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Controlled Solid Phase Peptide Bond Formation Using *N*-Carboxyanhydrides and PEG Resins in Water.

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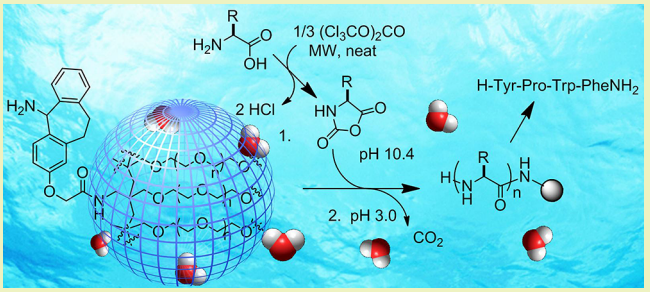
Supporting Information

ABSTRACT: The increasing need for large quantities of biologically active peptides for the market strongly raised the problem of the environmental sustainability of their synthesis. In this respect, we describe herein a solid phase procedure in water, using PEG peptide amide resins and NCAs under controlled conditions. This procedure afforded with reasonable yield and purity a short peptide amide, without the need of coupling reagents or protecting groups.



KEYWORDS: *PEG resin, Solid phase peptide synthesis, Peptide amide, Aqueous, N-carboxyanhydride, Microwave irradiation, Unprotected amino acids*

INTRODUCTION

The market for biologically active peptides is expected to grow rapidly in the next years. Besides the current interest in peptide as drugs and active pharmaceutical ingredients and as cosmeceuticals, recent findings suggest a wide range of novel applications in medicine, biotechnology, and surgery.[1−4] Peptides do not persist in the environment, giving innocuous degradation products; their components are the amino acids, which are renewable feedstocks available from biomasses[5,6] or agricultural byproduct streams.[7]

However, peptides are typical examples of products that impose an environmental burden during their manufacture. Both solution and solid phase (SP) syntheses[8] require large amounts of organic solvent, the use of reagents to activate the coupling between the residues, and the massive use of protecting groups (PGs), resulting in very poor atom economy and the release of harmful organic wastes.[9] As a consequence, the development of environmentally benign conditions has emerged as an important issue in green chemistry.[10,11]

One of the major problems concerns the huge amount of solvents needed, particularly for synthesis on solid supports. A convenient approach would be to carry out solvent-free reactions; this can be done with techniques such as mixing, grinding, or ball-milling.[12−14] So far, most of the efforts focused on the development of synthetic procedures in water. Because the common carbamate PGs are hydrophobic, reactivity can be improved by suspending in water nanoparticle reactants generated by using a planetary ball mill[15] or by MW activation.[16] Alternatively, new hydrophilic PGs have been proposed to improve water solubility.[17]

Also, the reagents for coupling and protection/deprotection steps should be reduced wherever possible. Significant reductions of organic wastes have been obtained using enzymatic catalysis for coupling reactions[18,19] or for PG removal.[20] The use of PGs was partially reduced on performing peptide coupling in aqueous solution utilizing the *N*-acylbenzotriazole derivatives and unprotected amino acids[21] or by using the native chemical ligation reaction of unprotected peptides in solution[22] on a water-compatible solid support.[23]

We report herein the SP preparation of peptide amides in water on a PEG resin by using *N*-carboxyanhydride (NCA) derivatives, conveniently prepared in turn under solvent-free conditions and MW irradiation. NCA, or Leuch's anhydrides, constitute a special class of α-amino acid-mixed anhydrides in which the amino group is protected and the carboxylate is activated at the same time.[24−26] The attractiveness of peptide synthesis by NCA lies in its simplicity and atom economy; the molecular bulk of the reactants remains with the newly formed peptide, the only byproduct being carbon dioxide, and the condensation sequence may be rapidly repeated to build polypeptides requiring only control to avoid NCA polymerization.

To test the efficacy of different kinds of PEG-based resins for peptide amides, we synthesized the endogenous opioid peptide endomorphin-1 (EM1), H-Tyr-Pro-Trp-Phe-NH_2. This tetrapeptide was isolated from mammalian brain, and it was found to activate μ-opioid receptors with high affinity and selectivity.[27,28]

EXPERIMENTAL METHODS

General Methods. Unless stated otherwise, chemicals were obtained from commercial sources and used without further

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Table 1. SPPS of EM-1 on Different PEG-Based ChemMatrix Peptide Amide Resins

entry	resin	loading (mmol/g)	bead size (mesh)[a]	cleavage mixture TFA/H_2O/Et_3SiH (%)	purity, crude (%)[b]	yield, isolated (%)[c,d]	purity, isolated (%)[d]
1	H-Rink amide	0.4−0.60	100−200	95.0/2.5/2.5	70	68	95
2	H-PAL	0.4−0.5	100−200	95.0/2.5/2.5	65	66	95
3	H-Ramage	0.25−0.50	100−200	5.0/2.5/2.5[e]	73	52	97

[a]Dry. [b]After crystallization, determined by analytical RP-HPLC. [c]Based on the average loading. [d]After semipreparative RP-HPLC. [e]The rest being DCM.

purification. The solid phase syntheses were conducted on ChemMatrix, 100% PEG-based resin from PCAS BioMatrix. The MW-assisted synthesis was performed using a Milestone Mycrosynth multimode labstation. Purities were determined by analytical RP-HPLC performed on a C18 column, 4.6 μm particle size, 100 Å pore diameter, 250 μm, DAD 210 nm, from a 9:1 H_2O/CH_3CN to a 2:8 H_2O/CH_3CN in 20 min, (for the analysis of EM-1: from a 9:1 H_2O/CH_3CN/0.1% TFA to 2:8 H_2O/CH_3CN/0.1% TFA) at a flow rate of 1.0 mL/min, followed by 10 min at the same composition. Semipreparative RP-HPLC was performed on a C18 column (7 μm particle size, 21.2 mm ×150 mm, from 8:2 H_2O/CH_3CN/0.1% TFA to 100% CH_3CN/0.1% TFA in 10 min) at a flow rate of 12 mL/min. Mass analysis was done by ESI. ^{1}H NMR spectra were recorded at 400 MHz, in 5 mm tubes, at rt. Chemical shifts are reported as δ values relative to the solvent peak.

Synthesis of NCAs. A limp mixture of triphosgene (0.35 mmol) and the amino acid (1.0 mmol) was mixed under MW irradiation in an open vessel equipped with a refrigerator under a fume hood, while gently blowing with nitrogen. The microwave-assisted reaction was performed keeping irradiation power fixed at 150W and monitoring the internal reaction temperature at 80 °C with a built-in ATC-FO advanced fiber optic automatic temperature control. After 20 min, the mixture was cooled at rt, and the resulting NCA (90−95%, 80−85% pure by analytical RP-HPLC) was used as a waxy solid without further purification.

Procedure for Synthesis of ProNCA. Proline (1.0 mmol) was treated with triphosgene as described above. After 20 min, the mixture was cooled at 0 °C, TEA (1.0 mmol) was added, and the limp mixture was mixed for additional 30 min. Crude ProNCA (90%, 70% pure by analytical RP-HPLC) was used without further purification.

SP Synthesis of EM-1. *NCA Coupling.* The PEG resin or PEG resin peptide (0.3 mmol in free amino group) was swollen with borate buffer pH 10.2 (4.0 mL). The NCA (0.9 mmol) was added at 5 °C, and the suspension was mechanically shaken for 3 h. Then the mixture was filtered, and the resin was washed three times with citrate buffer pH 3.0, and finally with borate buffer pH 10.2.

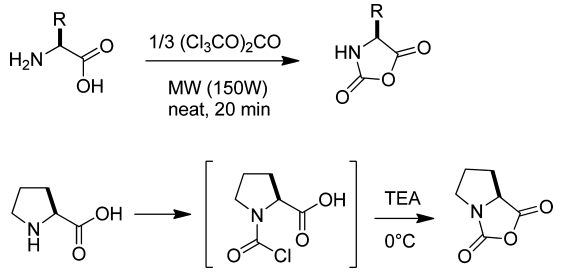
Cleavage Procedure. The resin peptide was suspended in a mixture of TFA and scavengers (5 mL, Table 1) and mechanically shaken at rt. After 2 h, the mixture was filtered, the resin was washed twice with 10% TFA in Et_2O (5 mL), and the collected filtrates were poured into 50 mL of ice-cold Et_2O. The precipitate was filtered, and the crude peptide TFA salt was recrystallized from EtOH/Et_2O and collected by centrifugation (65−73% pure by analytical RP-HPLC, Table 1). The peptide was isolated after semipreparative RP-HPLC (52−68% yield, 95−97% pure by analytical RP-HPLC, Table 1).

RESULTS AND DISCUSSION

In the last years, we have been involved in the synthesis and investigation of EM-1 and mimetics as potential remedies for pain relief devoid of unwanted side effects.[29,30] Because we became aware of the environmental impact of the classic syntheses, we faced the opportunity to develop a more sustainable procedure. We designed a very simple SP methodology in water, using PEG resins for the preparation of peptide amides and the NCAs of the amino acids Tyr, Pro, Trp, and Phe.

These NCAs were prepared in almost quantitative yields from the unprotected amino acids and one-third of triphosgene (Scheme 1). Though still hazardous (GHS05, GHS06), triphosgene can be used as a safe-to-handle phosgene

Scheme 1. MW-Assisted Synthesis of NCAs



substitute, and in recent years emerged as an important green chemical.[31,32] The classic procedure reported in the literature[33] was modified in that the condensation was performed under solvent-free conditions and MW irradiation. In the case of Pro, the *N*-carbamoyl intermediate did not cyclize spontaneously, and the use of a non-nucleophilic base was required; eventually, the use of polymer-supported bases have been also proposed.[34] The resulting NCAs were checked by ^{1}H NMR and ESI-MS, and the spectra are reported in the Supporting Information. Purities (70−85%) were determined by RP-HPLC. The analyses of the byproducts were compatible with the presence of amino acid salts, isocyanatoacyl chlorides, *N*-chloroformyl amino acids,[35] and for Pro triethylammonium chloride. Polymerization at this stage was excluded on the basis of the HPLC-ESI analyses. Interestingly, the crude NCAs were utilized without purification, a significant advantage consented by the SP approach.

The NCAs are prone to polymerization; indeed, poly amino acids can be conveniently synthesized from NCAs.[36] As a consequence, we operated under carefully controlled conditions; the key factor is the relative stability of the intermediate carbamic acid in an aqueous environment at a basic pH that prevents the formation of the free aminopeptide while the NCA is still present in the reaction mixture (Scheme 2).

The PEG-based resins are highly chemically stable and allow the use of almost any kind of solvent, including water.[16,37] PEG has a very low toxicity and is used in a variety of biocompatible products.[38] Three different peptide ChemMatrix amide resins were tested: H-Rink amide, H-PAL, and H-Ramage (Table 1). A suspension of the amino-free resin in borate buffer pH 10.2 was treated with a 3 equiv. of PheNCA, and the suspension was mechanically shaken at 5 °C. After 3 h, the resin was filtered and washed three times with citrate buffer pH 3.0 rapidly to avoid peptide cleavage.

The remaining NCAs were added in turn under the same conditions. The cleavage of the peptide amide from the resin was performed with TFA in the presence of scavengers. The crude peptides were analyzed by reversed phase HPLC and

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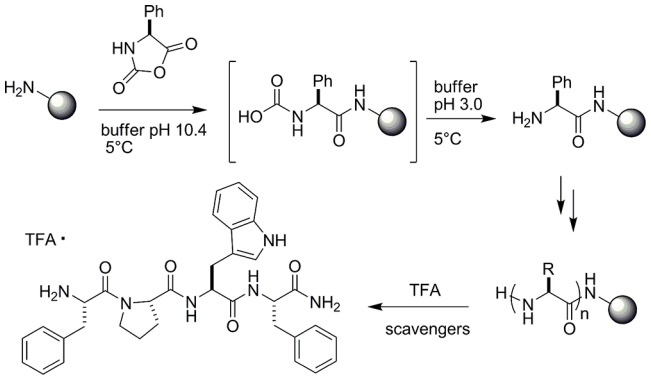
Scheme 2. SPPS of EM-1 in Aqueous Medium by Coupling NCAs on a PEG Peptide Amide Resin



ESI. The analyses were compatible with the presence of traces of peptide byproducts, in particular failure sequences, and sequences with duplicate amino acids, possibly arising from NCA polymerization during the coupling step, and excluded racemization.

Final purification was performed by semipreparative reversed phase HPLC, which afforded EM-1, ≥ 95% pure; EM-1 was identified by ESI-MS, ^{1}H NMR, and gCOSY spectroscopy (Supporting Information). The ^{1}H NMR spectra of the isolated peptides excluded the presence of epimers.

Figure 1 shows the chromatogram of the preparation performed with the H-Ramage resin (Table 1, entry 3). This

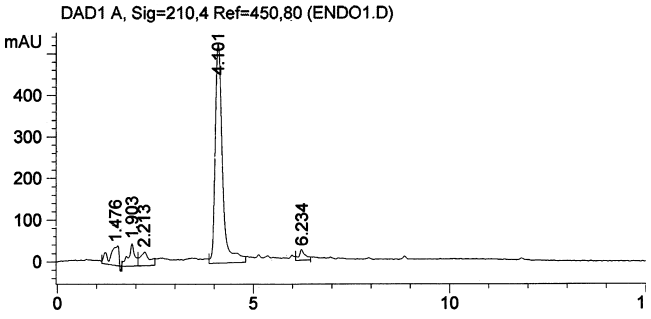


Figure 1. RP-HPLC analysis of crude EM-1 as obtained from entry 3 of Table 1.

afforded the highest purity but a slightly reduced yield, possibly due to the comparatively lower stability in the decarboxylation step (Scheme 2).

From the general point of view of sustainability, the proposed synthetic protocol gives some advantages with respect to the standard SP approach. The entire protocol was conducted in the almost complete absence of organic solvents. The solvent-free preparation of the NCAs required only the amino acids and one-third of triphosgene, apart from Pro which required 1 equiv. of TEA. Swelling and coupling were conducted in a borate buffer, and the washes were performed with citrate buffer. The high reactivity of the NCAs allowed using a reasonable excess of 3 equiv. for the couplings, comparable or inferior to classic SP synthesis.[8,16,37] The cleavage step was done as usual with TFA and scavengers. EtOH, Et_2O, and CH_3CN were utilized in the conclusive stages to recover and isolate the peptides.

CONCLUSIONS

A short peptide amide was synthesized in SP and in aqueous conditions by employing a PEG resin and NCAs as activated/protected derivatives of the amino acids. These NCAs have been prepared with triphosgene under MW irradiation, a very convenient method also for its efficient energy use. EM-1 was obtained with moderate yield and purity; so that for the moment, the method seems less competitive than the classic procedures for longer sequences. Nevertheless, the entire protocol is attractive for its environmental sustainability. Apart from the amino acids and some reagents used occasionally, the only bulk chemical was triphosgene, a relatively safe reagent for which danger and toxicity is justified by excellent atom economy and the absence of wastes of the overall protocol, including organic solvents.

ASSOCIATED CONTENT

Supporting Information

^{1}H NMR and ESI-MS of the NCAs and of EM-1, and references. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Author Contributions

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

SP, solid phase; PEG, polyethylene glycol; NCA, *N*-carboxyanhydride; PG, protecting group; TFA, trifluoroacetic acid; RP, reversed phase; MW, microwave; EM-1, endomorphin-1; gCOSY, gradient correlation spectroscopy

REFERENCES

(1) Hruby, V. J. Designing peptide receptor agonists and antagonists. *Nat. Rev. Drug Discovery* **2002**, *1* (11), 847−858.

(2) Gentilucci, L.; Tolomelli, A.; Squassabia, F. Peptides and peptidomimetics in medicine, surgery and biotechnology. *Curr. Med. Chem.* **2006**, *13* (20), 2449−2466.

(3) Zhang, L.; Falla, T. J. Cosmeceuticals and peptides. *Clin. Dermatol.* **2009**, *27* (5), 485−494.

(4) Stevenson, C. L. Advances in peptide pharmaceuticals. *Curr. Pharm. Biotechnol.* **2009**, *10* (1), 122−137.

(5) Scott, E.; Peter, F.; Sanders, J. Biomass in the manufacture of industrial products-the use of proteins and amino acids. *Appl. Microbiol. Biotechnol.* **2007**, *75* (4), 751−762.

(6) Williams, P. J. le B.; Laurens, L. M. L. Microalgae as biodiesel & biomass feedstocks: Review & analysis of the biochemistry, energetics & economics. *Energy Environ. Sci.* **2010**, *3*, 554−590.
(7) Lammens, T. M.; Franssen, M. C. R.; Scott, E. L.; Sanders, J. P. M. Availability of protein-derived amino acids as feedstock for the production of bio-based chemicals. *Biomass Bioenergy* **2012**, *44*, 168−181.
(8) Goodman, M., Felix, A., Moroder, L., Toniolo, C., Eds.; Synthesis of Peptides and Peptidomimetics; Thieme: Stuttgart, 2002; Vol. *E22*.
(9) Anastas, P. T., Williamson, T. C., Eds.; Green Chemistry: Frontiers in Benign Chemical Syntheses and Processes; Oxford University Press: New York, 1999.
(10) Dunn, P. J., Wells, A. S., Williams, M. T., Eds.; Green Chemistry in the Pharmaceutical industry; Wiley-VCH Verlag GmbH & Co.: Weinheim, 2010.
(11) Silpi, D.; Abha, S.; Torok, M. Steps toward green peptide synthesis. *Curr. Org. Synth.* **2011**, *8* (2), 262−280.
(12) Declerck, V.; Nun, P.; Martinez, J.; Lamaty, L. Solvent-free synthesis of peptides. *Angew. Chem., Int. Ed.* **2009**, *48* (49), 9318−9321.
(13) Štrukil, V.; Bartolec, B.; Portada, T.; Đilović, I.; Halaszc, I.; Margetić, D. One-pot mechanosynthesis of aromatic amides and dipeptides from carboxylic acids and amines. *Chem. Commun.* **2012**, *48*, 12100−12102.
(14) Bonnamour, J.; Métro, T.-X.; Martinez, J.; Lamaty, F. Environmentally benign peptide synthesis using liquid-assisted ballmilling: Application to the synthesis of Leu-enkephalin. *Green Chem.* **2013**, DOI: 10.1039/C3GC40302E.
(15) Hojo, K.; Ichikawa, H.; Onishi, M.; Fukumori, Y.; Kawasaki, K. Peptide synthesis 'in water' by a solution-phase method using water-dispersible nanoparticle Boc-amino acid. *J. Pept. Sci.* **2011**, *17* (7), 487−492 and references herein..
(16) Athanassios, S.; Albericio, F.; Grøtli, M. Solid-phase peptide synthesis in water using microwave-assisted heating. *Org. Lett.* **2009**, *11* (20), 4488−4491.
(17) Hojo, K.; Maeda, M.; Tanakamaru, N.; Mochida, K.; Kawasaki, K. Solid phase peptide synthesis in water VI: Evaluation of water-soluble coupling reagents for solid phase peptide synthesis in aqueous media. *Protein Pept. Lett.* **2006**, *13* (2), 189−192 and references herein..
(18) Schellenberger, V.; Jakubke, H. D. Protease-catalyzed kinetically controlled peptide synthesis. *Angew. Chem., Int. Ed. Engl.* **1991**, *30* (11), 1437−1449.
(19) Wong, C. H. Enzymatic catalysts in organic synthesis. *Science* **1989**, *244* (4909), 1145−1152.
(20) Góngora-Benítez, M.; Basso, A.; Bruckdorfer, T.; Royo, M.; Tulla-Puche, J.; Albericio, F. Eco-friendly combination of the immobilized PGA enzyme and the S-Phacm protecting group for the synthesis of Cys-containing peptides. *Chem.—Eur. J.* **2012**, *18* (50), 16166−16176.
(21) Katritzky, A. R.; Zhang, Y.; Singh, S. K. Efficient conversion of carboxylic acids into N-acylbenzotriazoles. *Synthesis* **2003**, *48* (18), 2795−2798.
(22) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. Synthesis of proteins by native chemical ligation. *Science* **1994**, *266* (5186), 776−779.
(23) Johnson, E. C. B.; Durek, T.; Kent, S. B. H. Total chemical synthesis, folding, and assay of a small protein on a water-compatible solid support. *Angew. Chem., Int. Ed.* **2006**, *45* (20), 3283−3287.
(24) Denkewalter, R. G.; Schwam, H.; Strachan, R. G.; Beesley, T. E.; veber, D. F.; Schoenwaldt, E. F.; Barkemeyer, H.; Paleveda, W. J., Jr.; Jacob, T. A.; Hirschmann, R. The controlled synthesis of peptides in aqueous medium. I. The use of α-amino acid *N*-carboxyanhydrides. *J. Am. Chem. Soc.* **1966**, *83* (11), 3163−3164.
(25) Montalbetti, C. A. G. N.; Falque, V. Amide bond formation and peptide coupling. *Tetrahedron* **2005**, *61* (5), 10827−10852.
(26) Spina, R.; Colacino, E.; Gabriele, B.; Salerno, G.; Martinez, J.; Lamaty, F. Synthesis of pyrrolin-4-ones by Pt-catalyzed cyclo-isomerization in PEG under microwaves. *J. Org. Chem.* **2013**, *78* (6), 2698−2702.
(27) Zadina, J. E.; Hackler, L.; Lin-Jun, G.; Kastin, A. J. A potent and selective endogenous agonist for the μ-opiate receptor. *Nature* **1997**, *386* (6624), 499−502.
(28) Keresztes, A.; Borics, A.; Tóth, G. Recent advances in endomorphin engineering. *Chem. Med. Chem.* **2010**, *5* (8), 1176−1196.
(29) Gentilucci, L. Addressing the Interactions between Opioid Ligands and Their Receptors. Classic and Nonclassic Cases. In *Neuropeptides in Neuroprotection and Neuroregeneration*; Nyberg, F., Ed.; CRC Press: Boca Raton, FL, 2012; pp 229−252.
(30) De Marco, R.; Tolomelli, A.; Spampinato, S.; Bedini, A.; Gentilucci, L. Opioid activity profiles of oversimplified peptides lacking in the protonable *N*-terminus. *J. Med. Chem.* **2012**, *55* (22), 10292−10296.
(31) Trotzki, R.; Nüchter, M.; Ondruschka, B. Microwave assisted phosgenation-alcoholysis using triphosgene. *Green Chem.* **2003**, *5* (3), 285−290.
(32) Fuse, S.; Tanabe, N.; Takahashi, T. Continuous in situ generation and reaction of phosgene in a microflow system. *Chem. Commun.* **2011**, *47* (44), 12661−12663.
(33) Wilder, R.; Mobashery, S. The use of triphosgene in preparation of *N*-carboxy α-amino acid anhydrides. *J. Org. Chem.* **1992**, *57* (9), 2755−2756.
(34) Gulin, P. O.; Rabanal, F.; Giralt, E. Efficient preparation of proline N-carboxyanhydride using polymer-supported bases. *Org. Lett.* **2006**, *8* (23), 5385−5388.
(35) Kramer, J. R.; Deming, T. J. General method for purification of α-amino acid-*N*-carboxyanhydrides using flash chromatography. *Biomacromolecules* **2010**, *11* (12), 3668−3672.
(36) Palacios, P.; Bussat, P.; Bichon, D. Synthesis of a thiol-terminated poly(L-glutamic acid) by polymerization of N-carboxyanhydrides on solid supports. *Angew. Chem., Int. Ed.* **1991**, *193* (1), 77−88.
(37) García-Ramos, Y.; Paradís-Bas, M.; Tulla-Puche, J.; Albericio, F. ChemMatrix® for complex peptides and combinatorial chemistry. *J. Pept. Sci.* **2010**, *16* (12), 675−678.
(38) Webster, R.; Elliott, V.; Park, B. K.; Walker, D.; Hankin, M.; Taupin, P. PEG and PEG Conjugates toxicity: Towards an Understanding of the Toxicity of PEG and Its Relevance to PEGylated Biologicals. In *PEGylated Protein Drugs: Basic Science and Clinical Applications. Milestones in Drug Therapy*; Veronese, F. M., Ed.; Birkhäuser: Basel, Switzerland, 2009.