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NF-31 color test uncovers 'hidden' alcohol functionalities in PEG-based resins for solid phase peptide synthesis

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ABSTRACT

Recently developed PEG-based resins have been shown to markedly improve the quality of the synthesis in case of the so-called 'difficult' peptide sequences. Difficult coupling reactions further require sensitive color tests for amines in the assessment of the completeness of coupling. We here describe how the use of PEG-based resins in combination with one of the more sensitive color tests in SPPS can lead to severe misinterpretation and unnecessary delays during solid phase peptide synthesis due to the presence of 'non-declared' free hydroxyl functionalities in aminomethyl-PEG-based resins.

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1. Introduction

Solid phase synthesis of highly hydrophobic peptide sequences is often problematic due to intramolecular aggregation of the peptide and hydrophobic interactions involving the solid support. Aggregation phenomena can have a severe negative influence on the reactivity of the free N-terminus, leading in subsequent reactions to deletion sequences and eventually premature termination.[1] The exact chemical composition of the resin material plays a major role and interaction with the solid support depends strongly on its hydrophobicity. With this in mind, the development of new and more performant solid supports has been and still is an active field of research. People have actively sought for solutions by the development of new advanced solid supports which consist of 100% polyethyleneglycol (PEG), like the example depicted in Figure 1. This type of resin is less hydrophobic than the traditional polystyrene (PS) and PS-based resins. Therefore the PEG-resins allow a better solvation of the growing peptide chains, making them perfectly suited for high purity synthesis of difficult hydrophobic, highly structured and also poly-arginine sequences.[1–4] These resins have good swelling properties in about any solvent. This springs from the vicinal arrangement of carbon oxygen bonds throughout the chain in the unique structure of PEG. It assumes helical structures with gauche interactions between the polarized bonds. Three helical arrangements of PEG featuring a low energy conformation exist. The first exposes the oxygen atoms to the environment and is thus quite hydrophilic. In the second low energy conformation the oxygen atoms are oriented towards the interior of the helix, which is therefore largely hydrophobic. The third possesses an intermediate polarity. For this reason PEG displays an amphipathic nature and swells in both polar and non polar solvents.[5] PEG-resins are also thermally, mechanically and chemically very stable as they are exclusively built up from primary ether bonds. Moreover, they are easy to handle in the dry state, thus facilitating weighing and transfer.[4]

The synthesis of peptides on solid phase could only be conveniently and generally applied with the development of colorimetric tests. These allow monitoring the coupling efficiency of amino acid coupling, without the need for cleavage from the solid phase.[8,9] Next to the widely used Kaiser[10] and TNBS[11] tests our group made a contribution in this area with the development of *p*-nitrophenylester 1 (see Fig. 2), which allows reliable monitoring of coupling reactions even with very sterically hindered primary and secondary amines.[12,13]

Combining the accuracy of sensitive color tests with the improved properties of new types of resins offers a way to improve the quality of difficult and not well-behaved peptide sequences. Clearly, next to the importance of the specific and optimized

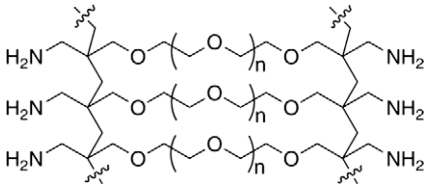

Figure 1. General representation of NovaPEG/ChemMatrix® resin structure: 100% cross-linked PEG.[4,6,7]

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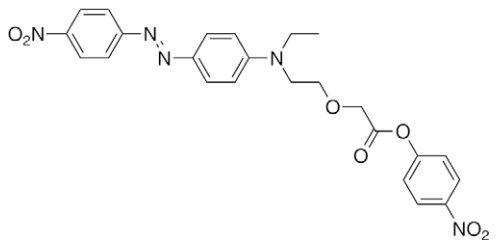


Figure 2. Structure of *p*-nitrophenylester NF31.

structure of the base polymer for improved properties of SPPS beads, the presence of a specific functionality on the resin is important for attachment of the desired sequence. Amine groups are generally used for this purpose, leading to stable amide bonds, but allowing for cleavage at the end of the synthesis by the introduction of suitable linker moieties. However it is important to be aware of residual functionality present on the beads, not deliberately introduced for attachment purposes but rather present as a result of the specific polymerization strategy used for construction of the resin. We here report on the presence of 'non-declared', residual, free hydroxyl groups in solid supports consisting of 100% PEG leading to background coloration with the sensitive NF-31 test.

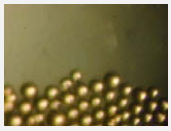
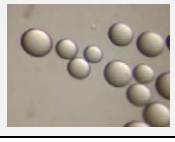
2. Results and discussion

In the course of our recent endeavors in the synthesis of large dipodal peptide constructs featuring long chains of more than 20 amino acids, we were faced with severe problems in achieving the desired purity. As described above, recent papers report on the exclusive capacities of PEG-based resins in 'difficult' peptide sequences.[1–4] This prompted us to turn our attention to this novel type of resins. Before embarking on the synthesis of the actually desired large peptide constructs, a series of test reactions was run to verify and establish suitable basic peptide synthesis conditions on this new type of resin. During the simple synthesis of a small peptide, when using NovaPEG, a 100% PEG-based resin from Novabiochem, TNBS tests were negative. But to our great surprise, our regularly applied NF-31 color test never indicated complete coupling. Despite prolonged reaction times or multiple couplings, red coloration of beads was continuously observed, pointing towards the presence of unreacted functionalities. Using identical conditions on TentaGel, a PS-based resin, color tests indicated complete couplings (see Table 1).

In view of the positive reports on the performance of these PEG-based resins, it was considered highly unlikely that it was impossible to obtain 100% efficiency on this novel resin during simple coupling reactions. Based on the structure of the resin, the presence of free hydroxyl groups was considered to offer a possible explanation for the unexpected positive color tests. Containing a reactive *p*-nitrophenyl ester functionality, NF-31 has shown to also allow for the detection of alcohols provided slightly different test conditions are applied.[14] Using these conditions, more intense red beads were obtained (see Table 1), as would be expected if hydroxyl groups were present. To further test this hypothesis, TNBS and NF-31 tests were carried out on the different types of resin, after applying a capping procedure with acetic acid anhydride, as illustrated in Table 2. To obtain a complete reaction of all present functionalities and associated disappearance of the red color, a longer reaction time was required than used in the standard protocol for the capping of amines, hinting toward the presence of another less nucleophilic functionality. In turn, after treatment of the resin with an aqueous 0.1 M NaOH solution, the originally observed red color reappeared. In view of the inherent difference in base sensitivity of an amide versus an ester bond, this experiment confirms the presence of free hydroxyl groups on the PEG-based resin.

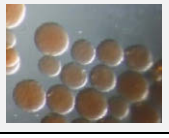
The specific and beneficial characteristics of the NF 31 test, especially its increased performance and sensitivity for secondary, sterically hindered and aromatic amine functionalities (as compared to more classical TNBS or Kaiser test) have initiated its use in a variety of cases where 'difficult' peptide sequences are desired.[15–17] Furthermore, in view of the growing importance of the incorporation of unnatural (and often difficult to couple) building blocks in peptide chains and the often improved results obtained when using the novel PEG-based resins, one should be aware that the combination of this novel well-performing resins with OH-sensitive color tests can give rise to false positive results, impeding a correct interpretation of coupling progress and leading to the entirely unnecessary application of double couplings and concurrent waste of precious building blocks. The presence of free hydroxyl groups can further also interfere in other reactions. When determining coupling efficiencies by UV absorbance of the piperidinebenzofulvene adduct[18] for example, amino acids coupled on the free hydroxyls and their subsequent Fmoc deprotection with piperidine, will give rise to wrong results.

Table 1
Color tests on resin after coupling reaction with *N*-Fmoc-glycine[a]

Resin	TNBS-test	NF-31-test	NF-31 + DMAP
Tenta-Gel			
Nova-PEG			
Wang[b]			

[a] *N*-Fmoc-glycine (4 equiv) was coupled with PyBOP (4 equiv) and DIPEA (8 equiv) for two times 2 h in DMF.
[b] No coupling reaction was performed on the Wang resin, the color tests were performed on the unmodified resin.

Table 2
Color tests after capping with acetic acid anhydride

Entry		TNBS-test	NF-31-test
1	30 min capping		
2	4 h capping		
3	After NaOH wash		

For the capping procedure: 6 equiv acetic acid anhydride and 6 equiv DIPEA were added to the solid support in DMF. For the washing procedure: the solid support was washed four times with 0.1 M NaOH.

Since schematic and graphical representations of aminomethylated NovaPeg and ChemMatrix resins in catalogs[6] and papers[4,7] describing the benefits of their use in difficult peptide synthesis, often omit to depict residual free hydroxyl functionalities (see Fig. 1), it is important to realize that they are present and might interfere with some of the processes carried out on the resin.

3. Conclusion

In conclusion, we have been able to understand and show why the application of the highly sensitive NF-31 test during SPPS on novel PEG-based resins (sold under popular brand names as NovaPEG or ChemMatrix®) gives rise to difficult interpretation of coupling progress. We have demonstrated that the presence of remaining free hydroxyl functionality leads to misleading background coloration of the resin when checking reaction progress.

Although the presence of free hydroxyl groups does not necessarily lead to associated problems during SPPS, one should be careful when following reactions using sensitive color tests as to avoid unnecessary application of multiple coupling procedures or use of increased excesses of precious reagents.

4. Experimental

4.1. Materials

NovaPEG Rink Amide resin LL, Wang resin, Fmoc-Gly-OH, and PyBOP were purchased from Novabiochem; dry dimethylformamide (DMF) and acetic anhydride from Acros Organics; diisopropylethylamine (DIPEA), MeCN, dichloromethane (DCM), and MeOH from Sigma–Aldrich; 2,4,6-trinitrobenzenesulphonic acid (TNBS, purum 1% in DMF) from Fluka and DMF from Biosolve. NF-31 was homemade.

4.2. Reactions

Prior to reaction dry resins are allowed to swell for minimum 15 min by suspending in an adequate volume (1.5 ml/mg) of DMF with occasional swirling.

For the coupling reaction 4 equiv of *N*-Fmoc-glycine (297.3 g/mol) and 8 equiv of DIPEA (129.3 g/mol, 0.782 g/ml) were added to the resin which was suspended in half of the total volume of DMF (1.5 ml/mg). After 15 min, 4 equiv of PyBOP (520.4 g/mol) was added together with the remaining necessary volume of DMF. The mixture was flushed with argon and vortexed at rt. This procedure was repeated twice for 2 h.

In the standard capping procedure, acetic acid anhydride (6 equiv) and DIPEA (6 equiv) were added to the resin in DMF, which was vortexed for 30 min at rt. To obtain complete capping of the hydroxyl groups, reaction time was prolonged to 4 h. To cleave formed ester bonds, the resin was washed four times for 20 s with an aqueous 0.1 M NaOH solution.

After each reaction, excess reagent was filtered off using vacuum. The resin was then thoroughly washed with DMF (4×), MeOH (4×), and DCM (4×). About 1 mL was added to the resin, which was shaken for 20 s and filtered.

4.3. Color tests

TNBS[11]: Few beads were transferred into a small glass tube and three drops of both 1% TNBS in DMF and 10% DIPEA in DMF were added. The color of the beads was immediately observed. Red beads indicate the presence of free amine functions while colorless beads indicate their absence.

NF-31[12]: Few beads were transferred into a small glass tube and 100 μL of a 2 mM solution of NF-31 in MeCN was added. The suspension was heated at 70° for 10 min. The excess dye was washed away with DMF, MeOH, and DCM. After washing, red beads indicate the presence of free amine functions, colorless beads the absence.

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