ACS Macro Letters

Letter

pubs.acs.org/macroletters

Convenient Routes to Efficiently N-PEGylated Peptides

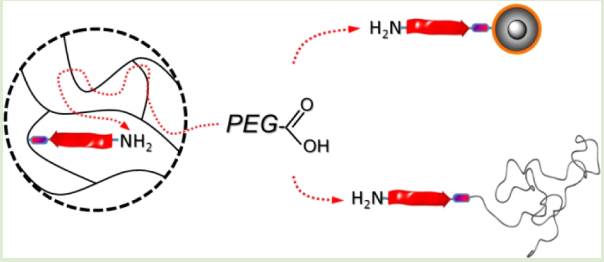
Christian Stutz,[†] Anna Meszynska,[‡] Jean-François Lutz,*[,‡] and Hans G. Börner*[,†]

[†]Department of Chemistry, Laboratory for Organic Synthesis of Functional Systems, Humboldt-Universität zu Berlin, Brook-Taylor-Str. 2, Berlin 12489, Germany

[‡]Precision Macromolecular Chemistry Group, Institut Charles Sadron, UPR-22 CNRS, 23 rue de Loess, 67034 Strasbourg, France

Supporting Information

ABSTRACT: Efficient routes to N-terminal PEGylated peptides are described. Alternative supports such as superparamagnetic core−shell nanoparticles as colloidal supports and end-functional poly(styrene) as homogeneous supports improve the available solid-phase supported coupling strategies preserving ease of purification. Poly(ethylene glycol) (PEG)−peptide bioconjugates are obtained in high yield despite the use of nearly stoichiometric PEGylation agents with respect to the supported peptides.



Peptide−polymer conjugates constitute a versatile class of biohybrid macromolecules, which proved to be applicable in various fields such as drug delivery, nanostructure assembly, surface-designed nanoparticles, biomineralization, composites, or molecular electronics.[1−11] Modern conjugation strategies enable the combination of peptides with a broad spectra of synthetic polymers.[12−16] Despite the fact that various synthetic polymer blocks could introduce interesting functionalities and functions to bioconjugates, poly(ethylene glycol) (PEG) still dominates the polymer segments of bioconjugates.[17−20] Covalent attachment of PEG to therapeutically active peptides or proteins (PEGylation) often improves solubility and reduces immunogenicity as well as toxicity.[21,22] This makes PEGylation one of the most generic and broadly used concepts for transport of bioactive peptides and proteins.

Solid-phase supported strategies constitute very convenient routes to peptide−PEG conjugates by either performing sequential synthesis on a PEG-preloaded resin (inverse conjugation)[23,24] or introducing end functional PEG to a supported peptide.[25] While the former generates C-terminal modifications (peptide−PEG), the latter enables N-terminal or sequence-specific PEGylation. On-support coupling strategies have strong advantages due to ease of purification and sequence specificity.[26] However, the reaction of end-functionalized PEG, e.g., α-methoxy-ω-carboxy-PEG (*m*PEG−COOH) with a supported peptide, occurs in a microgel resin and thus might suffer from diffusion limitations. This leads to a strong decrease of the overall coupling rates upon increasing molecular weight of the PEG.[26−28] The effect appears to be visible already with low molecular weight PEG. While *m*PEG−COOH with M_n = 760 could be coupled quantitatively, $M_{n,PEG}$ of 2000 leads with certain sequences despite the large excess of PEGylation agent to a conversion of only 40%.[28] For standard solid-phase supported peptide synthesis (SPPS) a cross-linked poly(styrene-*co*-divinyl benzene) microgel resin is commonly used. Recently, alternative supports occurred that might not suffer from inherent diffusion limitations if larger residues (e.g., *m*PEG-COOH with M_n > 1000) are attempted to be coupled to a supported peptide.

Here we evaluate two modern supports to improve the available solid-phase supported synthesis protocols to N-terminal PEGylated peptides, preserving ease of purification and leading to bioconjugates with high yields.

Two different supports, reflecting recent concepts for peptide synthesis, have been evaluated for the N-terminal PEGylation of peptides (Figure 1). 70 nm superparamagnetic core−shell nanoparticles with magnetite cores and amino functional silica shell proved to constitute a useful colloidal support for sequential assembly of peptides (Figures 1 and 2).[29] The peptide synthesis takes place not inside a microgel particle but at the permanent surface of the supports, and thus excellent accessibility of the surface-present peptides can be anticipated. Alternatively, end-functional poly(styrene)s, a recent development of soluble supports (Figures 1 and 2), have been investigated, where peptide assembly takes place homogeneously in solution.[30−32] Both support systems should not suffer from diffusion limitations during coupling of larger residues, and thus high yield PEGylation is expected. Moreover, both support concepts enable ease of purification via either magnetic sedimentation in the first or precipitation in the second system.

Surface amino-functionalized, core−shell nanoparticles were employed as colloidal supports (Figure 2).[29] The monodisperse particles exhibited sizes of 69 ± 8 nm, a 6−9 nm monodomain superparamagnetic magnetite core, and a silica shell (Supporting Information (SI) Figure S3). The Fe_3O_4-core particles were prepared from iron(III) acetylacetonate, following a microwave-assisted benzyl alcohol route (SI Figure S1).[33] The

Received: May 29, 2013
Accepted: July 10, 2013
Published: July 12, 2013

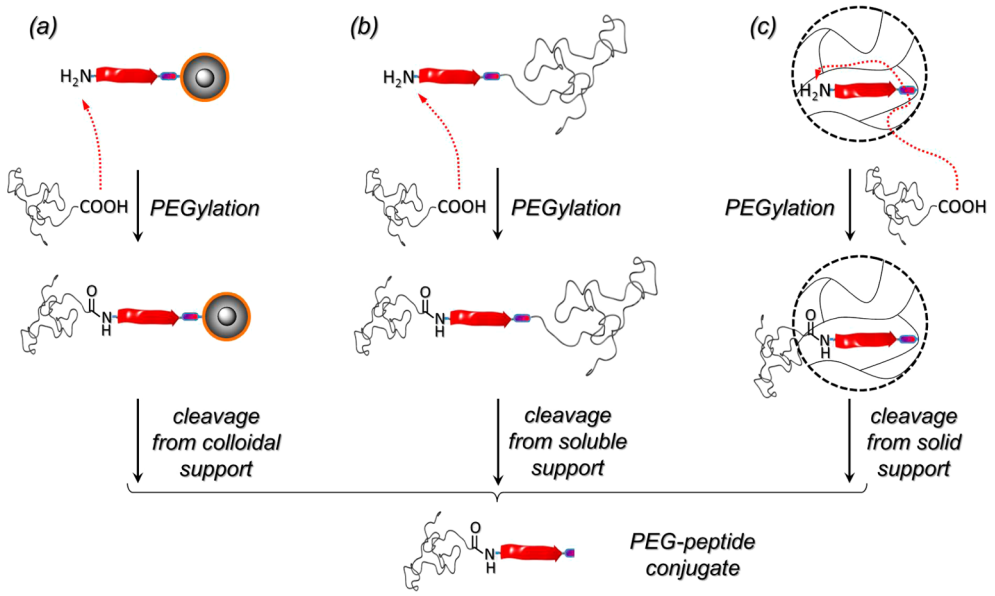


Figure 1. Strategies for N-terminal PEGylation of oligopeptides. Colloidal supports (a), soluble supports (b), and standard PS resins (c).

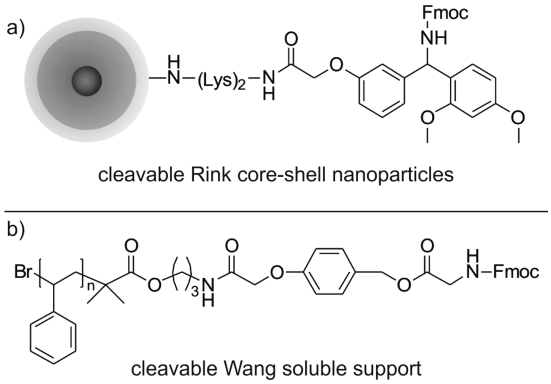


Figure 2. Supports for synthesis of PEG–peptide conjugates. Surface amino-functionalized, superparamagnetic core–shell nanoparticles with Rink–Linker as colloidal supports (a) and soluble poly(styrene) supports with Wang–Linker (b).

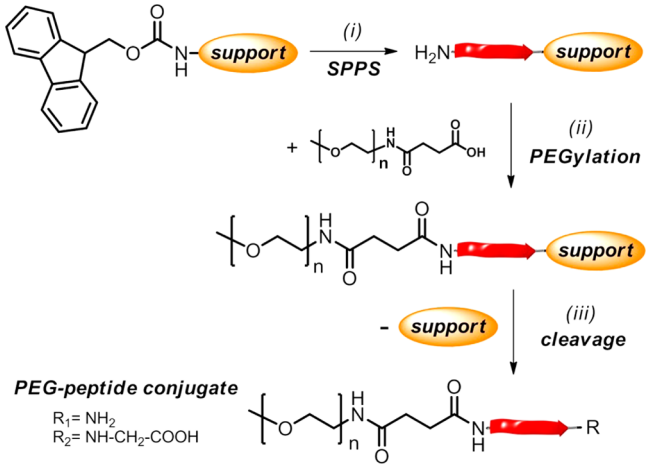
magnetite seeds were subsequently coated first with a silica shell followed by amino functionalization, using microwave-driven Stöber processes with tetraethyl orthosilicate first, followed by (3-aminopropyl)-trimethoxy-silane as precursors, respectively.[34] The particles were functionalized by twice coupling of Fmoc-Lys(Fmoc)OH to multimerize the amino functionalities to ultimately couple a Rink–Linker with a loading of 0.18 mmol/g (SI Figure S4).[35] The homogeneous support was prepared by atom transfer radical polymerization (ATRP) using a functional initiator.[30,36] The α-end-functional poly(styrene) was well-defined (M_n = 7100 g·mol^{-1}, PDI = 1.15) and presents a *para*-hydroxybenzyl alcohol Wang–Linker as anchor functionality for peptide synthesis (Figure 2).

Both supports (colloidal and soluble supports) were used to synthesize PEG–peptide conjugates (Figure 1 and Scheme 1). For that purpose, a mussel glue protein derived peptide (Tyr-Lys-Tyr-Lys-Gly) was synthesized, using standard SPPS activation, capping, and Fmoc-deprotection chemistry (Scheme 1 and SI).[35] In between each reaction step the colloidal supports could be conveniently separated from the reaction mixtures by magnetic sedimentation to isolate the intermediate products. Purification with the homogeneous supports used straightforward precipitation after each reaction step. In this particular case, the sequential growth of the pentapeptide was monitored by size exclusion chromatography (SEC) and NMR analysis (SI Figure S10). Following final Fmoc removal small aliquots of supported peptides from both synthesis procedures were removed for analysis purposes to prove the identity of the peptide before conjugation by ESI-MS (SI Figures S5 and S11). The residuals were used for PEGylation by coupling *m*PEG–COOH to the N-terminal amino groups of the supported peptides (Figure 1 and Scheme 1). To evaluate the potentials of both supports two PEGs with M_n = 2000 and 5000 (PEG2k and PEG5k) were investigated. The coupling to the colloidal supports was performed under an inert gas atmosphere for 5 days using only 2 equiv of PEG with respect to the amino functionalities. It should be noted that common on-support PEGylation strategies use usually larger excesses, e.g., 10 equiv, of PEGylation agents to drive the reaction to full conversion. Activation was facilitated by PyBOP/DIPEA (8/16 equiv) in a 9:1 DMF/DCM mixture. As control, PEGylation was also performed under similar conditions on a Rink polystyrene resin (1% cross-linked with 1,4-divinylbenzene) presenting the YKYKG sequence on a standard support for peptide synthesis (SI).[28] In the case of the homogeneous support, a near-equimolar amount of PEG was used (e.g., 1.15 equiv as compared to peptide) to avoid difficult macromolecular separations after ligation (i.e., separation of the unreacted PEG chains from the PEG-*b*-YKYKG-*b*-PS triblock adduct).[37]

Liberation of the PEG–peptide conjugates from the colloidal supports after PEGylation was performed by TFA/DCM (50 vol. %) for three hours. The conjugates could be isolated by ether precipitation from clear conjugate solutions after magnetic sedimentation of the empty supports. MALDI-TOF-MS proved the chemical identity of both conjugates with PEG2k and PEG5k (SI Figures S6 and S8). ^{1}H NMR analysis of the crude products confirmed a quantitative coupling for PEGylation with M_n = 2000 despite the fact that only 2 equiv of PEG compared to the peptide have been used. Even more remarkable, a conversion of 42% of the PEGylation with $M_{n,PEG}$ = 5000 could be observed (Table 1). The control experiments confirmed a strongly enhanced bioconjugate

dx.doi.org/10.1021/mz400260e | *ACS Macro Lett.* 2013, 2, 641–644

Scheme 1. Illustration of the Strategy for the Synthesis of PEG–Peptide Conjugates on Colloidal Nanoparticle Supports, Soluble PS Supports, and PS Microgel Supports[a]



[a](i) Standard solid-phase peptide synthesis by sequential Fmoc–amino acid coupling and Fmoc deprotection, (ii) PEGylation with *m*PEG–COOH using PyBOP/DIPEA, and (iii) liberation of the bioconjugate from the supports with TFA/CH_2Cl_2.

Table 1. Coupling Efficiencies of mPEG–COOH to Peptides Presented on Different Supports

	ratio of PEGylation agent to peptide[a]	coupling efficiency	
		$M_{n,PEO}$ = 2000	$M_{n,PEO}$ = 5000
colloidal supports	2:1	quantitative	42%
soluble supports	1.15:1	quantitative	39%
standard resin	2:1	6%	8%

[a]Excess of mPEG–COOH with respect to the supported peptides; reaction conditions cf. SI.

synthesis on the colloid supports, if compared to PEGylations of peptides on common PS Rink-amid resins. The latter yielded conversions below 10% for both of the peptide PEGylations, thus wasting 90% of the cost-intensive peptides. The colloidal supports illustrate that PEGylation with higher molecular weight PEGs can be realized on superparamagnetic core–shell nanoparticles with notable yields.

Figure 3 shows the SEC chromatograms recorded before and after peptide PEGylation on the homogeneous support. Although a small molar excess of mPEG–COOH was used as compared to the peptide, high coupling yields were observed in these experiments (Table 1). For instance, after coupling with PEG2k, a clear apparent molecular weight shift of about 3000 g·mol^{-1} was observed by SEC. It should be noted that these numbers are approximated since a PS calibration was used in SEC. Besides the main peak shift, a high molecular weight shoulder was also observed after reaction with PEG2k. This corresponds, most probably, to hexablocks (PEG-*b*-YKYKG-*b*-PS)$_2$ as dimerized supports formed as byproducts in the ATRP process by recombination termination.[36] Such structures are not problematic since they still allow cleavage and harvesting of the PEGylated peptide. PEGylation with PEG5k also led to a high degree of coupling. This was confirmed by an apparent molecular weight shift of approximately 7000 g·mol^{-1}

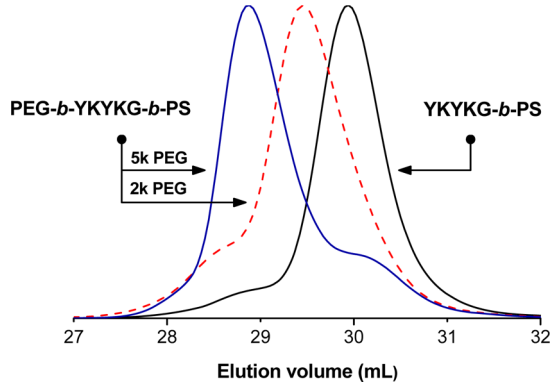


Figure 3. SEC chromatograms recorded in THF for the PEGylation of the pentapeptide YKYKG on the homogeneous support: initial chromatogram recorded for YKYKG-*b*-PS (black line), after reaction with PEG2k (dashed red line), and after reaction with PEG5k (blue line). Prior to analysis, the samples were precipitated in MeOH. Thus, the blue and red chromatograms show only the conjugate PEG-*b*-YKYKG-*b*-PS and the residual unreacted YKYKG-*b*-PS.

in SEC. Interestingly, no hexablocks were detected in these experiments. This is probably due to the fact that, after the first PEGylation of a symmetric dead chain, the coupling of a second PEG chain becomes slower and more unlikely due to the restricted accessibility of the remaining amino site in the formed random coil. The products were cleaved from the supports after synthesis was completed, and PEGylated conjugates could be separated by selective precipitation. NMR confirmed the full removal of the support and the formation of the PEG-*b*-YKYKG conjugates (SI).

In summary, two new support systems (superparamagnetic core–shell nanoparticles and well-defined soluble polystyrene supports) were investigated to synthesize N-terminal PEGylated peptides with high coupling efficiency. The supports enable ease of conjugation of PEG with M_n = 5000 as important for pharmacological applications. Where M_n = 2000 leads to quantitative conjugation reactions with only an excess of 1.15 equiv of mPEG–COOH with respect to the supported peptides, M_n = 5000 shows efficient coupling, leading to about 40% conversion. The supports investigated do not suffer from inherent diffusion limitations as compared to commonly established poly(styrene) microgel resins and thus prove particularly to be of value for coupling of larger residues, e.g., PEG with M_n = 5000. The presented supports are not limited to PEGylation. One can anticipate advantages in peptide modifications where reactions with sterically more demanding entities such as glycosylation, oligosaccharide ligation, fluorescence labeling, or even on-resin enzymatic transformations are required.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures and analytic data for all compounds are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: h.boerner@hu-berlin.de. Phone: (+49) 30-2093-7348. Fax: (+49) 30-2093-7500 (H.G.B.). E-mail: jlutz@unistra.fr (J.-F.L.).

dx.doi.org/10.1021/mz400260e | *ACS Macro Lett.* 2013, 2, 641–644

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge Felix Gandor (HU) for his contribution, M. Suess (ETH) for TEM, and C.E.M. for helpful assistance. H.G.B. acknowledges support by German Research Council DFG (CORE BO1762/4-1). J.F.L. and A.M. thank the European Research Council (Project SEQUENCES, ERC grant agreement n°258593) for financial support.

■ REFERENCES

(1) Wieczorek, S.; Krause, E.; Hackbarth, S.; Röder, B.; Hirsch, A. K. H.; Börner, G. H. *J. Am. Chem. Soc.* **2013**, 1711−1714.
(2) Dong, H.; Dube, N.; Shu, J. Y.; Seo, J. W.; Mahakian, L. M.; Ferrara, K. W.; Xu, T. *ACS Nano* **2012**, *6*, 5320.
(3) Kolodziej, C. M.; Kim, S. H.; Broyer, R. M.; Saxer, S. S.; Decker, C. G.; Maynard, H. D. *J. Am. Chem. Soc.* **2012**, *134*, 247.
(4) Shrestha, R.; Shen, Y. F.; Pollack, K. A.; Taylor, J. S. A.; Wooley, K. L. *Bioconjugate Chem.* **2012**, *23*, 574.
(5) Deacon, S. P. E.; Apostolovic, B.; Carbajo, R. J.; Schott, A. K.; Beck, K.; Vicent, M. J.; Pineda-Lucena, A.; Klok, H. A.; Duncan, R. *Biomacromolecules* **2011**, *12*, 19.
(6) Schwemmer, T.; Baumgartner, J.; Faivre, D.; Börner, H. G. *J. Am. Chem. Soc.* **2012**, *134*, 2385.
(7) Kühnle, R. I.; Börner, H. G. *Angew. Chem., Int. Ed.* **2011**, *50*, 4499.
(8) Verch, A.; Hahn, H.; Krause, E.; Cölfen, H.; Börner, H. G. *Chem. Commun.* **2010**, *46*, 8938.
(9) Kühnle, H.; Börner, H. G. *Angew. Chem., Int. Ed.* **2009**, *48*, 6431.
(10) Gentsch, R.; Pippig, F.; Schmidt, S.; Cernoch, P.; Polleux, J.; Börner, H. G. *Macromolecules* **2011**, *44*, 453.
(11) Jahnke, E.; Weiss, J.; Neuhaus, S.; Hoheisel, T. N.; Frauenrath, H. *Chem.—Eur. J.* **2009**, *15*, 388.
(12) Lutz, J.-F.; Börner, H. G. *Prog. Polym. Sci.* **2008**, *33*, 1.
(13) Broyer, R. M.; Grover, G. N.; Maynard, H. D. *Chem. Commun.* **2011**, *47*, 2212.
(14) Canalle, L. A.; Lowik, D.; van Hest, J. C. M. *Chem. Soc. Rev.* **2010**, *39*, 329.
(15) Tischer, T.; Goldmann, A. S.; Linkert, K.; Trouillet, V.; Börner, H. G.; Barner-Kowollik, C. *Adv. Funct. Mater.* **2012**, *22*, 3853.
(16) Pauloehrl, T.; Delaittre, G.; Winkler, V.; Welle, A.; Bruns, M.; Börner, H. G.; Greiner, A. M.; Bastmeyer, M.; Barner-Kowollik, C. *Angew. Chem., Int. Ed.* **2012**, *51*, 1071.
(17) Caliceti, P.; Veronese, F. M. *Adv. Drug Delivery Rev.* **2003**, *55*, 1261.
(18) Jevsevar, S.; Kunstelj, M.; Porekar, V. G. *Biotechnol. J.* **2010**, *5*, 113.
(19) Bailon, P.; Won, C. Y. *Expert Opin. Drug Delivery* **2009**, *6*, 1.
(20) Veronese, F. M.; Pasut, G. *Drug Discovery Today* **2005**, *10*, 1451.
(21) Greenwald, R. B.; Choe, Y. H.; McGuire, J.; Conover, C. D. *Adv. Drug Delivery Rev.* **2003**, *55*, 217.
(22) Veronese, F. M.; Harris, J. M. *Adv. Drug Delivery Rev.* **2002**, *54*, 453.
(23) Rapp, W. In *Combinatorial peptide and nonpeptide libraries. A Handbook*; Jung, G., Ed.; VCH: Weinheim, New York, Basel, 1996; pp 425−464.
(24) Eckhardt, D.; Groenewolt, M.; Krause, E.; Börner, H. G. *Chem. Commun.* **2005**, 2814.
(25) Zalipsky, S. *Adv. Drug Delivery Rev.* **1995**, *16*, 157.
(26) Börner, H. G. *Prog. Polym. Sci.* **2009**, *34*, 811.
(27) Becker, H.; Lucas, H.-W.; Maul, J.; Pillai, V. N. R.; Anzinger, H.; Mutter, M. *Makromol. Chem. Rapid Commun.* **1982**, *3*, 217.
(28) Vandermeulen, G. W. M.; Tziatzios, C.; Klok, H.-A. *Macromolecules* **2003**, *36*, 4107.
(29) Stutz, C.; Bilecka, I.; Thunemann, A. F.; Niederberger, M.; Börner, H. G. *Chem. Commun.* **2012**, *48*, 7176.
(30) Meszynska, A.; Badi, N.; Borner, H. G.; Lutz, J.-F. *Chem. Commun.* **2012**, *48*, 3887.
(31) Shemyakin, M. M.; Ovchinnikov, Y. A.; Kiryushkin, A. A.; Kozhevnikova, I. V. *Tetrahedron Lett.* **1965**, *6*, 2323.
(32) Mutter, M.; Uhmann, R.; Bayer, E. *Justus Liebigs Ann. Chem.* **1975**, 901.
(33) Bilecka, I.; Niederberger, M. *Nanoscale* **2010**, *2*, 1358.
(34) Stöber, W.; Fink, A.; Bohn, E. *J. Colloid Interface Sci.* **1968**, *26*, 62.
(35) Chan, W. C.; White, P. D. *Fmoc Solid-Phase Peptide Synthesis*; Oxford University Press: Oxford, 2000.
(36) Pfeifer, S.; Zarafshani, Z.; Badi, N.; Lutz, J.-F. *J. Am. Chem. Soc.* **2009**, *131*, 9195.
(37) Lutz, J.-F.; Börner, H. G.; Weichenhan, K. *Aust. J. Chem.* **2007**, *60*, 410.