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# CHAPTER 7

## Appendix

### 7.1. General Study Materials

Initially based on work performed by Dr. Shaw and an extensive literature search five GAS antigens (M1, MalE, SpyCEP, SpyAD, Shr) were selected for study as potential carriers for GAC. Based on recombinant protein yields, expression, localisation, and favourable immunogenic properties, three were selected for further investigation. These proteins were SpyAD and SpyCEP based on their favourable immunogenic properties included in the GSK combo preclinical GAS vaccine<sup>395</sup> and MalE as a novel protein to study.

#### 7.1.1. *GAS Protein Antigens – Nucleic Acid and Amino Acid Sequences – Chemical Conjugation*

##### **SpyCEP**

###### ➤ Nucleic Acid

```
GCGCCATGGGGCGATGAGCTAAGCACAATGAGCGAACCAACAATCACGAATCACGCTCAACAACAAGCGCAA
CATCTCACCAATACAGAGTTGAGCTCAGCTGAATCAAATCTCAAGACACATCACAATCACTCTCAAGACAAAT
CGTGAAAAAGAGCAATCACAAGATCTAGTCTCTGAGCCAACCACAACCTGAGCTAGCTGACACAGATGCAGCAT
CAATGGCTAATACAGGTTCTGATGCGACTCAAAAAAGCGCTTCTTTACCGCCAGTCAATACAGATGTTCCAGAT
TGGGTAAAAACCAAAGGAGCTTGGGACAAGGGATACAAAGGACAAGGCAAGGTTGTCCGAGTTATTGACACAG
GGATCGATCCGGCCCATCAAAGCATGCGCATCAGTGATGTATCAACTGCTAAAGTAAAAATCAAAAAGACATG
CTAGCACGCCAAAAAGCCGCCGGTATTAATTATGGGAGTTGGATAAATGATAAAGTTGTTTTGCGACATAATTAT
GTGGAAAAATAGCGATAATATCAAAGAAAATCAATTCGAGGATTTTGTATGAGGACTGGGAAAACTTTGAGTTTGA
TGAGAGGCCAGGCCAAAAGCCATCAAAAAACACAAGATCTATCGTCCCAATCAACCCAGGCACCGAAAGAA
ACTGTTATCAAAACAGAAAGAACAGATGGTTCACATGATATTGACTGGACACAAACAGACGATGACACCAAATA
CGAGTCACACGGTATGCATGTGACAGGTATTGTAGCCGGTAATAGCAAAGAAGCCGCTGCTACTGGAGAACGC
TTTTAGGAATTGCACCAGAGGCCCAAGTCATGTTTCATGCGTGTTTTTGGCAACGACATCATGGGATCAGCTGA
ATCACTCTTTATCAAAGCTATCGAAGATGCCGTGGCTTTAGGAGCAGATGTGATCAACCTGAGTCTTGGAAACC
CTAATGGGGCAGCTTAGTGGCAGCAAGCCCTAATGGAAGCAATTGAAAAAGCTAAAAAGCCGGTGTATC
AGTTGTTGTAGCAGCAGGAAATGAGCGCTCTATGGATCTGACCATGATGATCCATTGGCGACAAATCCAGAC
TATGGTTTTGGTCGGTCTCCCTCAACAGGTGCAACCAACATCAGTGGCAGCTATAAACAGTAAGTGGGTGA
TTCAACGTCTAATGACGGTCAAAGAATTAGAAAACCGTGCCGATTTAAATCATGGTAAAGCCATCTATTCAGAGT
CTGTGCACTTTAAAGACATAAAAGATAGCCTAGGTTATGATAAATCGCATCAATTTGCTTATGTCAAAGAGTCAA
CTGATGCGGGTTATAACGCACAAGACGTTAAAGGTTAAATTGCTTTAATTGAACGTGATCCCAATAAAACCTAT
GACGAAATGATTGCTTTGGCTAAGAAACATGGAGCTCTGGGAGTACTTATTTTTAATAACAAGCCTGGTCAATC
AAACCGCTCAATGCGTCTAACAGCTAATGGGATGGGGATACCATCTGCTTTTCATATCGCACGAATTTGGTAAGG
CCATGTCCCAATTAATGGCAATGGTACAGGAAGTTTAGAGTTTGACAGTGTGGTCTCAAAAAGCACCAGAGTCAA
AAAGGCAATGAAATGAATCATTTTTTCAAATTGGGGCCTAACTTCTGATCTCGAGGCC
```

###### ➤ Amino Acid

```
GDELSTMSEPTITNHAQQQAQHLNTELSSAESKSQDTSQITLKTNREKEQSQDLVSEPTTTELADTDAASMANTG
SDATQKSASLPPVNTDVHDWVKTKGAWDKGYKQGKVVAVIDTGIDPAHQSMRISDVSTAKVKSKEDMLARQKAA
GINYGSWINDKVVFAHNYVENSNDNIKENQFEDFDEDWENFEFDAEAEPKAIKHKIYRPQSTQAPKETVIKTEETDG
SHDIDWTQDDDTKYESHGMHVTGIVAGNSKEAAATGERFLGIAPEAQVMFMRVVFANDIMGSAESLFKAIEDAVAL
GADVINLSLGTANGAQLSGSKPLMEAIKAKKAGVSVVVAAGNERVYGSDDHDDPLATNPDYGLVGSPTGRTPTSV
AAINSKWVIQRLMTVKELENRADLNHGKAIYSESVDFDKIDSLGYDKSHQFAYVKESTDAGYNAQDVKGKIALIERD
PNKTYDEMIALAKKHGALGLVIFNFKPGQSNRSMRLTANGMGIPSAFISHEFGKAMSQNLNGNGTGSLEFDSVVSKA
PSQKGNEMNHFSNWGLTSD
```

## MalE

### ➤ Nucleic Acid

CGGCCATGGGGCGACTCAAAGACTATTAACCTTTGGGTACCGACTGGCTCTAAAAATCATATGCAGATACCAT  
TGCAAAATTTGAAAAAGATTCTGGTTACACCGTTAAAGTGGTTGAATCAGAAGATCCAAAAGCACAAGAAAAAAT  
CAAAAAAGATGCCTCAACTGCTGCCGATGTCTTTTCACTTCCTCATGATCAACTGGTCAACTTGTAGAATCTGG  
TACTATTCAAGAAGTTCAGAAAAGTACAATAAAGAAATGCTGCTACTTCTACTGATCAGGCGCTCGTGGTG  
CACAGTACAAAGGGAAAACATATGCCTTCCATTTGGTATTGAATCACAAGTCTTTTCTACAATAAATCAAAT  
AGCTGCTGAAGACGTTACTTCATATGATACAATTACTACAAAAGCAACTTTTGGTGGTACCTTTAAACAAGCTAA  
CACTTACGCTACTGGTCCATTATTCATGTCAAGTGGTAACACTTTATTTGGGGAAAACGGTGAAGATGTAAAAG  
GTACTAACTGGGGCAATGAAAAAGGCGCTGCTGTTCTTAAATGGATCGCAGACCAAGCTTCAAATAAAGTTTT  
GTTAGCTTAGATGCTAACAAACGTTATGTCTAAATTTGGTGACGGTTCAGTAGCTTCTTTGAATCAGGACCGTG  
GGACTATGAAGCTGCTCAAAAAGCTATCGGTAAGAAAACCTTGGTGTGCTATTTATCCAAAAGTAACTATTG  
GAGGCGAAACGGTTCAACAAAAGCATTCTTGGGTGTAAAACCTTACGCAGTCAACCAAGCACCAGCTAAAGG  
TGATACAAAACGTATCGCAGCTAGCTACAATTTGGCATCTACTTACTAATGTGAAAAGCCAAGAAAACCAAT  
TCAAAACTCGTAACATTGTCCAGCCAACAAGAAGTTCAATCTCAGAAGCTGTCAATCAAACGAACTTGTAA  
AAACCGTTATCACTATGGGTTCTTCTCAGACTACACTGTGTAATGCCAAAACCTAGCCAAATGGGCACATTCT  
GGACTGAAAGTGTGCTATTCTTAGTGATGCCTTCAACGGTAAAATCAAAGAAAACGACTACCTTACTAAGTTG  
CAACAATTCGACAAAGATATCGCTGCAACAAAACCTCGAGGGC

### ➤ Amino Acid

GDSKTIKLVWPTGSKKSYADTIKFEKDSGYTVKVVSEDPKAQEKIKKDASTAADVFLPHDQLGQLVESGTIQEV  
PEKYNKEIAATSTDQALVGAQYKGYAFPFGIESQVLFYNSKSLAAEDVTSYDTITTKATFGGTFKQANTYATGPLF  
MSVGNLTFGENGEDVKGTNWGNEKGAAVLKWADQASNKGFVSLDANNVMSKFGDGSVASFESGPWDYEAQK  
AIGKENLGVAIYPKVITIGGETVQQAFLGVKLYAVNQAPAKGDTKRIAASYKLASYLTNAESQENQFKTRNIVPANKE  
VQSSEAVQSNELAKTVITMGSSSDYTVVMPKLSQMGFTWTESAAILSDAFNGKIKENDYLTKLQFQDKDIAATK

## SpyAD

### ➤ Nucleic Acid

CGGCCATGGGATAGAGCCTCAGGAGAAAAGGGCGAGTAATACTCACGACGATAGTTTACAAAAACCGAGAAA  
CAATTC AAGAGGCAAAGGCAACTATTGATGCAGTTGAAAAACTCTCAGTCAACAAAAAGCAGAAGTACAGAG  
CTTGCTACCGCTCTGACAAAAACTACTGCTGAAATCAACCCTTAAAAGAGCAGCAAGATAATGAACAAAAAGC  
TTTAACTCTGCACAAGAAATTTACTAATACTCTTGAAGTAGTGAGGAGACGCTATTAGCCCAAGGAGCCG  
AACATCAAAGAGAGTTAACAGCTACTGAAACAGAGCTTCATAATGCCTAAGCAGATCAACATTCAAAAGAGACT  
GCATTGTCAGAACAAAAAGCTAGCATTTCAGCAGAAACTACTCGGGCTCAAGATTTAGTGGAAACAAGTCAAAC  
GTCTGAACAAAATATTGCTAAGCTCAATGCTATGATTAGCAATCCTGATGCTATCACTAAAGCAGCTCAAACGG  
CTAATGATAATACAAAAGCATTAAAGCTCAGAATTGGAGAAGGCTAAAAGCTGACTTAGAAAATCAAAAAGCTAAA  
GTTAAAAAGCAATTGACTGAAGAGTTGGCAGCTCAGAAAAGCTGCTCTAGCAGAAAAAGAGGCAGAAGTATGTC  
GTCTTAAATCCTCAGCTCCGCTACTCAAGATAGCATTGTGGTAATAATACCATGAAAGCACCAGCAAGGCTAT  
CCTCTTGAAGAACTTAAAAAATTAGAAGCTAGTGGTTATATTGGATCAGCTAGTTACAATAATTATTACAAAGAG  
CATGCAGATCAAATTTGCCAAAGCTAGTCCAGGTAATCAATTAATCAATACCAAGATATTCCAGCAGATCGT  
AATCGCTTTGTTGATCCCGATAATTTGACACCAGAAGTGCAAAATGAGCTAGCGCAGTTTGCAGCTCACATGAT  
TAATAGTGAAGAAGACAATTAGGTCTACCACCAAGTTACTGTTACAGCAGGATCACAGAATTTGCAAGATTACT  
TAGTACCAGCTATAAGAAAACCTCATGGTAATAACAAGACCATCATTTGTCTACGGACAGCCAGGGGTATCAGGGC  
ATTATGGTGTGGGCTCATGATAAACTATTATTGAAGACTCTGCCGGAGCGTCAGGGCTCATTGAAATGAT  
GATAACATGTACGAGAATATCGGTGCTTTTAAAGATGTGCATACTGTGAATGGTATTAAACGTGGTATTATGAC  
AGTATCAAGTATATGCTCTTTACAGATCATTTACACGGAAATACATACGGCCATGCTATTAACCTTTTACGTGTA  
GATAACATAACCCTAATGCGCCTGTTTACCTTGGATTTTCAACCAGCAATGTAGGATCTTTGAATGAACACTTT  
GTAATGTTTCCAGAGTCTAACATTGCTAACCATCAACGCTTTAATAAGACCCCTATAAAAAGCCGTTGGAAGTACA  
AAAGATTATGCCCAAAGAGTAGGCACTGTATCTGATACTATTGCAGCGATCAAAGGAAAAGTAAAGCTCATTAGA  
AAATCGTTTGTGGCTATTTCATCAAGAAGCTGATATTATGGCAGCCCAAGCTAAAGTAAAGTCAACTTCAAGGTA  
AATTAGCAAGCACACTTAAAGCAGTCAGACAGCTTAAATCTCCAAGTGAGACAATTAATGATACTAAAGTTTCTT  
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CTAGCATCGTTGAAAGCCGCACTGCACCAGACAGAAGCCTTAGCAGAGCAAGCCGAGCCAGAGTGACAGCA  
CTGGTGGCTAAAAAGCTCATTGCAATATCTAAGGGACTTTAAATGAATCCTAACCGCCTTCAAGTGATACGT  
GAGCGCATTGATAATACTAAGCAAGATTTGGCTAAAACCTCATCTTTGTTAAATGCACAAGAAAGCTTTAGCA  
GCCTTACAAAGCTAAACAAAAGCAGTCTAGAAGCTACTATTGCTACCACAGAACACCAGTTGACTTTGCTTAAAC  
CTTAGCTAACGAAAAGGAATATCGCCACTTAGACGAAGATATAGCTACTGTGCCTGATTTGCAAGTAGCTCCAC  
CTCTTACGGGCGTAAAACCGCTATCATATAGTAAAGATAGATACTACTCCGCTTGTTCAGAAATGGTTAAAGAAA  
CGAAACAACCTATTAGAAGCTTCAGCAAGATTAGCTGTGAAAATACAAGTCTTGTAGCAGAAGCGCTTGTGGC  
CAAACCTCTGAAATGGTAGCAAGTAATGCCATTGTGTCTAAAATCACATCTTCGATTACTCAGCCCTCATCTAAG  
ACATCTTATGGCTCAGGATCTTCTACAACGAGCAATCTCATTTCTGATGTTGATGAAAGTACTCAAAGACTCGAG  
GCG

➤ Amino Acid

DRASGETKASNTHDDSLPKPETIQEAKATIDAVEKTLSSQKAELELATALTKTTAEINHLKEQQDNEQKALTSAQEIY  
TNTLASSEETLLAQGAEHQRELTATETELHNAQADQHSKETALSEQKASISAETTRAQDLVEQVKTSEQNIAKLNAMI  
SNPDAITKAAQTANDNTKALSSELEKAKADLENQKAKVKKQLTEELAAQKAALAEKAELSRLKSSAPSTQDSIVGN  
NTMKAPQGYPLEELKKLEASGYIGSASYNYYKEHADQIIAKASPGNQLNQYQDIPADRNRFVDPDNLTPVEQNELA  
QFAAHMINSVRRQLGLPPVTVTAGSQEFARLLSTSYKKTGHGTRPSFVYGGQPGVSGHYGVGPHDKTIIEDSAGASG  
LIRNDDNMYENIGAFNDVHTVNGIKRGIYDSIKYMLFTDHLHGNTYGHAINFLRVDKHNPNAPVYLGFTSNVGLSNE  
HFVMPFESNIANHQRFNKTPIKAVGSKDYAQRVGTVSDTIAAIKGVSSLENRLSAIHQEADIMAAQAKVSQLQGGK  
ASTLKQSDSLNLQVRQLNDTKGSLRTELLAAKAKQAQLEATRDQSLAKLASLKAALHQTEALAEQAAARVLTALVAKK  
AHLQYLRDFKLNPNRLQVIRERIDNTKQDLAKTTSSLLNAQEALAAQAKQSSLEATIATTEHQLTLLKTLANEKEYRH  
LDEDIATVPDLQVAPPLTGKPLSYSKIDTTPLVQEMVKETKQLLEASARLAAENTSLVAEALVGQTSSEMVASNAIVS  
KITSSITQPSKTSYSGSGSSTSNLISDVDESTQR

7.1.2. GAS Protein Antigens – Nucleic Acid and Amino Acid Sequences –

Biological Conjugation

SpyCEP

➤ Nucleic Acid

GGAATTGCTAGCGCGGCGCAGGATGAGCTAAGCACAATGAGCGAACCAACAATCACGAATCACGCTCAACAA  
CAAGCGCAACATCTACCAATACAGAGTTGAGCTCAGCTGAATCAAAAATCTCAAGACACATCACAAATCACTCT  
CAAGACAAATCGTGAAAAAGAGCAATCACAAGATCTAGTCTCTGAGCCAACCACAACCTGAGCTTGCTGACACA  
GATGCAGCATCAATGGCTAATACAGTTCTGATGCGACTCAAAAAAGCGCTTCTTTACCGCCAGTCAATACAGA  
TGTTACGATTGGGTAAAAACCAAGGAGCTTGGGACAAGGGATACAAAGGACAAGGCAAGGTTGTGCGCAGTT  
ATTGACACAGGGATCGATCCGGCCCATCAAAGCATGCGCATCAGTGATGATCAACTGCTAAAGTAAAAATCAAA  
AGAAGACATGCTTGCACGCCAAAAAGCCGGTATTAATTTATGGGAGTTGGATAAAAGTTGTTTTTCG  
CACATAATTATGTGGAAAAATAGCGATAATCAAAAGAAAATCAATTCGAGGATTTTGTAGGACTGGGAAAACT  
TTGAGTTTGTAGCAGAGGCGAGGCCAAAAAGCCATCAAAAAACACAAGATCTATCGTCCCAATCAACCCAGGC  
ACCGAAAGAACTGTTATCAAAACAGAAAGAACAGATGGTTCACATGATATTGACTGGACACAAACAGACGATG  
ACACCAAAATACGATCACACGGTATGCATGTGACAGGTATTGTAGCCGGTAATAGCAAAGAAGCCGCTGCTAC  
TGGAGAACGCTTTTTAGGAATTGCACCAGAGGCCAAAGTCATGTTTCATGCGTGTTTTTGCCAACGACATCATGG  
GATCAGCTGAATCACTCTTTATCAAAGCTATCGAAGATGCCGTGGCTTTAGGAGCAGATGTGATCAACCTGAGT  
CTTGGAAACCGCTAATGGGGCACAGCTTAGTGGCAGCAAGCCTCTAATGGAAGCAATTGAAAAAGCTAAAAAAG  
CCGGTGTATCAGTTGTTGTAGCAGCAGGAAATGAGCGCGTCTATGGATCTGACCATGATGATCCATTGGCGAC  
AAATCCAGACTATGGTTTGGTCGGTCTCCCTCAACAGGTCGAACACCAACATCAGTGGCAGCTATAAACAGTA  
AGTGGGTGATTCAACGTCTAATGACGGTCAAAGAATTAGAAAACCGTGCCGATTTAAATCATGGTAAAGCCATC  
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AAAGAGTCAACTGATCGGGTTATAACGCACAAGACGTTAAAGGTAATAATTGCTTTAATTGAACGTGATCCCAA  
TAAAACCTATGACGAAATGATTGCTTTGGCTAAGAAACATGGAGCTCTGGGAGTACTTATTTTTAATAACAAGCC  
TGGTCAATCAAACCGCTCAATGCGTCTAACAGCTAATGGGATGGGGATACCATCTGCTTTCATATCGCACGAAT  
TTGGTAAGCCATGTCCCAATTAATGGCAATGGTACAGGAAGTTTAGAGTTTGACAGTGTGGTCTCAAAAAGCA  
CCGAGTCAAAAAGGCAATGAAATGAATCATTTTTCAAATGGGGCCTAACTTCTGATCATATGTAG

➤ Amino Acid

GIASAAQDELSTMSEPTITNHAQQQAHLTNTELSSAESKSQDTSQITLKTNREKEQSQDLVSEPTTTELADTDAAS  
MANTGSDATQKSASLPPVNTDVHDWVKTGAWDKGYKQGKVVAVIDTGIDPAHQSMRISDVSTAKVKSKEMLA  
RQKAAGINYGSWINDKVVFAHNYVENSNDNIKENQFEDFEDWENFEFDAEAPKAIKHKHIYRPQSTQAPKETVIKT  
EETDGSHDIDWTQDDDTKYESHGMHVTGIVAGNSKEAAATGERFLGIAPEAQVMFMRVFANDIMGSAESLFKAI  
DAVALGADVNLSTGTANGAQLSGSKPLMEIAIEKAKKAGVSVVVAAGNERVYGSDDHPLATNPDYGLVGPSTGR  
TPTSVAAINSKWVIQRLMTVKELNDRADLNHGKAIYSESVDFKDIKDSLGYDKSHQFAYVKESTDAGYNAQDVKGI  
ALIERDPNKTYDEMIALAKKHGALGVLFNFKPGQSNRSMRLTANGMGIPSAFISHEFGKAMSQNLNGTGSLEFDS  
VVKAPSQKGNEMNHFSNWGLTSDHM

MalE

➤ Nucleic Acid

GGAATTGCTAGCGCGGCGCAGGACTCAAAGACTATTAACCTTTGGGTACCGACTGGCTCTAAAAATCATACG  
CAGATACCAATTGCAAAAATTTGAAAAAGATTCTGGTTACACCGTTAAAGTGGTTGAATCAGAAGATCCAAAAGCA  
CAAGAAAAAATCAAAAAAGATGCCTCAACTGCTGCCGATGCTTTTTCACTTCCCTCATGATCAACTTGGTCAACTT  
GTAGAATCTGGTACTATTCAAGAAGTTCCAGAAAAGTACAATAAAGAAATTGCTGCTACTTCTACTGATCAGGC  
GCTCGTGGTGCACAGTACAAAGGGAAAAACATACGCCTTCCCAATTTGGTATTGAATCACAAGTTCTTTTTCTACA  
ATAAATCAAAATAGCTGCTGAAGACGTTACTTCATACGATACAATTACTACAAAAGCAACTTTTGGTGGTACCT  
TTAAACAAGCTAACACTTACGCTACTGGTCCATTATTCATGTGCTAGTTGGTAAACACTTTATTTGGGGAAAAAGCGGT  
AAGATGTAAAAGTACTAAGTGGGGCAATGAAAAAGCGCTGCTGTTCTTAAATGAGTCCGACACCAAGCTTC  
AAATAAAGGTTTTGTTAGCTTAGATGCTAAACAGTTATGCTAAATTTGGTGACGGTTCAAGTCTTCTTTGGA  
ATCAGGACCGTGGGACTATGAAGCTGCTCAAAAAGCTATCGGTAAAGAAAACCTTGGTGTGCTATTTATCCAA  
AAGTAACTATTGGAGGCGAAACGGTTCAACAAAAAGCATTCTTGGGTGTAATAACTTTACGAGTCAACCAAGCA

CCAGCTAAAGGTGATACAAAACGTATCGCAGCAAGCTACAAATTGGCATCCTACTTGACTAATGCTGAAAGCCA  
AGAAAACCAATTCAAAACCTCGTAACATTGTCCAGCCAACAAAGAAGTTCAATCTTCAGAAGCTGTTCAATCAA  
CGAACTTGCTAAAACCGTTATCACTATGGGTTCTTCTTCAGACTACACTGTCGTAATGCCAAAACCTTAGCCAAAT  
GGGCACATTCGGACTGAAAGTGCTGCTATTCTTAGTGATGCCTTCAACGGTAAAATCAAAGAAAACGACTACC  
TTACTAAGTTGCAACAATTCGACAAAAGATATCGCTGCAACAAAACATATGTAG

➤ Amino Acid

GIASAAQDSKTIKLWVPTGSKKSYADTIKFEKDSGYTVKVVESDPKAQEIKIKDASTAADVFLPHDQLGQLVES  
GTIQEVPEKYNKEIAATSTDQALVGAQYKGYAFPFGIESQVLFYKSKLAAEDVTSYDTITTKATFGGTFKQANTY  
ATGPLFMSVGNLTFGENGEDVKGTNWNWNEKGA AVLKWIADQASNKGFVSLDANNVMSKFGDGSVASFESGPWDY  
EAAQKAIGKENLGVAIYPKVITIGGETVQQKAFVLKLYAVNQAPAKGDTKRIIAASYKLASYLTNAESQENQFKTRNIV  
PANKEVQSSSEAVQSNELAKTVITMGSSSDYTVVMPKLSQMGTFWTESAAILSDAFNGKIKENDYLTKLQDFDKDIAA  
TKHM

**SpyAD**

➤ Nucleic Acid

GGAATTGCTAGCGCGCGCAGGATGATAGAGCCTCAGGAGAAAACGAAGGCGAGTAATACTCACGACGATAGT  
TTACCAAAAACCAGAAACAATTCAGAGGCCAAAGGCAACTATTGATGCAGTTGAAAAAAGCTCTCAGTCAACAAAA  
AGCAGAACTGACAGAGCTTGCTACCGCTCTGACAAAAACTACTGCTGAAATCAACCACTTAAAAGAGCAGCAA  
GATAATGAACAAAAGCTTTAACCTCTGCACAAGAAATTCACATAACTCTTGCAAGTAGTGAGGAGAGCGCTA  
TTAGCCCAAGGAGCCGAACATCAAAGAGAGTTAACAGCTACTGAAACAGAGCTTCATAATGCTCAAGCAGACTA  
ACATTCAAAAGAGACTGCATTGTGACAACAAAAGCAAGCATTTCAGCAGAACTACTCGAGCTCAAGATTTAG  
TGGAACAAGTCAAACCGTCTGAACAAAATATTGCTAAGCTCAATGCTATGATTAGCAATCCTGATGCTATCACTA  
AAGCAGCTCAAACGGCTAATGATAATAAAAAGCATTAAAGCTCAGAATTGGAGAAGGCTAAAGCTGACTTAGAA  
AATCAAAAAGCTAAAAGTTAAAAGCAATTGACTGAAGAGTTGGCAGCTCAGAAAGCTGCTAGCAGAAAAAGA  
GGCAGAACTTAGTCGTCTTAAATCCTCAGCTCCGCTACTCAAGATAGCATTGTGGGTAATAATACCATGAAAG  
CACCGCAAGGCTATCCTCTTGAAGAACTTAAAAATTAGAAGCTAGTGGTTATATTGGATCAGCTAGTTACAATA  
ATTATTACAAAGAGCATGCAGATCAAATTTATGCCAAAGCTAGTCCAGGTAATCAATTAATCAATACCAAGATA  
TTCCAGCAGATCGTAATCGCTTTGTTGATCCCGATAATTTGACACCAGAAGTGCAAAATGAGCTTGCCGAGTTT  
GCAGCTCATTAGAAAAATCGTTTAAAGAAGACAATTAGTCTACCACCAAGTTACTGTTACAGCAGGATCACAAGA  
ATTTGCAAGATTACTTAGTACCAGCTATAAGAAAACATGGAATAACAAGACCATCATTTGTCTACGGACAGCC  
AGGGGTATCAGGGCATTATGGTGTGGGCCCTCATGATAAAACTATTATTGAAGACTCTGCCGGAGCGTCAGGG  
CTCATTGCAATGATGATAACATGTACGAGAATATCGGTGCTTTAACGATGTGCATACTGTGAATGGTATTA  
CGTGGTATTTATGACAGTATCAAGTATATGCTCTTTACAGATCATTACACGGAATACATACGGCCATGCTATT  
AACTTTTTACGTGTAGATAAACATAACCCTAATGCGCCTGTTTACCTTGGATTTTCAACCAGCAATGTAGGATCT  
TTGAATGAACACTTTGTAATGTTTCCAGAGTCAACATTGCTAACCATCAACGCTTTAATAAGACCCCTATAAAA  
GCCGTTGGAAGTACAAAAGATTATGCCCAAAGAGTAGGCACTGTATCTGATACTATTGCAGCGATCAAAGGAAA  
AGTAAGCTCATTAGAAAAATCGTTTGTCCGGCTATTCAAGAAAGCTGATATTATGGCAGCCCAAGCTAAAGTAA  
GTCAACTTCAAGGTAATTAGCAAGCACACTTAAGCAGTCAGACAGCTTAAATCTCCAAGTGAGACAATTAAT  
GATACTAAAGTTCTTTGAGAACAGAACTACTAGCAGCTAAAGCAAAACAAGCACAACCTCGAAGCTACTCGTGA  
TCAATCATTAGCTAAGCTTGCATCGTTGAAAGCCGACTTGCAACAGACAGAAGCCTTAGCAGAGCAAGCCGCA  
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CCTTCAAGTGATACGTGAGCGCATTGATAACTAAGCAAGATTTGGCTAAAACCTACCTCATCTTTGTTAAATGC  
ACAAGAAGCTTTAGCAGCCTTACAAGCTAAACAAAGCAGTCTAGAAGCTACTATTGCTACCACAGAACACCAGT  
TGACTTTGCTTAAAACCTTAGCTAACGAAAAGGAATATCGCCACTTAGACGAAGATATAGCTACTGTGCCTGATT  
TGCAAGTAGCTCCACCTTACGGGCGTAAAACCGCTATCATATAGTAAAGATAGATACTACTCCGCTTGTTC  
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AGAAGCGCTTGTGGCCAAACCTCTGAAATGGTAGCAAGTAATGCCATTGTGTCTAAAATCACATCTTCGATTA  
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GTACTCAAAGACATATGTAG

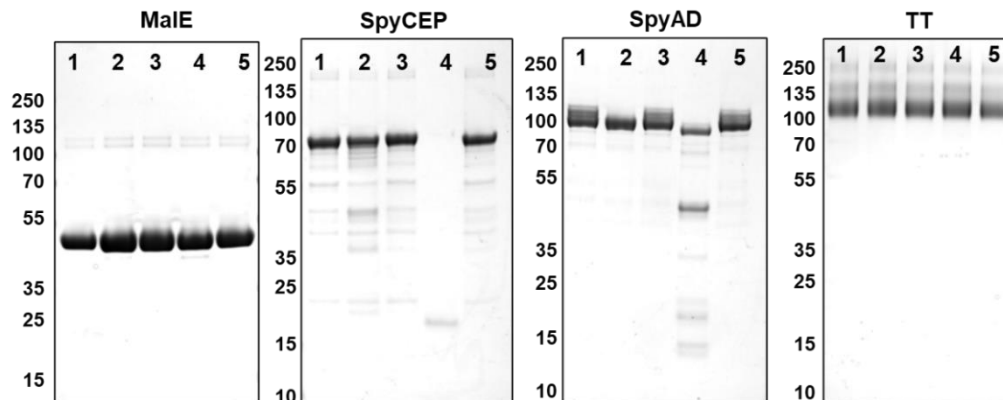
➤ Amino Acid

GIASAAQDDRASGETKASNTHDDSLPKPETIQEAKATIDAVEKTLSSQQAELTELATALTKTTAEINHLEKQQDNEQK  
ALTSAQEIYNTLASSEETLLAQGAHQRELTATELHNAQADQHSKETALSEQKASISAETTRAQDLVEQVKTSE  
QNIAKLNAMISNPDAITKAAQTANDNTKALSSELEKAKADLENQKAKVKKQLTEELAAQKAALAEKEAELSRLKSSAP  
STQDSIVGNNTMKAPQGYPLEELKKLEASGYIGSASYNYYKEHADQIIAKASPGNQLNQYQDIPADRNRFVDPDNL  
TPEVQNELAQFAAHMINSVRRQLGLPPVTVTAGSQEFARLLSTSYYKTHGNTRPSFVYQPGVSGHYGVGPHDKTI  
IEDSAGASGLIRNDDNMYENIGAFNDVHTVNGIKRGIYDSIKYMLFTDHLHGNTYGHAINFLRVDKHNPNAPVYLGFS  
TSNVGSLNEHFVMPFESNIANHQRFNKTPIKAVGSTKDYAQRVGTVSDTIAAIKGVSSLENRLSAIHQEAADIMAAQA  
KVSQKGLASTLKQSDSLNLQVRQLNDTKGSLRTELLAAKAKAQLEATRQSLAKLASLKAALHQTEALAEQAA  
ARVTALVAKKAHLQYLRDFKLPNRLQVIRERIDNTKQDLAKTTSSLLNAQEALAAALQAKQSSLEATITTEHQLTLK  
TLANEKEYRHLDEDIATVPLQVAPPLTGKPLSYSKIDITPLVQEMVKETKQLLEASARLAAENTSLVAEALVGQTS  
EMVASNAIVSKITSSITQPSKTSYSGSSTTNSLISDVDESTQRHM

## 7.2. Chemical Conjugation

### 7.2.1. *Component Preparation and Analysis*

Prior to chemical conjugation a storage stability study was performed for protein carriers in buffers used for reactions left for one month at different temperatures. The aim was to determine storage conditions for conjugate samples during analysis. MalE and TT protein were the most stable, whereas SpyCEP and SpyAD showed some protein degradation especially in MES buffer when stored at 4 °C (Figure 7.1).



**Figure 7.1: 10 µg of purified recombinant proteins separated by SDS-PAGE and Coomassie stained after storage in chemical conjugation reaction buffers for one month at various temperatures.**

Conditions: **1)** PBS pH 7.2 -80 °C, **2)** PBS pH 5.5 4 °C, **3)** MES pH 5.5 -20 °C, **4)** MES pH 7.2 4 °C and **5)** MES pH 7.2 -20 C.

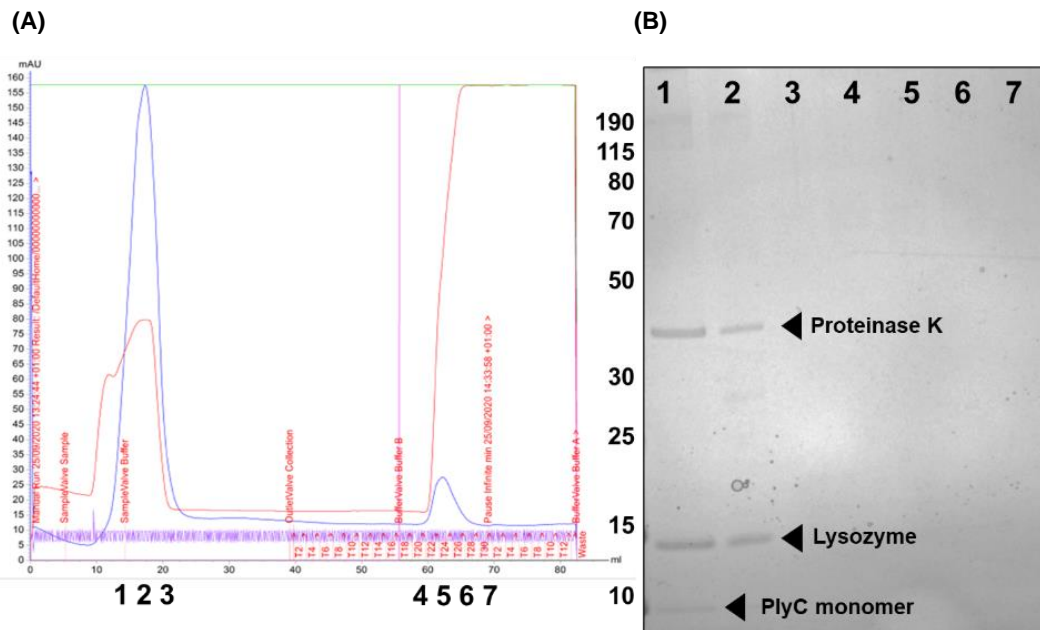
### 7.2.2. *PlyC Purification for Polysaccharide Extraction*

Recombinant PlyC was used to extract polysaccharide from GAS cell walls, and its activity was determined for each preparation according to GAS cell lysis calculated by OD<sub>600nm</sub> reduction.  $\Delta gacI$  mutant cultures appeared less sensitive to PlyC lysis than the parent GAS M1 NCTC-8198 strain (Table 7.1). Such reduction in activity was accounted for when calculating the amount of PlyC required per GAS cell pellet for extraction. Following digestion and extraction of polysaccharide, PlyC was removed through purification using a HiTrap™ Capto™ adhere column attached to ÄKTA start protein purification system. Bound GAC was eluted from the column using a salt gradient (Figure 7.2a), whereas protein contaminants such as PlyC flowed through the chromatography system as shown by Coomassie stained SDS PAGE of collected fractions (Figure 7.2b).

**Table 7.1: PlyC cell activity on GAS M1 NCTC-8198 and mutant  $\Delta gacI$  GAS cells.**

Purified recombinant PlyC was diluted 1 : 5 and added to 1 ml of mid-log GAS bacterial liquid cultures. Time 0 represents average OD<sub>600nm</sub> before incubation with PlyC.

Time (mins)	WT GAS	$\Delta gacI$ GAS
0	0.695	0.855
1	0.090	0.773
5	0.073	0.765
10	0.072	0.757
20	0.070	0.707

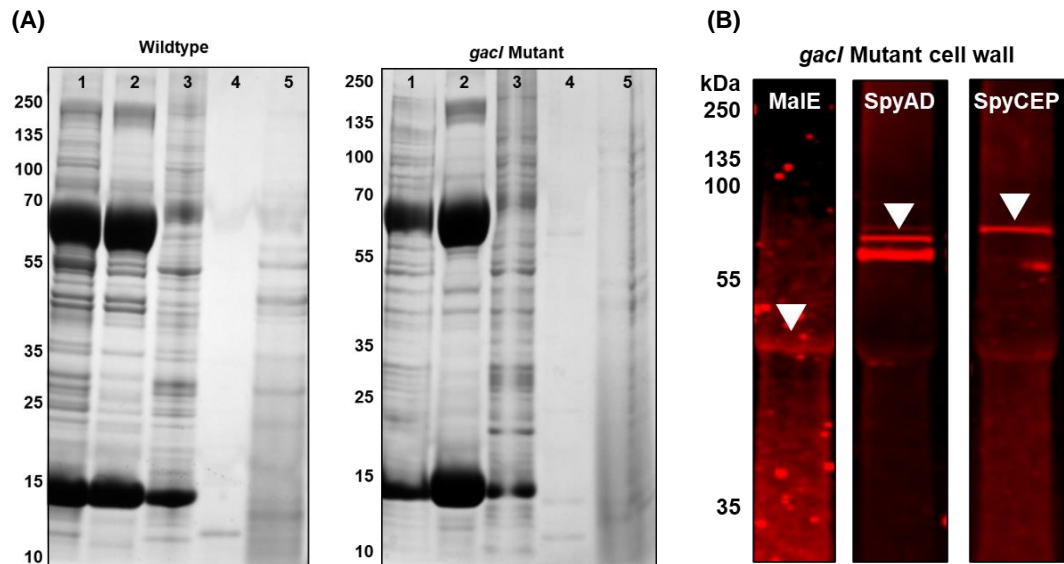


**Figure 7.2: PlyC purification from GAC polysaccharide.**

(A) Chromatogram of PlyC extracted GAC and resulting eluted fractions. UV<sub>280nm</sub> absorbance shows removal of contaminating proteins (1 – 3) and elution of GAC (4 – 7).

(B) Limited protein species are present in the eluted GAC fractions as shown by Coomassie stained SDS-PAGE. Numbers above denote the fractions in the chromatograph (A) over time.

Due to differences in sensitivity to PlyC as well as other phenotype differences stated in the thesis it was of interest to assess protein profiles between parent GAS NCTC-8198 strain and mutant  $\Delta gacI$  GAS cells. Coomassie stained SDS-PAGE show similarities in protein profiles (Figure 7.3a), and generated murine protein antisera (Chapter 3 section 3.4.2.2) is able to recognise native antigens within isolated  $\Delta gacI$  GAS cell walls (Figure 7.3b).



**Figure 7.3: WT and  $\Delta gacI$  GAS protein profiles and recognition by murine GAS protein antigen specific antisera.**

**(A)** WT and  $\Delta gacI$  cells were found to share similar cellular fraction profiles when separated by SDS-PAGE and stained by Coomassie. Samples; **1**) whole cell lysate, **2**) cell wall, **3**) cytoplasm, **4**) insoluble fraction and **5**) culture supernatants.

**(B)** Cell wall extracts from  $\Delta gacI$  GAS cells were transferred onto nitrocellulose membranes and incubated with antigen-specific mouse sera from day 28 (terminal). Mouse sera dilutions; 1 : 500 Anti MaIE, 1 : 750, Anti SpyAD, and 1 : 1,000 Anti SpyCEP. Detected with Goat anti-Mouse IgG IRDye®- 680RD.

As part of polysaccharide characterisation extracted wildtype GAC and GAS\_Rha polymers were sized. Size Exclusion Chromatography (SEC) required UV detection. Prior to sample analysis, UV signals were evaluated for GlcNAc and rhamnose monosaccharides independently. GlcNAc can be read at 214 nm wavelength, however, rhamnose could not be read until 187 nm (Table 7.2).



**Table 7.2: Evaluation of UV signal for GAC components rhamnose and GlcNac using UV-Vis spectrophotometer.**

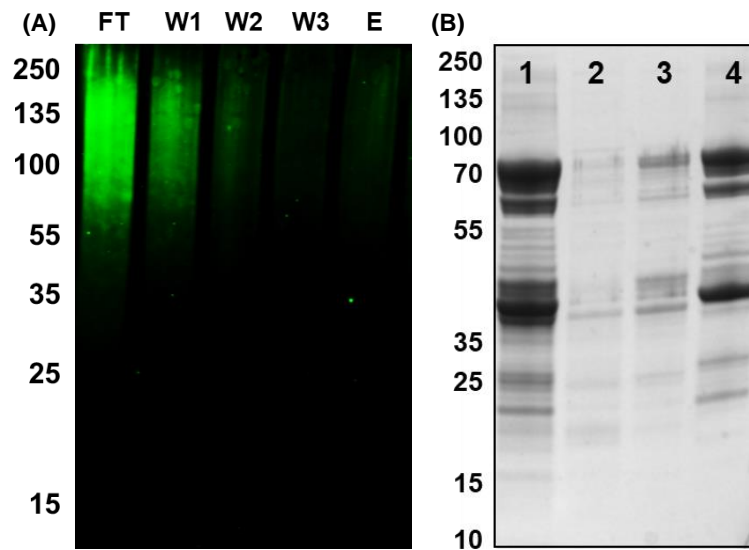
Wavelength (nm)	Absorbance	Sample
280	-0.1149	Rhamnose
	-0.1496	GlcNac
	-0.1577	Mixture
214	0.1489	Rhamnose
	1.2796	GlcNac
	0.3982	Mixture
202	> 10	GlcNac
	3.2546	Mixture
191	> 10	GlcNac
	10.0001	Mixture
187	1.882	Rhamnose
	3.012	Mixture

### 7.2.3. Chemical Conjugation Reaction Analysis

Successful glycoconjugate samples were routinely purified by using a 50 kDa MWCO spin filter to remove unconjugated GAC. The same ultracentrifugation spin filters were tested with purified GAC alone to ensure the absence of non-specific interactions. Although the majority of GAC was removed a faint anti-GAC signal was observed in the elution therefore washes were increased from 3 to 5 to minimise any carry over of unconjugated GAC into purified conjugate samples (Figure 7.4a). Chemical conjugation was not possible for all reaction components tested despite attempts at pH optimisation to balance EDC activation and stability (Table 7.3). Possible reasons for this could be degradation of SpyCEP which was frequently unstable in solution, precipitating in the conjugation reaction buffers, with degradation products coming through washes of 50 kDa MWCO filter (Figure 7.4b).

**Table 7.3: Chemical conjugation reaction summary.**

<b>Protein Carrier</b>	<b>Reactant Ratios</b>	<b>Conditions</b>
<b>MaIE</b>	14 : 1 <i>m/m</i> GAC : MaIE 1 : 0.5 <i>w/w</i> GAC : MaIE  (repeated with increased quantities)	EDC in water EDC + GAC 5 mins Protein incubated room temp 4 hrs Spin filter buffer exc. PBS
	1 : 0.5 <i>m/m</i> GAC : MaIE 1 : 3.6 <i>w/w</i> GAC : MaIE  (repeated with 2-ME inactivation and NaHCO <sub>3</sub> for pH increase)	Sulfo-NHS 2 step reaction EDC in water EDC + Sulfo NHS + GAC 15 mins / 1 hour Protein incubated room temp 4 hrs Spin filter buffer exc. PBS
<b>SpyCEP</b>	1 : 9.2 <i>m/m</i> GAC : SpyCEP 1 : 1 <i>w/w</i> GAC : SpyCEP	Lyophilised GAC EDC 50 mM MES Protein buffer exc. into MES Protein incubated room temp overnight Hydrophobicity purification – C-18
	1 : 2.7 <i>m/m</i> GAC : SpyCEP 1 : 24 <i>w/w</i> GAC : SpyCEP	
	1 : 2 <i>m/m</i> GAC : SpyCEP 1 : 17.9 <i>w/w</i> GAC : SpyCEP	Sulfo-NHS 2 step reaction 10 fold molar excess EDC in MES 2 hr GAC activation 2-ME inactivation SpyCEP pH 6.4 Reaction pH 7.2 Protein incubated 2 hr and overnight 4 °C Spin filter buffer exc. PBS
	5 : 1 <i>m/m</i> GAC : SpyCEP 1 : 17.9 <i>w/w</i> GAC : SpyCEP	
	8.75 : 1 <i>m/m</i> GAC : SpyCEP 1 : 1 <i>w/w</i> GAC : SpyCEP	As above pH increase with NaHCO <sub>3</sub> 2-ME inactivation Reaction left 2 hrs and overnight 4 °C Affinity purification – His tag
<b>TT</b>	2 : 1 <i>m/m</i> GAC : TT 6.4 : 1 <i>w/w</i> GAC : TT	Sulfo-NHS 2 step reaction pH increase with NaHCO <sub>3</sub> 2-ME inactivation Reaction left 3 hrs and overnight SEC purification
	12.8 : 1 <i>m/m</i> GAC : TT 1 : 1 <i>w/w</i> GAC : TT	Sulfo-NHS 2 step reaction pH increase with NaHCO <sub>3</sub> 2-ME inactivation Reaction left 3 hrs and overnight Spin filter buffer exc. PBS
<b>SpyAD</b>	23.3 : 1 <i>m/m</i> GAC : SpyAD 1 : 0.62 <i>w/w</i> GAC : SpyAD	Sulfo-NHS 2 step reaction 10 fold molar excess EDC in MES 2 hr GAC activation 2-ME inactivation pH increase with NaHCO <sub>3</sub> Reaction left 3 hrs and overnight 4 °C Spin filter buffer exc. PBS

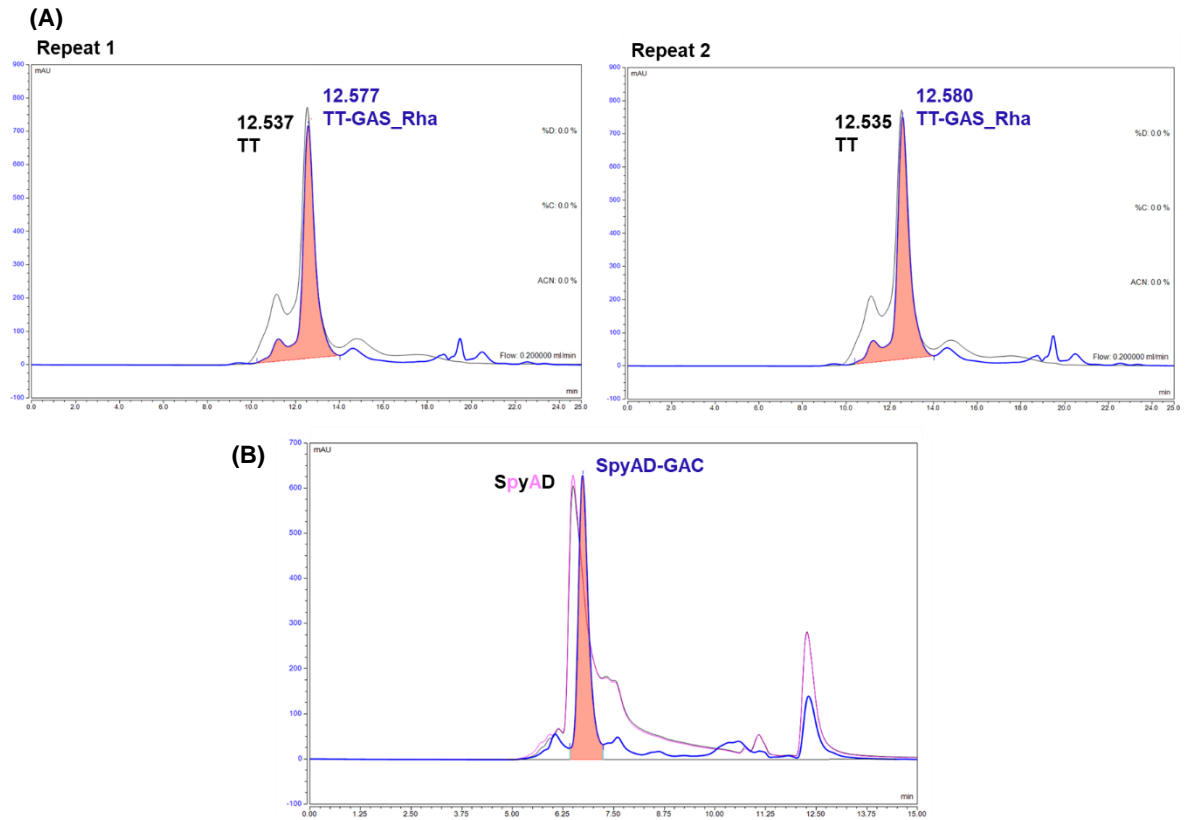


**Figure 7.4: Analysis of chemical conjugation reactions and clean up approach.**

**(A)** GAC interaction with 50 kDa MWCO spin filter. Flowthrough (**FT**), three washes (**W 1 – 3**) and final elution (**E**) separated by SDS-PAGE and stained with anti-GAS antibodies detected with Rabbit anti-Goat IgG IRDye® - 800CW.

**(B)** SpyCEP-GAC chemical reaction. Total reaction (**1**) was purified using the same 50 kDa MWCO spin filter and flowthrough (**2**), pooled wash samples (**3**), and purified conjugate (**4**) were collected and separated by SDS-PAGE and stained by Coomassie.

Successful conjugation reactions were analysed using high pressure liquid chromatography (HPLC) techniques. Conditions were optimised so that baselines were stable. The TT-GAS\_Rha conjugate consistently eluted at the same retention time during different runs of the same sample performed on different days with the prepared mobile phase buffer (Figure 7.5a). Additionally, the column integrity was confirmed through SpyAD protein alone run both before and after the SpyAD-GAC glycoconjugate sample on the same run showing the same retention times, indistinguishable when chromatographs are superimposed (Figure 7.5b – pink and black lines). This shows that changes to retention times observed in Chapter 3 are not due to lack of column integrity or buffer inconsistency.

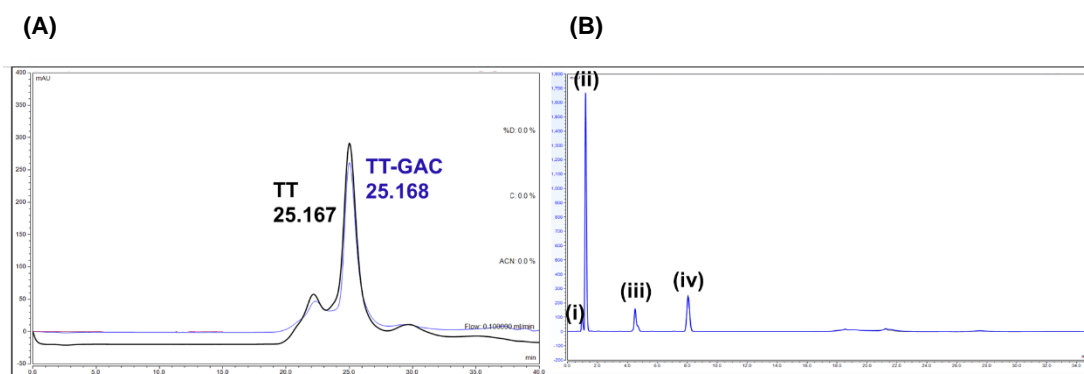


**Figure 7.5: TT-GAS\_Rha and SpyAD-GAC chemical glycoconjugate HPLC-SEC testing.**

**(A)** TT-GAS\_Rha (blue) showed consistent retention times (RT) between two independent runs (RT = 12.577 and 12.580 for repeat 1 and 2 respectively) with a minor shift from TT protein alone (black) (RT = 12.537 and 12.535 for repeat 1 and 2 respectively).

**(B)** SpyAD protein alone run in duplicate (superimposed black and pink lines) before and after the conjugate SpyAD-GAC sample (blue) consistently eluted earlier.

However, the same technique could not be applied to all conjugates, with TT-GAC glycoconjugate not showing any size differences using HPLC-SEC with both the conjugate and TT showing the same retention time (Figure 7.6a). Instead, reverse phase HPLC was adopted showing changes to TT hydrophobicity when conjugated (Chapter 3 Figure 3.13). The column used in this analysis showed no peak shifts following two standard injections before and after TT-GAC test samples demonstrating column integrity (Figure 7.6b – superimposed blue and black lines).



**Figure 7.6: TT-GAC physicochemical testing using HPLC-SEC and RP-HPLC.**

**(A)** TT-GAC (blue) did not show any difference in retention time compared to TT alone (black) using HPLC-SEC techniques with UV 280 nm as a detection method. RT = 25.167 (TT) and 25.168 (TT-GAC).

**(B)** RP-HPLC was used to analyse TT-GAC and vaccine components TT and GAC alone. A standard was injected before and after runs containing (i) uracil, (ii) benzamide, (iii) benzophenone, and (iv) biphenyl. The standard injections showed consistent retention times without any peak shifting when overlaid demonstrating column integrity (blue and black lines).

### 7.3. Overall Glycoconjugate Characterisation

This study used a variety of analytical methods (Table 7.4) to characterise protein and polysaccharide component composition and identity. Methods to prove successful conjugation, quantify and characterise the physicochemical glycoconjugate properties, including the ratio of the two components, and separation and quantification of unconjugated components are also stated.

**Table 7.4: Summary of physicochemical approaches used to characterise glycoconjugate vaccines and associated components.**

Yellow – polysaccharide (PS), blue – carrier protein, and green – glycoconjugate.

Test	Method	Description	Tested
Component Identity / Composition	SDS-PAGE - Coomassie / Western Blot	Dual PS and protein staining with specific antibodies	
	Proton NMR	PS specific unique regions of spectra	
	H-PAD	Identity and estimation of PS content based on monosaccharide references	
	HPLC-SEC (MALS)	PS size and heterogeneity (molar mass, hydrodynamic radius, molecular distribution)	
	Tandem Mass Spectrometry	Amino acid sequence and purity	
Conjugation Proof	ELISA	Antibodies specific to PS to capture, detected with antibodies specific to protein or vice versa	
	SDS-PAGE - Coomassie / Western Blot	Increase in protein molecular weight and / or PS laddering of increased size via dual staining	
	Proteinase K	Absence of protein and PS signal after proteinase K treatment	
Separation and Quantification Free PS / Protein	Gel filtration	Separation of unconjugated / conjugated PS based on size - gel filtration	Data not shown
	Ultra-filtration	Separation of unconjugated / conjugated PS based on size using centrifugal filters with MWCO	Small scale only
	Affinity Chromatography	Separation of unconjugated PS based on presence of His tag on conjugated protein carrier – HisTrap / NiNTA	
	Reverse Phase Chromatography	Separation of unconjugated PS based on purification of conjugated protein using hydrophobicity - C18 column	Data not shown
	Immunoppt.	Use of immune sera and PEG to precipitate and separate one component to quantify other unconjugated component	
	Anthrone	Colourmetric acid based assay for hydrolysed PS concentration determination	
	BCA	Colourmetric copper reduction based assay for protein concentration determination	
Protein : PS Ratio	H-PAD	Quantification of PS based on monosaccharide references	
	HPLC-SEC (RI/UV)	Separation based on size with dual monitoring such as refractive index (RI) and UV	
	Proton NMR	NMR spectra - ratio by integration of sets of resonances from protein and PS	
Stability	HPLC-SEC/UV/MALS	Changes in size and percentage of unconjugated PS / protein as above	
	SDS-PAGE - Coomassie / Western Blot	Size and degradation products	
Immunogenicity	Animal Immunisation	Serum antibody titres – Serum IgG Antibody functionality – cell binding and opsonophagocytosis Antibody isotypes B and T cell responses – Cytokine profiles and T cell stimulation assay Protein specific blocking of function – e.g. IL-8 cleavage and binding to ECM	