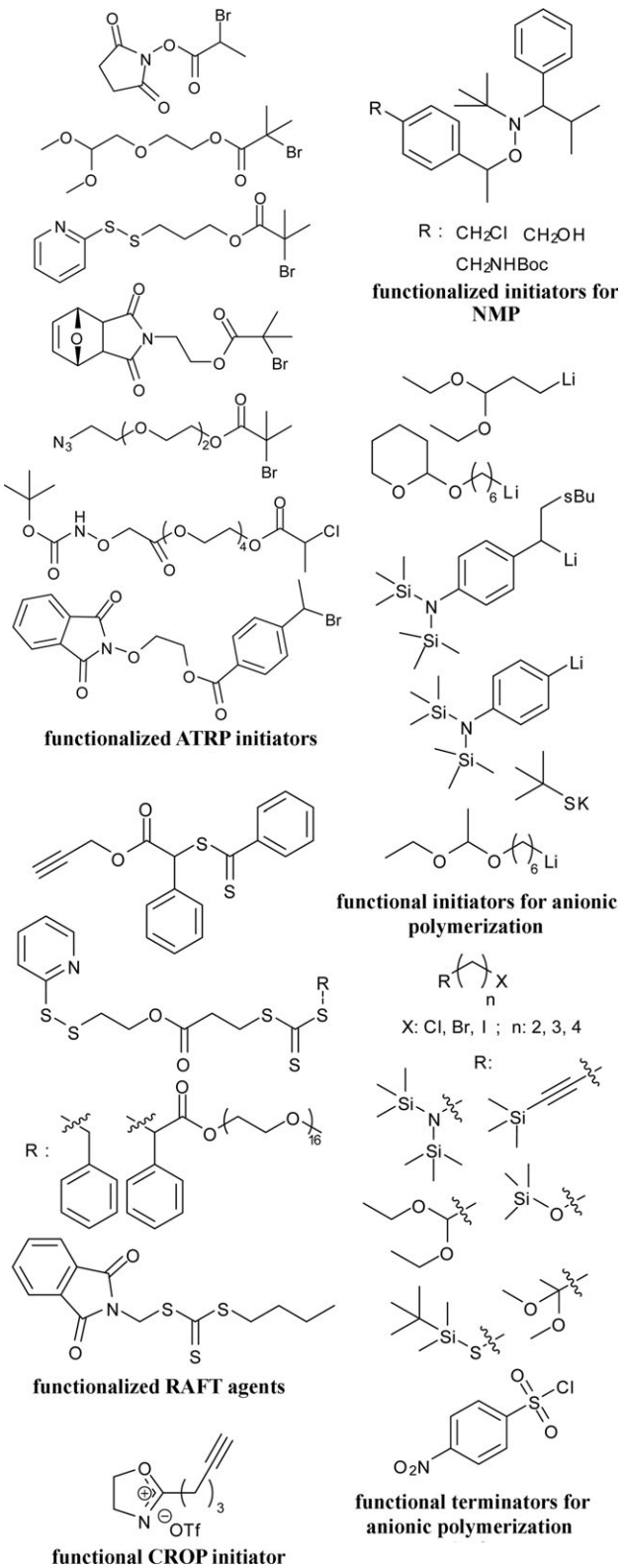
3.2 Strategies for the preparation of end-group *peptide/protein-reactive polymers*[82,129–131]

End-group *peptide/protein-reactive polymers* can be prepared by a variety of controlled or living polymerization techniques using appropriate functional initiators (α-functionalization). These initiators are designed to be inert with respect to the polymerization reaction either in their native form or in a protected state, which may be activated following polymerization. Alternatively, the polymerization process may be terminated by a functional terminator, thereby conferring functionality to the end of the polymer (ω-functionalization). A non-exhaustive list of functional initiators and terminators for various polymerization reactions can be found in Table 6.

Synthesis of α-functionalized polymers. Preparing polymers *via* living radical polymerization techniques has become routine. These methods are generally very tolerant to monomer functionality and the end-groups of such polymers can be controlled through a careful choice of initiator. For atom transfer radical polymerization, a wide range of initiators has been reported, which also contain functional groups suitable for peptide/protein conjugation. A comprehensive list of functional ATRP initiators is given in the review by Coessens *et al.*[82] In another, more recent article, Heredia and Maynard show several specific examples of *peptide/protein-reactive* ATRP initiators.[129] It has been reported that the nature of the targeting functional group as well as its proximity to the initiator functional group can significantly influence the efficiency of ATRP initiation through stabilization or destabilization of the C–X bond. More specifically, as initiation of ATRP occurs through the homolytic scission of a C–X bond alpha to an electron-withdrawing group, the nature of this group will influence its reactivity through steric and electronic effects. While many examples of functional initiators with ester groups alpha to the C–X bond can be found in the literature, polymerization from amide functional initiators has also been shown to be possible, though initiation conditions must be carefully chosen to avoid premature termination in the early stages of the polymerization.[132] ATRP can also be carried out using active ester functionalized initiators. Lecolley *et al.* have shown that the hydrolytic stability of the resulting α-functionalized polymers decreased with increasing substitution of the carbon atom from which polymerization is initiated.[133] Maleimide functionalized initiators must always be used in a protected form due to the participation of this group in the polymerization reaction.[134] Examples of heterobifunctional ATRP initiators include acetal initiators, which can be used to target the N-terminus or lysine residues,[135] pyridyl disulfide[136] and protected-maleimide[134] functionalized initiators for coupling with thiol groups on cysteine residues, protected aminooxy initiators for oxime chemistry[137–139] and azide[140] functionalized initiators for coupling to a modified peptide residue bearing a terminal alkyne group (Table 6).

Functionalized initiators have also been developed for other forms of living radical polymerization. Recent examples include initiators for nitroxide-mediated polymerization bearing protected amino, hydroxyl and *p*-aryl halide groups, which are susceptible to conversion into azido groups following polymerization.[141,142] α-Functionalization of polymers prepared by RAFT polymerization can be achieved by introducing the desired functionality in the free radical leaving group of the RAFT agent.[130] Examples of α-functional groups that have been introduced into polymers prepared by RAFT

Table 6 Examples of functionalized initiators, terminators or mediating agents for polymerization methods discussed in section 3.2



polymerization include primary amino[130] and alkynyl groups.[141] Polymers bearing a α-carboxylic acid group have also been prepared by iniferter-mediated polymerization and subsequently activated with *N*-hydroxysuccinimide in a post-polymerization reaction.[143–145]

α-Functionalized polymers may also be prepared by non-radical polymerization methods by judicious choice of initiating species. Polymers with hydroxyl, thiol, amino, aldehyde or carboxylic acid α-end-groups can be prepared by anionic polymerization using appropriately protected functionalized initiators, some of which are shown in Table 6.[131,146,147] Alcohols bearing functional groups protected against nucleophilic attack (such as those used for anionic polymerization) offer the possibility of introducing functional groups at the α-terminus of polylactides prepared by ROP.[148,149] Recently, the methyl triflate salt of 2-(pent-4-ynyl)-2-oxazoline was shown to be an excellent initiator for the cationic ring opening polymerization of 2-oxazolines, affording polymers with a α-alkynyl group suitable for click chemistry.[128]

Synthesis of ω-functionalized polymers. Living radical polymerizations such as ATRP and RAFT polymerization produce polymers with labile end-groups, which may be chemically modified into useful functionalities.

The terminal halogen groups on polymers prepared by ATRP have been successfully transformed by nucleophilic substitution, electrophilic addition and radical addition reactions as described by Coessens *et al.*[82] Essentially, the halogen end group of polymers produced by ATRP can be readily transformed into azido or amino groups assuming of course the reactions involved are orthogonal to the reactivity of the repeat units of the polymer.

ω-Functionalization of polymers produced by RAFT polymerization can be accomplished either by incorporating the functionality of choice onto the "activating" *Z* position of the RAFT agent or by performing a post-polymerization modification reaction on the RAFT end-group. For example, pyridyl disulfide groups have been introduced onto the *Z* position of a RAFT agent in order to prepare a bovine serum albumin macro-RAFT agent.[150,151] In general, introduction of a functional group on the *Z* position of the RAFT agent may be problematic for producing ω-functionalized polymers due to the lability of the C–S bond, which would lead to incomplete functionalization.[130] However, the RAFT end-group may be thermally eliminated to produce an unsaturated end-group or reacted with a nucleophile to produce a thiol end-group.[130]

ω-Functionalization of polymers produced by anionic polymerization may be accomplished by introduction of terminators bearing masked functional groups. The ω-functionalization of polymers with appropriately protected haloalkanes has led to the production of hydroxy, amino, thiol, aldehyde, carboxylic acid and terminal alkyne functionalized polymers.[131] Alternatively, termination of polymerization by means of nosylchloride followed by subsequent reaction with $N_3SiMe_3(Bu)_4N^+F^-$ has led to the production of azide end-functionalized polymers suitable for orthogonal coupling reactions.[152] ω-Functionalization of polymers produced by anionic polymerization is attractive because many functional terminators are commercially available, protecting

groups for most functional groups towards anionic polymerization exist, and the use of a small amount of terminator is sufficient to functionalize all polymers (barring pre-mature termination of polymer chains due to impurities in the reaction vessel).

Polyesters prepared by ROP possess a ω-terminal hydroxyl group, which may be modified with electrophilic reagents such as acid chlorides or carboxylic acids bearing (potentially) protected functional groups.[153,154] ADMET polymerization of diene monomers carried out in the presence of a suitably functionalized alkene chain transfer agent can be used to prepare α,ω-functionalized polyolefins.[155] The living character of the ruthenium carbene-initiated ROMP provides possibilities to prepare ω-functionalized polymers. Reaction of the metal alkylidene end-group with *e.g.* molecular oxygen[156] or various enol ethers[157] has been used to generate aldehyde, masked acid, ketone and masked amino end-functional polymers. ω-End functionalized polyoxazolines can be obtained by terminating the polymerization reaction with an appropriately functionalized nucleophilic reagent.[158]

4. Preparation of *peptide/protein–polymer conjugates*

Sections 2 and 3 have presented different approaches to introduce chemoselective handles into peptides/proteins or synthetic polymers, which can allow for the preparation of *peptide/protein–polymer conjugates*. This section discusses the different strategies, which can be employed for preparing the final *peptide/protein–polymer conjugates*.

4.1 Convergent synthesis of *peptide/protein–polymer conjugates*[2,8,159–161]

The direct coupling of a functionalized peptide/protein with a complementary functionalized synthetic polymer is the classical solution to preparing *peptide/protein–polymer conjugates*. In general, the difficulty of this approach lies, on the one hand, in the reduced accessibility of functional groups on macromolecules relative to small molecules, which can limit reaction conversion, and on the other hand in the isolation of the desired conjugate from a reaction mixture containing macromolecular starting materials and/or by-products. In this section, an overview is presented of the different reactions that have been used to prepare *peptide/protein–polymer conjugates*. A summary of the reaction conditions that will be discussed below can be found in Table 7.

Peptide coupling chemistry. The most straightforward method for conjugating a polymer and a peptide/protein relies on well-established protocols for coupling carboxylic acids and amines. This coupling chemistry is directly used in SPPS because of the high levels of conversion attainable by this method. The introduction of a carboxylic acid modified polymer, for example the commercially available PEG-COOH, in the final coupling step of SPPS of a peptide directly yields the desired *peptide/protein–polymer conjugate*. This strategy has been used to prepare N-terminal PEGylated peptides bearing elastin-like peptides,[162] amphiphilic β-strands,[76] coiled-coil motifs[72–74] and "switch" peptides.[74] In a similar fashion, conjugates of a short $(Thr–Val)_5$ peptide sequence and poly(*n*-butyl acrylate) have also been prepared.[163]

Pd^0 catalyzed coupling reactions. A very attractive approach for conjugating peptides/proteins to small molecules or synthetic polymers involves the use of Pd^0 catalysts to promote the formation of stable C–C bonds. In the reported cases where these reactions have been used on peptides/proteins, these coupling reactions have been found insensitive to all side groups, though interference of thiol groups due to catalyst poisoning is expected.[164] Examples of Heck, Sonogashira and Suzuki reactions exist for the coupling of small molecules as well as macromolecules to appropriately modified peptides/proteins. The application of these reactions has been reviewed extensively elsewhere[165–168] and therefore only precedence for their use on peptides/proteins will be discussed. While the Heck and Sonogashira coupling of small molecules, including short peptides, has been reported to proceed quantitatively, coupling to proteins lead to moderate yields and was accompanied by significant dehalogenation side-reactions.[52,169] Furthermore, the use of these coupling reactions in aqueous media generally required the use of a complex mixture of additives to assure solubility of the protein and to improve conversion.[52] Sonogashira coupling of small molecules to proteins was found to result in higher conversion in comparison to the Heck reaction.[52] Reaction conditions for Suzuki coupling in water close to room temperature have recently been established for conjugation to peptides. In particular, it was found that the addition of glycerol to the reaction mixture significantly improved conversion through suppression of side-reactions.[54] These mild conditions have been shown to permit protein modification without change of protein tertiary structure.

Staudinger ligation. Staudinger ligation provides many opportunities for the synthesis of *peptide/protein–polymer conjugates*. This reaction generates an amide bond between two species, one bearing an azide and the other a phosphinothioester group. This ligation strategy has been used for the site-specific PEGylation of proteins,[170] for fluorescently labeling proteins,[171] for immobilization of proteins onto gold surfaces[172] and for coupling carbohydrate haptens to immunogenic protein carriers.[173] In the latter example, phosphino-functionalized active esters were developed for the residue-specific Staudinger ligation at lysine residues. Until recently, Staudinger ligation was carried out in organic or mixed organic/aqueous media, which limits the applicability of this reaction for the conjugation of peptides/proteins. However, a series of acidic and basic water soluble phosphinothiols has been recently developed, which enables Staudinger ligation to be carried out in mild aqueous media with reaction yields ranging from 17–90% depending on pH.[32]

Cycloaddition reactions. The Diels–Alder cycloaddition reaction also offers possibilities for the preparation of *peptide/protein–polymer conjugates*. Using a *trans*,*trans*-2,4-hexadienyl ester moiety as diene and the maleimide group as dienophile, this reaction has been successfully used in peptide/protein ligation and immobilization.[174,175] This reaction is

Table 7 Reactions suitable for coupling small molecules or synthetic polymers to peptides/proteins

Name	Reaction conditions
Heck	**Small molecule to protein:** 1.6 M DMSO, 80 mM $MgCl_2$, HEPES-KOH (10 mM, pH 8), 1.1 M glycerol, 20 mM NaCl, TAPS-NaOH (100 mM, pH 8.3), 1.6 mM DG, 50 mM TBAC, 1 M NaOAc, 10 mM tyramine HCl, protein (285 μg mL^{-1}), 4 mM alkene reagent, 0.5 mM $Pd(OAc)_2$, 0.5 mM TPPTS, 5 °C, 50 h. Yield: 2% functionalized protein, 28% dehalogenation side-reaction.[52]
Sonogashira	**Small molecule to protein:** 2.3 M DMSO, 0.5 mM $Mg(OAc)_2$, 0.2 mM sodium ascorbate, TAPS-NaOH (90 mM, pH 8.3), Tris-HCl (2.5 mM, pH 7.9), 15 mM NaCl, Triton X-100 (0.4% v/v), imidazole HCl (13 mM, pH 7), protein (60 μg mL^{-1}), 15 mM alkyne reagent, 1.7 mM $Pd(OAc)_2$, 8.3 mM TPPTS, 0.7 M copper(I) trifluoromethanesulfonate, 6 °C, 80 min. Yield: 25% functionalized protein, 13% dehalogenation side-reaction.[52] **Small molecule to peptide (9 amino acids):** 0.2 M TAPS buffer in H_2O (pH 8.3), 10 mM peptide, 20 mM alkyne reagent, 10 mol% $Pd(OAc)_2$, guanidinophosphane hydrochloride ligand (4 eq *vs.* Pd), 10–19.2 mol% CuI, 50 °C, 240 min. Yield: 75% functionalized peptide.[184] **Peptide to peptide (17–33 amino acids):** 6 M guanidine/50 mM phosphate buffer (pH 7.5) or 50 mM NaOAc (pH 5.5), 4–10 mM peptide (final concentration not given), 1.3–3.3 mM alkyne, 5 mol% Pd/CuI, trisulfonated phosphine ligand (5 eq. *vs.* Pd), r.t., 0.5–3 h. Yield: 0–100%.[53]
Suzuki	**Small molecule to peptide (34 amino acids):** 10 mM Tris-HCl (pH 8.0), 0–50% v/v glycerol, 1 mM peptide, 3–5 mM boronic acid, 1–2.5 mM Na_2PdCl_4, 25–40 °C, 18–20 h. Yield: 38–>90%.[54]
Staudinger ligation	**Small molecule model reactions in water:** 0.4 M sodium phosphate buffer (pH 6.5–9), 0.1 mM phosphinothioester, 10.7 mM azide, 16 h. Yield: 17–90% yield.[32] (Study also describes the preparation of protein phosphinothioesters for Staudinger ligation in aqueous media.) **Protein to polymer:** phosphate-buffered saline (pH 7.4), 300–500 nM azide protein, large excess methyl-PEG-triarylphosphine, 37 °C, 36 h. Yield: 100%.[170]
Diels–Alder cycloaddition	**Peptide ligation (3–10 amino acids):** H_2O, 10 mM diene, 10 mM dienophile, 25 °C, 47 h. Yield: 92%.[175] H_2O : MeOH (20 : 1–3 : 2) or H_2O : DMF (4 : 1–1 : 0), 10 mM diene, 10–14 mM dienophile, r.t., 20–48 h. Yield: 84–100%.[174] **Small molecule to protein:** 10 mM sodium phosphate buffer (pH 5.5–6.5), 100 μM diene, 3 mM dienophile, 25 °C, 24 h. Yield: ~90%.[174,175]
Huisgen 1,3-dipolar cycloaddition	**Oligonucleotide to protein:** 0.1 M sodium phosphate buffer (pH 7.9), 1 μM protein, 50 μM alkyne, 2 mM $CuSO_4$, 3 mM tris(2-carboxyethyl(phosphine)), 37 °C, 6 h. Yield: 50–100%.[178] **Peptide coupling on a solid support (4–12 amino acids):** 20% DIEA in DMF, 26 mM CuI, r.t., 48 h. Yield: 6–90% (lower yields for sequential reactions).[177] **Protein to polymer:** 20 mM phosphate buffer (pH 7.2) containing 16% THF, 0.252 μM alkyne protein, 12.6 μM azide polymer, 0.8 μM $CuSO_4{\cdot}5H_2O$, 8 μM ascorbic acid, r.t., 24 h. No yield given.[181]
Reductive alkylation	**Protein to polymer:** 100 mM sodium acetate (pH 5), 20 mM sodium cyanoborohydride, 5 mg mL^{-1} protein, 5 molar eq. polymer aldehyde, ice bath, 10 h. Yield: 92%.[185] **Protein to polymer:** 25 mM sodium formate, 50 mM potassium phosphate buffer (pH 7.4), 20 μM iridium complex, 100 μM protein, 1 mM aldehyde polymer, 37 °C, 15 h. Yield: 59%.[12]
Hydrazone formation	**Polymer to protein:** 30 mM sodium acetate (pH 5): H_2O (pH 6) (71:1), 0.2 μM aldehyde protein, 0.14 μM hydrazine polymer, 4 °C, 16 h. Yield: 27%.[188]
Oxime formation	**Polymer to protein:** 50% aq. acetonitrile (+0.1% TFA), 1.2–1.5 eq. protein, 1 eq. aminooxy polymer, r.t., no reaction time given. Yield: 30–50%.[191] **Protein to polymer:** 50 mM sodium phosphate buffer (pH 6.5), 1 mg mL^{-1} ketone protein (0.056 μM ketone), 28.3 μM alkoxyamine polymer, r.t., 24 h. Yield: ~60%.[21]
Michael addition	**Polymer to peptide (5 amino acids):** 100 mM HEPES (pH 7.4), 2.5 mM acrylate, 2.5 mM thiol, 37 °C, variable time (kinetic experiments). No yield given.[193] **Peptide (13 amino acids) to acrylate functionalized solid support:** phosphate buffer saline (10 mM, pH 7.4), 20 mg mL^{-1} acrylate functionalized particles (loading 0.26–0.48 mmol g^{-1} of acrylate groups), 20 eq. of cysteine (within peptide), 1 eq. TCEP, r.t., overnight, brief sonication. Yield: 9%.[195]
Radical thiol addition	**Short peptide to polymer (2 amino acids):** dry degassed NMP, ~3 wt% solution containing 1:2.5:0.33 (molar ratio) of alkenyl groups: peptide thiols: AIBN, 70 °C, 24–48 h. Yield: 55–75%.[197]
Oxidative coupling	**Polymer to protein:** 25 mM sodium phosphate (pH 6.5), 50 μM aniline labeled protein, 250 μM phenylenediamine derivative, 500 μM $NaIO_4$, r.t., 4 h. Yield: 45%.[58]

Abbreviations: AIBN: 2,2-azoisobutyronitrile; DG: decyl-β-D-glucopyranoside; DIEA: diisopropyl ethyl amine; NMP: *N*-methylpyrrolidone; HEPES: *N*-[2-Hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; TAPS: *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; TBAC: tetrabutyl ammonium chloride; TCEP: tris(2-carboxyethyl)phosphine; TFA: trifluoroacetic acid; TPPTS: triphenylphosphine-3,3′,3″-trisulfonate; Tris-HCl: 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride.

compatible with all amino acid side groups except for the thiol group on cysteine, which may react with the maleimide moiety *via* Michael addition. The thiol group must therefore be protected. When performed in mixed aqueous media it has been reported that the use of DMF as the co-solvent slows down the rate of formation of the cycloadduct. In contrast, the use of excess dienophile considerably reduces reaction time and increases conversion.[175] Recently, a transition-metal free spontaneous tandem [3+2] cycloaddition-retro-Diels–Alder ligation method has been reported for the coupling of small

molecules to proteins and polymers to peptides by means of azides and oxanorbornadienes.[176] The chemoselectivity of this reaction may be compromised by Michael addition reactions, which have been observed to occur between lysine, arginine and histidine and the oxonorbornadiene in model experiments.

The Cu(I)-mediated Huisgen 1,3-dipolar cycloaddition reaction has been extensively explored for the preparation of peptide/protein–polymer, or small molecule, conjugates. This reaction has been shown to give high yields in mild, basic conditions in solution as well as on solid supports.[177–179] Azide or alkyne functionalized peptides/proteins and synthetic polymers can be easily synthesized using the methods discussed in the previous sections. While, in general, Cu(I) mediated cycloadditions proceed more rapidly under basic conditions, it has recently been shown that histidine derivatives strongly accelerate this reaction, possibly due to strong coordination of histidine to the copper ion. This aspect has been taken advantage of for the coupling of "self-activated" peptides containing modified histidine residues.[180] In contrast, however, it has been observed that copper coordination by guanidinium side-chains of arginine residues may prevent conjugation of peptides/proteins to synthetic polymers.[181] This problem may be overcome using a strong copper binding ligand such as pentamethyldiethylenetriamine (PMDETA). Finally, it is noteworthy to mention that for solubility reasons, it may be desirable to perform 1,3-dipolar cycloaddition on side-chain protected peptides rather than unprotected ones.[182]

Reductive alkylation, oxime and hydrazone formation. Aldehydes and ketones can react with amines, alkoxyamines and hydrazides to form imines, oximes and hydrazones, respectively. Each of these three reactions has found use for the preparation of *peptide/protein–polymer conjugates*. Probably due to the fact that amine groups, in contrast to alkoxyamine and hydrazide groups, are present in amino acids, their reaction with aldehydes/ketones has been most intensively used for the synthesis of *peptide/protein–synthetic polymer conjugates*. As the imine bond, which is initially generated upon reaction of the aldehyde/ketone and amine groups, is hydrolytically labile, a subsequent reduction step is necessary to produce a stable secondary amine bond. Typical reducing agents for reductive alkylation are $NaBH_4$ and $NaCNBH_4$ which can be used at basic or neutral pH, respectively.[183] Recently, water-stable iridium complexes have been shown to be interesting mild reagents for reductive alkylation of imines.[12] This reagent, however, was unable to reduce imines formed from ketones. Reductive alkylation is extensively used for the preparation of PEGylated proteins.[185,186,187] In contrast to imine bonds, which require an additional reduction step to generate a stable linkage, oximes and hydrazones formed by reaction between ketones/aldehydes and alkoxyamines or hydrazides, respectively, are hydrolytically stable from pH 2–7 and 5–7, respectively,[188,189] but decompose rapidly above pH 9.0.[190] Pons *et al.* have prepared a hydrazino poly(ethylene imine), which they have successfully conjugated to oxidized human serum transferrin.[188] Kochendoerfer and co-workers have very elegantly used oxime chemistry to prepare PEGylated analogues of erythropoiesis protein[191] and the chemokine CCL-5 (RANTES).[192] In the former example, the human erythropoietin analogue was chemically synthesized using SPPS and native chemical ligation. This allowed the introduction of N^{ε}-levulinyl lysine residues at specific positions in the protein, which subsequently served as conjugation sites for the attachment of branched aminooxy PEG derivatives. The preparation of the PEGylated CCL-5 analogue followed a similar strategy, but in this case, aminooxy groups were site-selectively introduced into the protein, which was subsequently modified with PEG aldehyde derivatives.

Thiol addition reactions. Michael addition of thiols onto activated alkenes such as maleimides, vinyl sulfones, acrylates, *etc.*, is a common method for preparing *peptide/-protein–polymer conjugates* due to the rarity of free cysteine residues in natural proteins (Table 1) and the possibility for introducing these in a site-selective manner by protein engineering.[159] In fact, many polymeric reagents for this reaction, such as PEG-maleimide, are commercially available. Furthermore, this type of reaction occurs in mild (physiological) conditions, does not require the presence of organometallic catalysts and the extent of reaction may be monitored by the decrease of the absorption bands at 233 nm that are associated with the thiolate and the alkene groups, assuming of course that additional underlying absorption bands are absent.[193] At physiological pH, it has been reported that the rate of Michael addition of thiols is one order of magnitude higher than that of amines, highlighting the kinetic selectivity of this coupling method for cysteines *versus* all other amino acids in proteins.[194] In model studies, it has been shown that amino acids bearing positive charges close to the thiol group accelerate the Michael addition significantly, while the opposite is true for negatively charged amino acids.[193] Michael addition has been used to immobilize a 13 amino acid peptide containing a cysteine residue onto PEG-acrylate functionalized nanoparticles. It was however observed that the yield of this reaction ($\sim$9%) was significantly lower than the equivalent experiment performed with cysteine (65%) due to diffusion related considerations.[195]

Aside from Michael-type addition, thiols may be coupled to alkenes by a radical addition mechanism. This reaction is currently receiving considerable interest for the modification of side-chain functional polymers due to its compatibility with functional groups such as carboxylic acids, amines, alcohols, *etc.*[124,196] While there is little precedence for the preparation of *peptide/protein–polymer conjugates* using this reaction, a model dipeptide containing a cysteine residue has been successfully added to the pendant alkenyl groups of a 1,2 poly(butadiene) produced by anionic polymerization.[197] The radical thiol addition of poly(1,2-butadiene)s, however, suffers from a side-reaction, which leads to the formation of six-membered cyclic units. This side reaction can be effectively suppressed, however, by increasing the distance between pendant alkenyl groups, as was demonstrated by the modification of poly-(2-(3-butenyl)-2-oxazoline).[198]

Oxidative coupling. Aniline groups introduced by methods discussed in section 2.3 present another method for orthogonal site-selective coupling of peptides/proteins and polymers. These functionalities can be selectively modified under mild

oxidative conditions with a phenylenediamine derivative. The reaction conditions have been shown to be insensitive to the presence of lysine residues. This strategy has been used to site-selectively PEGylate a C-terminal aniline modified GFP derivative.[58]

4.2 Divergent synthesis of peptide/protein–polymer conjugates[7,81,129,161]

Two main strategies can be distinguished for the divergent synthesis of *peptide/protein–polymer conjugates*: (i) polymerization from a peptide/protein macroinitiator; and (ii) synthesis of a peptide segment on a soluble or solid supported synthetic polymer. These two approaches will be highlighted in this section.

Polymerization directly from peptide/protein macroinitiators has been accomplished by ATRP, NMP and RAFT polymerization. One advantage of this approach for preparing *peptide/protein–polymer conjugates* is that isolation does not involve the separation of macromolecular species, but of a high molecular weight *peptide/protein–polymer conjugate* from low molecular weight monomers. Of concern when using any of the radical based polymerization techniques is the presence of thiol groups on cysteine residues, which may act as chain transfer agents.

Monomers such as monomethoxy poly(ethylene glycol) methacrylate,[199,200] 4-styrenesulfonate[199] and *N*-isopropylacrylamide[201,202] have been successfully polymerized by ATRP from protein macroinitiators derived from chymotrypsin,[199] bovine serum albumin,[200,201] streptavidin[202] or lysozyme.[200,201] Lele *et al*. have noticed that the initiation efficiency of protein macroinitiators bearing multiple copies of the initiator moiety was reduced due to steric hindrance of the growing polymer chains and have suggested that proteins bearing 1–2 initiator groups produced conjugates with the greatest uniformity, an argument in favor of site-specific protein modification.[199] The use of pyridyl disulfide modified ATRP initiators to modify the free cysteine residues of proteins results in polymers that are attached *via* reversible disulfide bonds.[201] This offers the advantage that the synthetic polymer can be cleaved from the protein and characterized. A possible drawback with the direct polymerization from peptide/protein macroinitiators may be the small quantities in which these molecules are often available. At very low macroinitiator concentrations, controlled polymerization can be difficult to achieve. This problem, however, can be overcome by the use of a ''sacrificial'' (resin-bound) initiator.[201] In addition to protein macroinitiators, solution ATRP has also been successfully used to graft synthetic polymer segments from low molecular weight linear and cyclic peptide ATRP initiators.[203–207] In a recent study by Broyer *et al*., two ATRP initiators based on serine and tyrosine have been developed for initiation of polymerization of methacrylates and styrenes, respectively.[208] These initiators can be introduced site-selectively into peptides by SPPS using standard Fmoc protocols. The serine-based initiator, Fmoc-*O*-(2-bromoisobutyryl)-serine (S*) was successfully incorporated in a model peptide, VMS*VVQTK, which was subsequently used to initiate the ATRP of a sugar-modified 2-hydroxyethyl methacrylate derivative. ATRP has also been performed on a resin-bound peptide initiator to produce *peptide–polymer conjugates via* a tandem SPPS-ATRP strategy.[71,209] Solid-supported peptide initiators have also been successfully used to prepare *peptide–polymer conjugates via* NMP.[209,210] It has been suggested, however, that the high concentration of growing polymer chains and a diffusion limited access of the deactivating species may make it difficult to accurately control polymer chain length and chain length distribution when solid supported controlled radical initiators are used.[211]

In addition to ATRP and NMP, RAFT polymerization has also been used to prepare *peptide/protein–polymer conjugates*. Boyer *et al*. have developed a cysteine targeting RAFT agent to initiate the polymerization of *N*-isopropylacrylamide and (hydroxyethyl)acrylate from bovine serum albumin in aqueous media at 25 °C.[151] Instead of using a conventional free radical initiator, polymerization from protein macro-RAFT agents can also be initiated using γ-irradiation.[150] This strategy has been used to prepare BSA-poly(oligoethylene glycol methacrylate) conjugates. ten Cate *et al*. have prepared *peptide–polymer conjugates via* RAFT polymerization of *n*-butyl acrylate using a peptide macrotransfer agent.[212] RAFT polymerization in aqueous media using peptide/protein transfer agents bears the risk of hydrolysis and aminolysis of the RAFT agent and/or active chain ends. These side reactions, however, can be minimised by careful control of the reaction conditions, especially the pH.[213] Hydrolysis may be minimised at neutral to moderately acidic pH. Suppressing aminolysis requires acidic conditions.

Instead of grafting a synthetic polymer from a peptide initiator or chain transfer agent, the peptide domain of a *peptide–polymer conjugate* can also be synthesized from a soluble[214–216] or solid-supported polymer.[76] Peptide synthesis on solid supported synthetic polymers can be conveniently carried out on commercially available Tentagel resins in which poly(ethylene glycol) chains are attached to a solid support *via* an acid labile linker.[76,217–219] Reynhout *et al*. reported an interesting strategy for the synthesis of polystyrene–peptide conjugates.[220] The synthesis starts with reductive alkylation of an aldehyde functionalized resin with an amino functionalized polystyrene. This results in a secondary amine bond between the polystyrene and the solid support, which is subsequently used to grow the peptide segment *via* SPPS.

In addition to solid supported synthetic polymers, soluble synthetic polymers such as poly(ethylene glycol) have been used for peptide synthesis, which offer advantages of both solution and solid phase chemistry.[221] In addition to poly(ethylene glycol), poly(styrene), poly(vinyl alcohol), poly(ethylene imine) and poly(acrylic acid) have also been used as soluble supports for peptide synthesis.[221] Using these supports, isolation of the growing peptide is performed by making use of the differential solubility of the polymer support with respect to all reagents and catalysts used for the synthesis. An important consideration in the use of soluble synthetic polymer supports is that with increasing length of the peptide segment, the solubility properties of the conjugate may be no longer dictated by the synthetic polymer support but rather by the peptide, which can hamper

isolation and purification at the different steps in the peptide synthesis process.

4.3 "Grafting-through" strategies for preparing *peptide/protein–polymer conjugates*[81,211]

The final approach for preparing *peptide/protein–polymer conjugates* involves the polymerization of peptide/protein macromonomers in the so-called "grafting through" strategy. Peptides have been prepared that contain a broad range of functional groups allowing polymerization using a variety of methods. Compared to the post-polymerization modification of a side-chain functional polymer, this strategy has the advantage that all repeat units on the conjugate will bear a pendant peptide/protein moiety.

The conjugation of a β-sheet forming $(Ala)_4$ peptide sequence to diacetylene has been used to drive the self-assembly of these peptide functionalized monomers into a highly ordered arrangement, which, in a subsequent step, allowed topochemical polymerization of the diacetylene groups.[222,223] In a similar fashion, the self-assembly properties of the Gly–Ala–Ala–Asn–Pro–Asn–Ala–Gly peptide sequence have been exploited to form nanoribbons susceptible to alignment in a strong magnetic field, and which can be further polymerized by diacetylene polymerization.[224] Murata *et al.* reported the free radical polymerization of a series of *N*-methacryloyl-(L-leucyl-L-alanyl)$_n$ (n = 2–4) methyl esters.[225] It was found that monomer conversion and polymer molecular weight decreased with increasing peptide chain length. Free-radical polymerization has also been successfully used to prepare copolymers of *N*-(2-hydroxypropyl)methacrylamide with methacrylamide functionalized amphiphatic Tyr–Ile–Leu–Ile–His–Arg–Asn[226] peptides or the enzymatically degradable Gly–Phe–Lys–Gly peptide.[227] Ring-opening metathesis polymerization of peptide functionalized norbornenes or cyclooctenes has been used to generate polymers that incorporate cell adhesion peptide sequences,[228] to prepare novel polyelectrolytes[229] or to prepare polymers with ACE inhibitory[230] or immunostimulant properties.[230] The RAFT polymerization of methacrylate monomers bearing elastin-like Val–Pro–Gly–Val–Gly peptide side chains afforded polymers with narrow polydispersity indexes and degrees of polymerization up to 88.[231] ATRP of these monomers has also lead to the production of high molecular weight polymers.[232]

5. Outlook

The field of *peptide/protein–polymer conjugate* design and synthesis is booming as new applications and new properties are sought out for such hybrid materials. The importance of producing site-specific modifications on both peptides/proteins and synthetic polymers should be clear when one considers applications related to stimuli-responsiveness, self-assembly and bioactivity. Continued efforts towards scaling up and improving the efficiency of incorporating non-canonical amino acids, whether by chemical or biological means, may open the way to new *peptide/protein–polymer conjugates* with exciting new properties or functions. The demonstration that controlled polymerization can be performed directly from protein macroinitiators will no doubt contribute significantly to the production of a wide range of well-defined conjugates, and potentially serve as an alternative to PEGylation for modifying protein properties.

Acknowledgements

MAG gratefully acknowledges a post-doctoral scholarship from the FQRNT (Quebec, Canada).

Notes and references

1 G. Pasut and F. M. Veronese, *Prog. Polym. Sci.*, 2007, **32**, 933–961.
2 P. Thordarson, B. Le Droumaguet and K. Velonia, *Appl. Microbiol. Biotechnol.*, 2006, **73**, 243–254.
3 http://expasy.org/sprot/.
4 H. O. Villar and R. T. Koehler, *Biopolymers*, 2000, **53**, 226–232.
5 H. O. Villar and L. M. Kauvar, *FEBS Lett.*, 1994, **349**, 125–130.
6 S. Moelbert, E. Emberly and C. Tang, *Protein Sci.*, 2004, **13**, 752–762.
7 H.-A. Klok, *J. Polym. Sci., Part A: Polym. Chem.*, 2005, **43**, 1–17.
8 J. M. Antos and M. B. Francis, *Curr. Opin. Chem. Biol.*, 2006, **10**, 253–262.
9 D. R. W. Hodgson and J. M. Sanderson, *Chem. Soc. Rev.*, 2004, **33**, 422–430.
10 H. B. F. Dixon, *J. Protein Chem.*, 1984, **3**, 99–108.
11 J. H. van Maarseveen, J. N. H. Reek and J. W. Back, *Angew. Chem., Int. Ed.*, 2006, **45**, 1841–1843.
12 J. M. McFarland and M. B. Francis, *J. Am. Chem. Soc.*, 2005, **127**, 13490–13491.
13 G. Levesque, P. Arsène, V. Fanneau-Bellenger and T.-N. Pham, *Biomacromolecules*, 2000, **1**, 387–399.
14 G. Levesque, P. Arsène, V. Fanneau-Bellenger and T.-N. Pham, *Biomacromolecules*, 2000, **1**, 400–406.
15 S. Shaunak, A. Godwin, J.-W. Choi, S. Balan, E. Pedone, D. Vijayarangam, S. Heidelberger, I. Teo, M. Zloh and S. Brocchini, *Nat. Chem. Biol.*, 2006, **2**, 312–313.
16 S. Balan, J.-W. Choi, A. Godwin, I. Teo, C. M. Laborde, S. Heidelberger, M. Zloh, S. Shaunak and S. Brocchini, *Bioconjugate Chem.*, 2007, **18**, 61–76.
17 L. Lorand and S. M. Conrad, *Mol. Cell. Biochem.*, 1984, **58**, 9–35.
18 B.-H. Hu and P. B. Messersmith, *J. Am. Chem. Soc.*, 2003, **125**, 14298–14299.
19 S. D. Tilley and M. B. Francis, *J. Am. Chem. Soc.*, 2006, **128**, 1080–1081.
20 N. S. Joshi, L. R. Whitaker and M. B. Francis, *J. Am. Chem. Soc.*, 2004, **126**, 15942–15943.
21 T. L. Schlick, Z. Ding, E. W. Kovacs and M. B. Francis, *J. Am. Chem. Soc.*, 2005, **127**, 3718–3723.
22 P. G. Holder and M. B. Francis, *Angew. Chem., Int. Ed.*, 2007, **46**, 4370–4373.
23 J. M. Hooker, E. W. Kovacs and M. B. Francis, *J. Am. Chem. Soc.*, 2004, **126**, 3718–3719.
24 G. Espuña, G. Arsequell, G. Valencia, J. Barluenga, M. Pérez and J. M. González, *Chem. Commun.*, 2000, 1307–1308.
25 G. Espuña, G. Arsequell, G. Valencia, J. Barluenga, J. M. Alvarez-Gutiérrez, A. Ballesteros and J. M. González, *Angew. Chem., Int. Ed.*, 2004, **43**, 325–329.
26 S. Y. Wong and D. Putnam, *Bioconjugate Chem.*, 2007, **18**, 970–982.
27 S.-J. Xiao, M. Wieland and S. Brunner, *J. Colloid Interface Sci.*, 2005, **290**, 172–183.
28 T. Tanaka, N. Kamiya and T. Nagamune, *FEBS Lett.*, 2005, **579**, 2092–2096.
29 J. M. Antos and M. B. Francis, *J. Am. Chem. Soc.*, 2004, **126**, 10256–10257.
30 *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*, ed. W. C. Chan and P. D. White, Oxford University Press, New York, 2000.
31 L. C. Chan and B. G. Cox, *J. Org. Chem.*, 2007, **72**, 8863–8869.
32 A. Tam, M. B. Soellner and R. T. Raines, *J. Am. Chem. Soc.*, 2007, **129**, 11421–11430.

33 P. E. Dawson, M. J. Churchill, M. R. Ghadiri and S. B. H. Kent, *J. Am. Chem. Soc.*, 1997, **119**, 4325–4329.
34 R. Wetzel, R. Halualani, J. T. Stults and C. Quan, *Bioconjugate Chem.*, 1990, **1**, 114–122.
35 S. D. Mikolajczyk, D. L. Meyer, J. J. Starling, K. L. Law, K. Rose, B. Dufour and R. E. Offord, *Bioconjugate Chem.*, 1994, **5**, 636–646.
36 H. Deng, *J. Pept. Sci.*, 2007, **13**, 107–112.
37 W.-K. Chan, C.-M. Ho, M.-K. Wong and C.-M. Che, *J. Am. Chem. Soc.*, 2006, **128**, 14796–14797.
38 J. M. Gilmore, R. A. Scheck, A. P. Esser-Kahn, N. S. Joshi and M. B. Francis, *Angew. Chem., Int. Ed.*, 2006, **45**, 5307–5311.
39 J. P. Tam, J. Xu and K. D. Eom, *Biopolymers*, 2001, **60**, 194–205.
40 K. F. Geoghegan and J. G. Stroh, *Bioconjugate Chem.*, 1992, **3**, 138–146.
41 D. Chelius and T. A. Shaler, *Bioconjugate Chem.*, 2003, **14**, 205–211.
42 L. Zhang and J. P. Tam, *Anal. Biochem.*, 1996, **233**, 87–93.
43 K. Rose, W. Zeng, P.-O. Regamey, I. V. Chernushevich, K. G. Standing and H. F. Gaertner, *Bioconjugate Chem.*, 1996, **7**, 552–556.
44 H. F. Gaertner, K. Rose, R. Cotton, D. Timms, R. Camble and R. E. Offord, *Bioconjugate Chem.*, 1992, **3**, 262–268.
45 J. Wilken and S. B. H. Kent, *Curr. Opin. Biotechnol.*, 1998, **9**, 412–426.
46 S.-Y. Chen, S. Cressman, F. Mao, H. Shao, D. W. Low, H. S. Beilan, E. N. Cagle, M. Carnevali, V. Gueriguian, P. J. Keogh, H. Porter, S. M. Stratton, M. C. Wiedeke, L. Savatski, J. W. Adamson, C. E. Bozzini, A. Kung, S. B. H. Kent, J. A. Bradburne and G. G. Kochendoerfer, *Chem. Biol.*, 2005, **12**, 371–383.
47 M. Taki and M. Sisido, *Biopolymers*, 2007, **88**, 263–271.
48 X. Li, L. Zhang, S. E. Hall and J. P. Tam, *Tetrahedron Lett.*, 2000, **41**, 4069–4073.
49 R. E. Connor and D. A. Tirrell, *Polym. Rev.*, 2007, **47**, 9–28.
50 A. J. Link, M. L. Mock and D. A. Tirrell, *Curr. Opin. Biotechnol.*, 2003, **14**, 603–609.
51 H. E. Blackwell and R. H. Grubbs, *Angew. Chem., Int. Ed.*, 1998, **37**, 3281–3284.
52 K. Kodama, S. Fukuzawa, H. Nakayama, K. Sakamoto, T. Kigawa, T. Yabuki, N. Matsuda, M. Shirouzu, K. Takio, S. Yokoyama and K. Tachibana, *ChemBioChem*, 2007, **8**, 232–238.
53 D. T. Bong and M. R. Ghadiri, *Org. Lett.*, 2001, **3**, 2509–2511.
54 A. Ojida, H. Tsutsumi, N. Kasagi and I. Hamachi, *Tetrahedron Lett.*, 2005, **46**, 3301–3305.
55 A. Deiters, T. A. Cropp, M. Mukherji, J. W. Chin, J. C. Anderson and P. G. Schultz, *J. Am. Chem. Soc.*, 2003, **125**, 11782–11783.
56 A. Deiters, T. A. Cropp, D. Summerer, M. Mukherji and P. G. Schultz, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 5743–5745.
57 M. Paris, C. Douat, A. Heitz, W. Gibbons, J. Martinez and J.-A. Fehrentz, *Tetrahedron Lett.*, 1999, **40**, 5179–5182.
58 J. M. Hooker, A. P. Esser-Kahn and M. B. Francis, *J. Am. Chem. Soc.*, 2006, **128**, 15558–15559.
59 D. Crich and A. Banerjee, *J. Am. Chem. Soc.*, 2007, **129**, 10064–10065.
60 B. P. Duckworth, Z. Zhang, A. Hosokawa and M. D. Distefano, *ChemBioChem*, 2007, **8**, 98–105.
61 B. P. Duckworth, J. Xu, T. A. Taton, A. Guo and M. D. Distefano, *Bioconjugate Chem.*, 2006, **17**, 967–974.
62 H. T. ten Brink, J. T. Meijer, R. V. Geel, M. Damen, D. W. P. M. Löwik and J. C. M. van Hest, *J. Pept. Sci.*, 2006, **12**, 686–692.
63 G. T. Dolphin, *Chem.–Eur. J.*, 2006, **12**, 1436–1447.
64 P. J. Duggan and D. A. Offermann, *Aust. J. Chem.*, 2007, **60**, 829–834.
65 U. Hoffmanns and N. Metzler-Nolte, *Bioconjugate Chem.*, 2006, **17**, 204–213.
66 M. J. O'Donnell, M. D. Drew, R. S. Pottorf and W. L. Scott, *J. Comb. Chem.*, 2000, **2**, 172–181.
67 Y. Shin, K. A. Winans, B. J. Backes, S. B. H. Kent, J. A. Ellman and C. R. Bertozzi, *J. Am. Chem. Soc.*, 1999, **121**, 11684–11689.
68 P. E. Dawson, T. W. Muir, I. Clark-Lewis and S. B. H. Kent, *Science*, 1994, **266**, 776–779.
69 B. L. Nilsson, L. L. Kiessling and R. T. Raines, *Org. Lett.*, 2000, **2**, 1939–1941.
70 P. Wang and L. P. Miranda, *Int. J. Pept. Res. Therapeut.*, 2005, **11**, 117–123.
71 Y. Mei, K. L. Beers, H. C. M. Byrd, D. L. VanderHart and N. R. Washburn, *J. Am. Chem. Soc.*, 2004, **126**, 3472–3476.
72 G. W. M. Vandermeulen, C. Tziatzios and H.-A. Klok, *Macromolecules*, 2003, **36**, 4107–4114.
73 G. W. M. Vandermeulen, C. Tziatzios, R. Duncan and H.-A. Klok, *Macromolecules*, 2005, **38**, 761–769.
74 H.-A. Klok, G. W. M. Vandermeulen, H. Nuhn, A. Rösler, I. W. Hamley, V. Castelletto, H. Xu and S. S. Sheiko, *Faraday Discuss.*, 2005, **128**, 29–41.
75 I. W. Hamley, I. A. Ansari, V. Castelletto, H. Nuhn, A. Rösler and H. A. Klok, *Biomacromolecules*, 2005, **6**, 1310–1315.
76 A. Rösler, H.-A. Klok, I. W. Hamley, V. Castelletto and O. O. Mykhaylyk, *Biomacromolecules*, 2003, **4**, 859–863.
77 J. T. I. V. Lundquist and J. C. Pelletier, *Org. Lett.*, 2001, **3**, 781–783.
78 L. E. Canne, S. J. Bark and S. B. H. Kent, *J. Am. Chem. Soc.*, 1996, **118**, 5891–5896.
79 P. Botti, M. R. Carrasco and S. B. H. Kent, *Tetrahedron Lett.*, 2001, **42**, 1831–1833.
80 L. Wang and P. G. Schultz, *Angew. Chem., Int. Ed.*, 2005, **44**, 34–66.
81 J. Nicolas, G. Mantovani and D. M. Haddleton, *Macromol. Rapid Commun.*, 2007, **28**, 1083–1111.
82 V. Coessens, T. Pintauer and K. Matyjaszewski, *Prog. Polym. Sci.*, 2001, **26**, 337–377.
83 J. C. M. van Hest, *Polym. Rev.*, 2007, **47**, 63–92.
84 A. Godwin, M. Hartenstein, A. H. E. Müller and S. Brocchini, *Angew. Chem., Int. Ed.*, 2001, **40**, 594–597.
85 M. J. Yanjarappa, K. V. Gujraty, A. Joshi, A. Saraph and R. S. Kane, *Biomacromolecules*, 2006, **7**, 1665–1670.
86 J. Hwang, R. C. Li and H. D. Maynard, *J. Controlled Release*, 2007, **122**, 279–286.
87 C. Cheng, G. Sun, E. Khoshdel and K. L. Wooley, *J. Am. Chem. Soc.*, 2007, **129**, 10086–10087.
88 R. C. Li, R. M. Broyer and H. D. Maynard, *J. Polym. Sci., Part A: Polym. Chem.*, 2006, **44**, 5004–5013.
89 G. Sun, C. Cheng and K. L. Wooley, *Macromolecules*, 2007, **40**, 793–795.
90 T. Dispinar, R. Sanyal and A. Sanyal, *J. Polym. Sci., Part A: Polym. Chem.*, 2007, **45**, 4545–4551.
91 M. Malkoch, R. J. Thibault, E. Drockenmuller, M. Messerschmidt, B. Voit, T. P. Russell and C. J. Hawker, *J. Am. Chem. Soc.*, 2005, **127**, 14942–14949.
92 D. Hua, W. Bai, J. Xiao, R. Bai, W. Lu and C. Pan, *Chem. Mater.*, 2005, **17**, 4574–4576.
93 B. S. Sumerlin, N. V. Tsarevsky, G. Louche, R. Y. Lee and K. Matyjaszewski, *Macromolecules*, 2005, **38**, 7540–7545.
94 J. Qiu and K. Matyjaszewski, *Macromolecules*, 1997, **30**, 5643–5648.
95 M. Camail, A. Margaillan, J. C. Maesano, S. Thuret and J. L. Vernet, *Polymer*, 1998, **39**, 3187–3192.
96 F. Sanda, T. Abe and T. Endo, *J. Polym. Sci., Part A: Polym. Chem.*, 1997, **35**, 2619–2629.
97 F. Sanda and T. Endo, *Macromol. Chem. Phys.*, 1999, **200**, 2651–2661.
98 A. Bentolila, I. Vlodavsky, R. Ishai-Michaeli, O. Kovalchuk, C. Haloun and A. J. Domb, *J. Med. Chem.*, 2000, **43**, 2591–2600.
99 H. Mori, M. Matsuyama, K. Sutoh and T. Endo, *Macromolecules*, 2006, **39**, 4351–4360.
100 S. Tugulu, P. Silacci, N. Stergiopulos and H.-A. Klok, *Biomaterials*, 2007, **28**, 2536–2546.
101 J. Yang, C. Xu, P. Kopečková and J. Kopeček, *Macromol. Biosci.*, 2006, **6**, 201–209.
102 K. K. L. Cheuk, J. W. Y. Lam, L. M. Lai, Y. Dong and B. Z. Tang, *Macromolecules*, 2003, **36**, 9752–9762.
103 H. Zhao, F. Sanda and T. Masuda, *J. Macromol. Sci., Pure Appl. Chem.*, 2007, **44**, 389–394.
104 K. Okoshi, K. Sakajiri, J. Kumaki and E. Yashima, *Macromolecules*, 2005, **38**, 4061–4064.
105 G. Gao, F. Sanda and T. Masuda, *Macromolecules*, 2003, **36**, 3932–3937.
106 F. Sanda, H. Araki and T. Masuda, *Macromolecules*, 2004, **37**, 8510–8516.

107 H. Zhao, F. Sanda and T. Masuda, *Macromol. Chem. Phys.*, 2005, **206**, 1653–1658.
108 F. Sanda, K. Terada and T. Masuda, *Macromolecules*, 2005, **38**, 8149–8154.
109 T. M. Trnka and R. H. Grubbs, *Acc. Chem. Res.*, 2001, **34**, 18–29.
110 C. W. Bielawski and R. H. Grubbs, *Prog. Polym. Sci.*, 2007, **32**, 1–29.
111 A. Carrillo, M. J. Yanjarappa, K. V. Gujraty and R. S. Kane, *J. Polym. Sci., Part A: Polym. Chem.*, 2006, **44**, 928–939.
112 C. Kluger and W. H. Binder, *J. Polym. Sci., Part A: Polym. Chem.*, 2007, **45**, 485–499.
113 W. H. Binder and C. Kluger, *Macromolecules*, 2004, **37**, 9321–9330.
114 A. Demonceau, A. W. Stumpf, E. Saive and A. F. Noels, *Macromolecules*, 1997, **30**, 3127–3136.
115 R. Revanur, B. McCloskey, K. Breitenkamp, B. D. Freeman and T. Emrick, *Macromolecules*, 2007, **40**, 3624–3630.
116 T. E. Hopkins and K. B. Wagener, *Macromolecules*, 2004, **37**, 1180–1189.
117 X. Lou, C. Detrembleur and R. Jérôme, *Macromol. Rapid Commun.*, 2003, **24**, 161–172.
118 B. Parrish, R. B. Breitenkamp and T. Emrick, *J. Am. Chem. Soc.*, 2005, **127**, 7404–7410.
119 R. Riva, S. Schmeits, F. Stoffelbach, C. Jérôme, R. Jérôme and P. Lecomte, *Chem. Commun.*, 2005, 5334–5336.
120 A. Hirao, S. Loykulnant and T. Ishizone, *Prog. Polym. Sci.*, 2002, **27**, 1399–1471.
121 M. Kobayashi, Y. Matsumoto, M. Uchiyama and T. Ohwada, *Macromolecules*, 2004, **37**, 4339–4341.
122 M. Tsuji, A. K. M. F. Azam, M. Kamigaito and Y. Okamoto, *Macromolecules*, 2007, **40**, 3518–3520.
123 M. Tanaka, A. Sudo, F. Sanda and T. Endo, *J. Polym. Sci., Part A: Polym. Chem.*, 2003, **41**, 853–860.
124 J. Justynska, Z. Hordyjewicz and H. Schlaad, *Polymer*, 2005, **46**, 12057–12064.
125 S. Cesana, J. Auernheimer, R. Jordan, H. Kessler and O. Nuyken, *Macromol. Chem. Phys.*, 2006, **207**, 183–192.
126 C. Taubmann, R. Luxenhofer, S. Cesana and R. Jordan, *Macromol. Biosci.*, 2005, **5**, 603–612.
127 W. H. Binder and H. Gruber, *Macromol. Chem. Phys.*, 2000, **201**, 949–957.
128 R. Luxenhofer and R. Jordan, *Macromolecules*, 2006, **39**, 3509–3516.
129 K. L. Heredia and H. D. Maynard, *Org. Biomol. Chem.*, 2007, **5**, 45–53.
130 G. Moad, Y. K. Chong, A. Postma, E. Rizzardo and S. H. Thang, *Polymer*, 2005, **46**, 8458–8468.
131 A. Hirao and M. Hayashi, *Acta Polym.*, 1999, **50**, 219–231.
132 A. Limer and D. M. Haddleton, *Macromolecules*, 2006, **39**, 1353–1358.
133 F. Lecolley, L. Tao, G. Mantovani, I. Durkin, S. Lautru and D. M. Haddleton, *Chem. Commun.*, 2004, 2026–2027.
134 G. Mantovani, F. Lecolley, L. Tao, D. M. Haddleton, J. Clerx, J. J. L. M. Cornelissen and K. Velonia, *J. Am. Chem. Soc.*, 2005, **127**, 2966–2973.
135 L. Tao, G. Mantovani, F. Lecolley and D. M. Haddleton, *J. Am. Chem. Soc.*, 2004, **126**, 13220–13221.
136 D. Bontempo, K. L. Heredia, B. A. Fish and H. D. Maynard, *J. Am. Chem. Soc.*, 2004, **126**, 15372–15373.
137 H. D. Maynard, K. L. Heredia, R. C. Li, D. P. Parra and V. Vázquez-Dorbatt, *J. Mater. Chem.*, 2007, **17**, 4015–4017.
138 K. L. Heredia, Z. P. Tolstyka and H. D. Maynard, *Macromolecules*, 2007, **40**, 4772–4779.
139 J. T. Kopping, Z. P. Tolstyka and H. D. Maynard, *Macromolecules*, 2007, **40**, 8593–8599.
140 S. Sen Gupta, K. S. Raja, E. Kaltgrad, E. Strable and M. G. Finn, *Chem. Commun.*, 2005, 4315–4317.
141 R. K. O'Reilly, M. J. Joralemon, C. J. Hawker and K. L. Wooley, *J. Polym. Sci., Part A: Polym. Chem.*, 2006, **44**, 5203–5217.
142 A. W. Bosman, R. Vestberg, A. Heumann, J. M. J. Fréchet and C. J. Hawker, *J. Am. Chem. Soc.*, 2003, **125**, 715–728.
143 D. Miyamoto, J. Watanabe and K. Ishihara, *J. Appl. Polym. Sci.*, 2004, **91**, 827–832.
144 D. Miyamoto, J. Watanabe and K. Ishihara, *Biomaterials*, 2003, **25**, 71–76.
145 D. Miyamoto, J. Watanabe and K. Ishihara, *J. Appl. Polym. Sci.*, 2005, **95**, 615–622.
146 M.-H. Dufresne, M. A. Gauthier and J.-C. Leroux, *Bioconjugate Chem.*, 2005, **16**, 1027–1033.
147 S. K. Varshney, Z. Song, J. X. Zhang and R. Jérôme, *J. Polym. Sci., Part A: Polym. Chem.*, 2006, **44**, 3400–3405.
148 S. Caillol, S. Lecommandoux, A.-F. Mingotaud, M. Schappacher, A. Soum, N. Bryson and R. Meyrueix, *Macromolecules*, 2003, **36**, 1118–1124.
149 M. Gotsche, H. Keul and H. Höcker, *Macromol. Chem. Phys.*, 1995, **196**, 3891–3903.
150 J. Liu, V. Bulmus, D. L. Herlambang, C. Barner-Kowollik, M. H. Stenzel and T. P. Davis, *Angew. Chem., Int. Ed.*, 2007, **46**, 3099–3103.
151 C. Boyer, V. Bulmus, J. Liu, T. P. Davis, M. H. Stenzel and C. Barner-Kowollik, *J. Am. Chem. Soc.*, 2007, **129** 7145–7154.
152 D. Taton, S. Angot, Y. Gnanou, E. Wolert, S. Setz and R. Duran, *Macromolecules*, 1998, **31**, 6030–6033.
153 M. J. Stanford and A. P. Dove, *Polym. Prepr. (Am. Chem. Soc., Div. Polym. Chem.)*, 2007, **48**, 888–889.
154 M. Schappacher, A. Soum and S. M. Guillaume, *Biomacromolecules*, 2006, **7**, 1373–1379.
155 K. R. Brzezinska and T. J. Deming, *Macromolecules*, 2001, **34**, 4348–4354.
156 S. C. G. Biagini, R. Gareth Davies, V. C. Gibson, M. R. Giles, E. L. Marshall and M. North, *Polymer*, 2001, **42**, 6669–6671.
157 R. M. Owen, J. E. Gestwicki, T. Young and L. L. Kiessling, *Org. Lett.*, 2002, **4**, 2293–2296.
158 C. J. Waschinski and J. C. Tiller, *Biomacromolecules*, 2005, **6**, 235–243.
159 A. S. Hoffman and P. S. Stayton, *Prog. Polym. Sci.*, 2007, **32**, 922–932.
160 J.-F. Lutz, *Angew. Chem., Int. Ed.*, 2007, **46**, 1018–1025.
161 H. G. Börner and H. Schlaad, *Soft Matter*, 2007, **3**, 394–408.
162 M. Pechar, J. Brus, L. Kostka, Č. Koňák, M. Urbanová and M. Šlouf, *Macromol. Biosci.*, 2007, **7**, 56–69.
163 J. Hentschel and H. G. Börner, *J. Am. Chem. Soc.*, 2006, **128**, 14142–14149.
164 T. Itoh and T. Mase, *J. Org. Chem.*, 2006, **71**, 2203–2206.
165 R. Chinchilla and C. Nájera, *Chem. Rev.*, 2007, **107**, 874–922.
166 L. Yin and J. Liebscher, *Chem. Rev.*, 2007, **107**, 133–173.
167 F. Alonso, I. P. Beletskaya and M. Yus, *Tetrahedron*, 2005, **61**, 11771–11835.
168 S. Bräse, J. H. Kirchhoff and J. Köbberling, *Tetrahedron*, 2003, **59**, 885–939.
169 K. Kodama, S. Fukuzawa, H. Nakayama, T. Kigawa, K. Sakamoto, T. Yabuki, N. Matsuda, M. Shirouzu, K. Takio, K. Tachibana and S. Yokoyama, *ChemBioChem*, 2006, **7**, 134–139.
170 C. S. Cazalis, C. A. Haller, L. Sease-Cargo and E. L. Chaikof, *Bioconjugate Chem.*, 2004, **15**, 1005–1009.
171 M.-L. Tsao, F. Tian and P. G. Schultz, *ChemBioChem*, 2005, **6**, 2147–2149.
172 J. Kalia, N. L. Abbott and R. T. Raines, *Bioconjugate Chem.*, 2007, **18**, 1064–1069.
173 C. Grandjean, A. Boutonnier, C. Guerreiro, J.-M. Fournier and L. A. Mulard, *J. Org. Chem.*, 2005, **70**, 7123–7132.
174 A. Dantas de Araújo, J. M. Palomo, J. Cramer, O. Seitz, K. Alexandrov and H. Waldmann, *Chem.–Eur. J.*, 2006, **12**, 6095–6109.
175 A. Dantas de Araújo, J. M. Palomo, J. Cramer, M. Köhn, H. Schröder, R. Wacker, C. Niemeyer, K. Alexandrov and H. Waldmann, *Angew. Chem., Int. Ed.*, 2006, **45**, 296–301.
176 S. S. van Berkel, A. J. Dirks, M. F. Debets, F. L. van Delft, J. J. L. M. Cornelissen, R. J. M. Nolte and F. P. J. T. Rutjes, *ChemBioChem*, 2007, **8**, 1504–1508.
177 R. Franke, C. Doll and J. Eichler, *Tetrahedron Lett.*, 2005, **46**, 4479–4482.
178 M. Humenik, Y. Huang, Y. Wang and M. Sprinzl, *ChemBioChem*, 2007, **8**, 1103–1106.
179 H.-J. Musiol, S. Dong, M. Kaiser, R. Bausinger, A. Zumbusch, U. Bertsch and L. Moroder, *ChemBioChem*, 2005, **6**, 625–628.

180 K. Tanaka, C. Kageyama and K. Fukase, *Tetrahedron Lett*., 2007, **48**, 6475–6479.
181 A. J. Dirks, S. S. van Berkel, N. S. Hatzakis, J. A. Opsteen, F. L. van Delft, J. J. L. M. Cornelissen, A. E. Rowan, J. C. M. van Hest, F. P. J. T. Rutjes and R. J. M. Nolte, *Chem. Commun*., 2005, 4172–4174.
182 J.-F. Lutz, H. G. Börner and K. Weichenhan, *Aust. J. Chem*., 2007, **60**, 410–413.
183 G. E. Means, *J. Protein Chem*., 1984, **3**, 121–130.
184 H. Dibowski and F. P. Schmidtchen, *Angew. Chem., Int. Ed*., 1998, **37**, 476–478.
185 O. Kinstler, G. Molineux, M. Treuheit, D. Ladd and C. Gegg, *Adv. Drug Delivery Rev*., 2002, **54**, 477–485.
186 M. J. Roberts, M. D. Bentley and J. M. Harris, *Adv. Drug Delivery Rev*., 2002, **54**, 459–476.
187 S. Zalipsky, *Adv. Drug Delivery Rev*., 1995, **16**, 157–182.
188 B. Pons, L. Mouhoubi, A. Adib, P. Godzina, J.-P. Behr and G. Zuber, *ChemBioChem*, 2006, **7**, 303–309.
189 K. Rose, *J. Am. Chem. Soc*., 1994, **116**, 30–33.
190 J. Shao and J. P. Tam, *J. Am. Chem. Soc*., 1995, **117**, 3893–3899.
191 G. G. Kochendoerfer, S.-Y. Chen, F. Mao, S. Cressman, S. Traviglia, H. Shao, C. L. Hunter, D. W. Low, E. N. Cagle, M. Carnevali, V. Gueriguian, P. J. Keogh, H. Porter, S. M. Stratton, M. C. Wiedeke, J. Wilken, J. Tang, J. J. Levy, L. P. Miranda, M. M. Crnogorac, S. Kalbag, P. Botti, J. Schindler-Horvat, L. Savatski, J. W. Adamson, A. Kung, S. B. H. Kent and J. A. Bradburne, *Science*, 2003, **299**, 884–887.
192 H. Shao, M. M. Crnogorac, T. Kong, S.-Y. Chen, J. M. Williams, J. M. Tack, V. Gueriguian, E. N. Cagle, M. Carnevali, D. Tumelty, X. Paliard, L. P. Miranda, J. A. Bradburne and G. G. Kochendoerfer, *J. Am. Chem. Soc*., 2005, **127**, 1350–1351.
193 M. P. Lutolf, N. Tirelli, S. Cerritelli, L. Cavalli and J. A. Hubbell, *Bioconjugate Chem*., 2001, **12**, 1051–1056.
194 M. Friedman, J. F. Cavins and J. S. Wall, *J. Am. Chem. Soc*., 1965, **87**, 3672–3682.
195 M. Heggli, N. Tirelli, A. Zisch and J. A. Hubbell, *Bioconjugate Chem*., 2003, **14**, 967–973.
196 J. Justynska, Z. Hordyjewicz and H. Schlaad, *Macromol. Symp*., 2006, **240**, 41–46.
197 Y. Geng, D. E. Discher, J. Justynska and H. Schlaad, *Angew. Chem., Int. Ed*., 2006, **45**, 7578–7581.
198 A. Gress, A. Völkel and H. Schlaad, *Macromolecules*, 2007, **40**, 7928–7933.
199 B. S. Lele, H. Murata, K. Matyjaszewski and A. J. Russell, *Biomacromolecules*, 2005, **6**, 3380–3387.
200 J. Nicolas, V. San Miguel, G. Mantovani and D. M. Haddleton, *Chem. Commun*., 2006, 4697–4699.
201 K. L. Heredia, D. Bontempo, T. Ly, J. T. Byers, S. Halstenberg and H. D. Maynard, *J. Am. Chem. Soc*., 2005, **127**, 16955–16960.
202 D. Bontempo and H. D. Maynard, *J. Am. Chem. Soc*., 2005, **127**, 6508–6509.
203 H. Rettig, E. Krause and H. G. Börner, *Macromol. Rapid Commun*., 2004, **25**, 1251–1256.
204 L. Ayres, P. Hans, J. Adams, D. W. P. M. Löwik and J. C. M. van Hest, *J. Polym. Sci., Part A: Polym. Chem*., 2005, **43** 6355–6366.
205 M. G. J. ten Cate, N. Severin and H. G. Börner, *Macromolecules*, 2006, **39**, 7831–7838.
206 J. Couet and M. Biesalski, *Macromolecules*, 2006, **39**, 7258–7268.
207 J. Couet, J. D. Jeyaprakash, S. Samuel, A. Kopyshev, S. Santer and M. Biesalski, *Angew. Chem., Int. Ed*., 2005, **44**, 3297–3301.
208 R. M. Broyer, G. M. Quaker and H. D. Maynard, *J. Am. Chem. Soc*., 2008, **130**, 1041–1047.
209 M. L. Becker, J. Liu and K. L. Wooley, *Biomacromolecules*, 2005, **6**, 220–228.
210 M. L. Becker, J. Liu and K. L. Wooley, *Chem. Commun*., 2003, 180–181.
211 D. W. P. M. Löwik, L. Ayres, J. M. Smeenk and J. C. M. van Hest, *Adv. Polym. Sci*., 2006, **202**, 19–52.
212 M. G. J. ten Cate, H. Rettig, K. Bernhardt and H. G. Börner, *Macromolecules*, 2005, **38**, 10643–10649.
213 D. B. Thomas, A. J. Convertine, R. D. Hester, A. B. Lowe and C. L. McCormick, *Macromolecules*, 2004, **37**, 1735–1741.
214 S. Drioli, I. Adamo, M. Ballico, F. Morvan and G. M. Bonora, *Eur. J. Org. Chem*., 2002, 3473–3480.
215 V. N. R. Pillai, M. Mutter, E. Bayer and I. Gatfield, *J. Org. Chem*., 1980, **45**, 5364–5370.
216 E. Bayer and M. Mutter, *Nature*, 1972, **237**, 512–513.
217 J. Hentschel, E. Krause and H. G. Börner, *J. Am. Chem. Soc*., 2006, **128**, 7722–7723.
218 T. S. Burkoth, T. L. S. Benzinger, D. N. M. Jones, K. Hallenga, S. C. Meredith and D. G. Lynn, *J. Am. Chem. Soc*., 1998, **120**, 7655–7656.
219 T. S. Burkoth, T. L. S. Benzinger, V. Urban, D. G. Lynn, S. C. Meredith and P. Thiyagarajan, *J. Am. Chem. Soc*., 1999, **121**, 7429–7430.
220 I. C. Reynhout, D. W. P. M. Löwik, J. C. M. van Hest, J. J. L. M. Cornelissen and R. J. M. Nolte, *Chem. Commun*., 2005, 602–604.
221 D. J. Gravert and K. D. Janda, *Chem. Rev*., 1997, **97**, 489–509.
222 E. Jahnke, A.-S. Millerioux, N. Severin, J. P. Rabe and H. Frauenrath, *Macromol. Biosci*., 2007, **7**, 136–143.
223 E. Jahnke, I. Lieberwirth, N. Severin, J. P. Rabe and H. Frauenrath, *Angew. Chem., Int. Ed*., 2006, **45**, 5383–5386.
224 D. W. P. M. Löwik, I. O. Shklyarevskiy, L. Ruizendaal, P. C. M. Christianen, J. C. Maan and J. C. M. van Hest, *Adv. Mater*., 2007, **19**, 1191–1195.
225 H. Murata, F. Sanda and T. Endo, *Macromol. Chem. Phys*., 2001, **202**, 759–764.
226 H. Ding, P. Kopečková and J. Kopeček, *J. Drug Target*., 2007, **15**, 465–474.
227 Y. Kasuya, Z.-R. Lu, P. Kopeckova, T. Minko, S. E. Tabibi and J. Kopecek, *J. Controlled Release*, 2001, **74**, 203–211.
228 H. D. Maynard, S. Y. Okada and R. H. Grubbs, *Macromolecules*, 2000, **33**, 6239–6248.
229 R. B. Breitenkamp, Z. Ou, K. Breitenkamp, M. Muthukumar and T. Emrick, *Macromolecules*, 2007, **40**, 7617–7624.
230 S. C. G. Biagini and A. L. Parry, *J. Polym. Sci., Part A: Polym. Chem*., 2007, **45**, 3178–3190.
231 F. Fernández-Trillo, A. Duréault, J. P. M. Bayley, J. C. M. van Hest, J. C. Thies, T. Michon, R. Weberskirch and N. R. Cameron, *Macromolecules*, 2007, **40**, 6094–6099.
232 L. Ayres, M. R. J. Vos, P. J. H. M. Adams, I. O. Shklyarevskiy and J. C. M. van Hest, *Macromolecules*, 2003, **36**, 5967–5973.