# New cellulose membranes for the *in situ* synthesis of peptide arrays with improved stability towards acid and aqueous base

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## Abstract

We now disclose a series of new amino-PEG functionalised cellulose membranes with improved properties in chemical synthesis and biological assays. The new membranes are stable to strong aqueous base and neat TFA for days. Due to the high acid stability more efficient side chain cleavage protocols were applied which yielded peptides of improved purity and allowed for the use of special amino acid building blocks and side chain protecting groups such as phosphoamino acids. The drastically improved stability of the anchor to the cellulose against aqueous bases prevents the loss of membrane bound peptides in assays at pH higher than 7. Additionally, for the first time thicker amino-PEG functionalised cellulose membranes with a loading as high as 5 µmol/cm² became accessible and were used in the manual SPOT synthesis of soluble peptides in larger quantities.

## Introduction

SPOT-Synthesis is a well established and widely used method for parallel synthesis and biological screening of peptides and other compounds in an array format on membrane supports.[1] Cellulose is still the major membrane material used, primarily because of its excellent compatibility with biological assay conditions. The esterification of free hydroxy functions of the cellulose fibres with an Fmoc amino acid is a convenient method to introduce a free amino function for the SPPS of peptide arrays.[2]

On the other hand however, cellulose and cellulose membranes show only a limited acid stability which severely restricts side chain deprotection conditions that can be applied, even for the most stable commercially available material. We recommend 50% TFA in DCM for 2 x 1 h for this type of membrane. This limitation can result in incomplete deprotection. A further drawback of ester bound peptides is the lability towards aqueous base not uncommon for bio-assay and stripping conditions. The ester bond is rapidly cleaved and the peptides are removed from the membrane. Membranes with a ß-alanine linkage are readily hydrolysed at pH 9 or higher (Fig. 1). Cellulose synthesis membranes with a base resistant linkage were reported, introducing the amino functionality to the membrane via an ether bond in an acid-catalysed reaction.[3,4] These not commercially available membranes were successfully used for the synthesis of small peptides and organic molecules but no data concerning their stability to acids or aqueous base were presented.

We now disclose a series of new amino-PEG functionalised cellulose membranes with improved properties in chemical synthesis and biological assays.

## Results and Discussion

### Stability towards aqueous base

We developed a mild proprietary derivatisation chemistry for the amination of membranes via a PEG-spacer. The mechanical stability of the new AC-S membranes was not effected at all by this procedure. The new AC-S membranes showed a drastically improved stability against hydrolysis when compared to the standard cellulose membrane, esterified with Fmoc-ß-alanine. Fig. 1 shows the residual amino functions on both types of membranes after treatment with phosphate buffer of pH 9 or pH 10 respectively, quantified with bromophenol blue.[5] While the ß-alanine membranes were hydrolysed almost completely, the new AC-S membranes showed no decrease of amino-functionalisation at both pH. No hydrolysis was observed even after treatment with conc. ammonia over night at room temperature (data not shown).

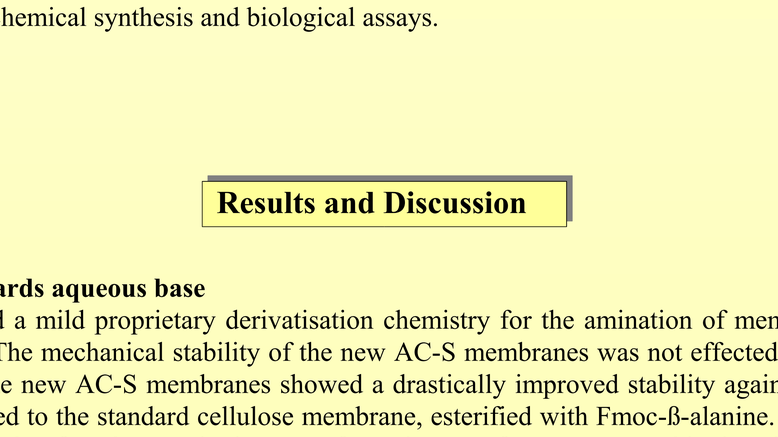


**Fig.1:** Hydrolysis of AC-S and ß-alanine modified membranes (starting functionality set to 100%)

### Acid-Stability

Next, we examined the acid stability of the AC-S membrane to neat TFA and compared it to ß-alanine esterified membranes. The results of a 24 h treatment at room temperature are summarized in Fig. 2. The cellulose membrane, which was used as starting material for both types of amino-functionalisation, was completely dissolved (Fig. 2a). The "ß-Ala"-membrane was only marginally more stable (Fig. 2b). Mechanical stress like shaking or manipulation with tweezers resulted in the membrane falling to pieces. In contrast, the new standard AC-S membrane was unaltered by this treatment (Fig. 2c). The improved manufacturing procedure also made high-load (Fig. 2d) and ultra high-load membranes (Fig. 2d) with high mechanical stability after TFA treatment accessible.


**Fig. 2**: Cellulose membranes in neat TFA after 24 h: a) Starting cellulose membrane; b) "ß-Ala" membrane; c) AC-S membrane (0.4 µmol/cm²); d) AC-L membrane (2 µmol/cm²); e) AC-X membrane (5 µmol/cm²).

### Peptide synthesis on the new membranes

To prove proper synthesis performance with the new membrane, a phosphorylated nonapeptide was assembled on the HMBA-Linker. The phosphotyrosine residue was introduced as Fmoc-Tyr ($PO(NMe_2)_2$)-OH.[6] Side-chain deprotection required over-night treatment with 92% TFA. The main product was the expected phosphopeptide as shown by HPLC and MALDI-MS analysis (Fig. 3).

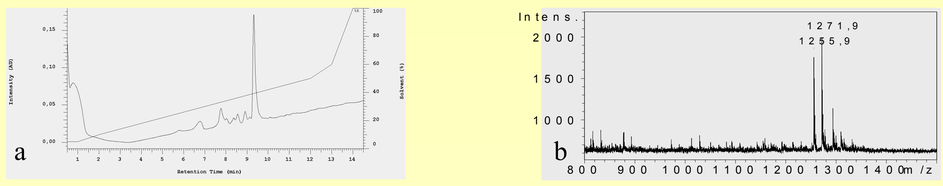


**Fig. 3**: Phosphopeptide synthesis of crude Ac-YV**Y***IDPTQL-OH: side chain deprotection with 92% TFA , 3% TIBS and 5% water, 16 h; cleavage from solid support with $NEt_3$/THF/water 1/3/6, 16 h; a) HPLC analysis; b) MALDI analysis$[M+Na]^+$= 1256.3

The area specific loading with free amino groups of the new AC-S membrane is 0.4 µmol/cm². This is more than sufficient for most of the applications of immobilized peptide arrays. A higher loading is beneficial only for the parallel synthesis of soluble peptides as a higher amount of peptide can be synthesised per area. To examine the performance of the new high load membranes in peptide synthesis the N-terminally bromo-acetylated peptide BrAc-NYGKYE-$NH_2$ was assembled manually with the SPOT method on an AC-L membrane. The array was formed by spotting the Fmoc protected Rink linker. In each cycle 1 µl of activated amino acid solution in NMP was spotted 10 times. After N-terminally bromoacetylation, the efficiency of several different deprotection method with increasingly harsher TFA treatment was examined. The results are summarized in Fig.4 and clearly demonstrate the need for acid stable membranes to ensure complete deprotection particularly for immobilized arrays. One spot of 0.5 cm² yielded 350 nmol pure soluble peptide as determined by amino acid analysis.

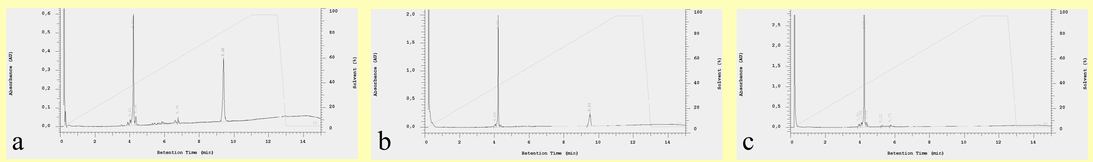


**Fig. 4**: HPLC analysis of a differently deprotected hexapetide: a) 50% TFA, 3% TIBS, 2% water, 45% DCM, 2 h; b) 92% TFA , 3% TIBS, 5% water, 2 h; c) 92% TFA , 3% TIBS and 5% water, 16 h

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