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[395] 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
CATCTCACCAATACAGAGTTGAGCTCAGCTGAATCAAAATCTCAAGACACATCACAAATCACTCTCAAGACAAAT
CGTGAAAAAGAGCAATCACAAGATCTAGTCTCTGAGCCAACCACAACTGAGCTAGCTGACACAGATGCAGCAT
CAATGGCTAATACAGGTTCTGATGCGACTCAAAAAAGCGCTTCTTTACCGCCAGTCAATACAGATGTTCACGAT
TGGGTAAAAACCAAAGGAGCTTGGGACAAGGGATACAAAGGACAAGGCAAGGTTGTCGCAGTTATTGACACAG
GGATCGATCCGGCCCATCAAAGCATGCGCATCAGTGATGTATCAACTGCTAAAGTAAAATCAAAAGAAGACATG
CTAGCACGCCAAAAAGCCGCCGGTATTAATTATGGGAGTTGGATAAATGATAAAGTTGTTTTCGCACATAATTAT
GTGGAAAATAGCGATAATATCAAAGAAAATCAATTCGAGGATTTTGATGAGGACTGGGAAAACTTTGAGTTTGA
TGCAGAGGCAGAGCCAAAAGCCATCAAAAAACACAAGATCTATCGTCCCCAATCAACCCAGGCACCGAAAGAA
ACTGTTATCAAAACAGAAGAAACAGATGGTTCACATGATATTGACTGGACACAAACAGACGATGACACCAAATA
CGAGTCACACGGTATGCATGTGACAGGTATTGTAGCCGGTAATAGCAAAGAAGCCGCTGCTACTGGAGAACGC
TTTTTAGGAATTGCACCAGAGGCCCAAGTCATGTTCATGCGTGTTTTTGCCAACGACATCATGGGATCAGCTGA
ATCACTCTTTATCAAAGCTATCGAAGATGCCGTGGCTTTAGGAGCAGATGTGATCAACCTGAGTCTTGGAACCG
CTAATGGGGCACAGCTTAGTGGCAGCAAGCCTCTAATGGAAGCAATTGAAAAAGCTAAAAAAGCCGGTGTATC
AGTTGTTGTAGCAGCAGGAAATGAGCGCGTCTATGGATCTGACCATGATGATCCATTGGCGACAAATCCAGAC
TATGGTTTGGTCGGTTCTCCCTCAACAGGTCGAACACCAACATCAGTGGCAGCTATAAACAGTAAGTGGGTGA
TTCAACGTCTAATGACGGTCAAAGAATTAGAAAACCGTGCCGATTTAAATCATGGTAAAGCCATCTATTCAGAGT
CTGTCGACTTTAAAGACATAAAAGATAGCCTAGGTTATGATAAATCGCATCAATTTGCTTATGTCAAAGAGTCAA
CTGATGCGGGTTATAACGCACAAGACGTTAAAGGTAAAATTGCTTTAATTGAACGTGATCCCAATAAAACCTAT
GACGAAATGATTGCTTTGGCTAAGAAACATGGAGCTCTGGGAGTACTTATTTTTAATAACAAGCCTGGTCAATC
AAACCGCTCAATGCGTCTAACAGCTAATGGGATGGGGATACCATCTGCTTTCATATCGCACGAATTTGGTAAGG
CCATGTCCCAATTAAATGGCAATGGTACAGGAAGTTTAGAGTTTGACAGTGTGGTCTCAAAAGCACCGAGTCAA
AAAGGCAATGAAATGAATCATTTTTCAAATTGGGGCCTAACTTCTGATCTCGAGGCG

- Amino Acid

GDELSTMSEPTITNHAQQQAQHLTNTELSSAESKSQDTSQITLKTNREKEQSQDLVSEPTTTELADTDAASMANTG
SDATQKSASLPPVNTDVHDWVKTKGAWDKGYKGQGKVVAVIDTGIDPAHQSMRISDVSTAKVKSKEDMLARQKAA
GINYGSWINDKVVFAHNYVENSDNIKENQFEDFDEDWENFEFDAEAEPKAIKKHKIYRPQSTQAPKETVIKTEETDG
SHDIDWTQTDDDTKYESHGMHVTGIVAGNSKEAAATGERFLGIAPEAQVMFMRVFANDIMGSAESLFIKAIEDAVAL
GADVINLSLGTANGAQLSGSKPLMEAIEKAKKAGVSVVVAAGNERVYGSDHDDPLATNPDYGLVGSPSTGRTPTSV
AAINSKWVIQRLMTVKELENRADLNHGKAIYSESVDFKDIKDSLGYDKSHQFAYVKESTDAGYNAQDVKGKIALIERD
PNKTYDEMIALAKKHGALGVLIFNNKPGQSNRSMRLTANGMGIPSAFISHEFGKAMSQLNGNGTGSLEFDSVVSKA
PSQKGNEMNHFSNWGLTSD

MalE

- Nucleic Acid

GCGCCATGGGGCGACTCAAAGACTATTAAACTTTGGGTACCGACTGGCTCTAAAAAATCATATGCAGATACCAT
TGCAAAATTTGAAAAAGATTCTGGTTACACCGTTAAAGTGGTTGAATCAGAAGATCCAAAAGCACAAGAAAAAAT
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AGCTGCTGAAGACGTTACTTCATATGATACAATTACTACAAAAGCAACTTTTGGTGGTACCTTTAAACAAGCTAA
CACTTACGCTACTGGTCCATTATTCATGTCAGTTGGTAACACTTTATTTGGGGAAAACGGTGAAGATGTAAAAG
GTACTAACTGGGGCAATGAAAAAGGCGCTGCTGTTCTTAAATGGATCGCAGACCAAGCTTCAAATAAAGGTTTT
GTTAGCTTAGATGCTAACAACGTTATGTCTAAATTTGGTGACGGTTCAGTAGCTTCCTTTGAATCAGGACCGTG
GGACTATGAAGCTGCTCAAAAAGCTATCGGTAAAGAAAACCTTGGTGTTGCTATTTATCCAAAAGTAACTATTG
GAGGCGAAACGGTTCAACAAAAAGCATTCTTGGGTGTAAAACTTTACGCAGTCAACCAAGCACCAGCTAAAGG
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TCAAAACTCGTAACATTGTCCCAGCCAACAAAGAAGTTCAATCTTCAGAAGCTGTTCAATCAAACGAACTTGCTA
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GGACTGAAAGTGCTGCTATTCTTAGTGATGCCTTCAACGGTAAAATCAAAGAAAACGACTACCTTACTAAGTTG
CAACAATTCGACAAAGATATCGCTGCAACAAAACTCGAGGCG

- Amino Acid

GDSKTIKLWVPTGSKKSYADTIAKFEKDSGYTVKVVESEDPKAQEKIKKDASTAADVFSLPHDQLGQLVESGTIQEV
PEKYNKEIAATSTDQALVGAQYKGKTYAFPFGIESQVLFYNKSKLAAEDVTSYDTITTKATFGGTFKQANTYATGPLF
MSVGNTLFGENGEDVKGTNWGNEKGAAVLKWIADQASNKGFVSLDANNVMSKFGDGSVASFESGPWDYEAAQK
AIGKENLGVAIYPKVTIGGETVQQKAFLGVKLYAVNQAPAKGDTKRIAASYKLASYLTNAESQENQFKTRNIVPANKE
VQSSEAVQSNELAKTVITMGSSSDYTVVMPKLSQMGTFWTESAAILSDAFNGKIKENDYLTKLQQFDKDIAATK

SpyAD

- Nucleic Acid

GCGCCATGGGATAGAGCCTCAGGAGAAACGAAGGCGAGTAATACTCACGACGATAGTTTACCAAAACCAGAAA
CAATTCAAGAGGCAAAGGCAACTATTGATGCAGTTGAAAAAACTCTCAGTCAACAAAAAGCAGAACTGACAGAG
CTTGCTACCGCTCTGACAAAAACTACTGCTGAAATCAACCACTTAAAAGAGCAGCAAGATAATGAACAAAAAGC
TTTAACCTCTGCACAAGAAATTTACACTAATACTCTTGCAAGTAGTGAGGAGACGCTATTAGCCCAAGGAGCCG
AACATCAAAGAGAGTTAACAGCTACTGAAACAGAGCTTCATAATGCTCAAGCAGATCAACATTCAAAAGAGACT
GCATTGTCAGAACAAAAAGCTAGCATTTCAGCAGAAACTACTCGGGCTCAAGATTTAGTGGAACAAGTCAAAAC
GTCTGAACAAAATATTGCTAAGCTCAATGCTATGATTAGCAATCCTGATGCTATCACTAAAGCAGCTCAAACGG
CTAATGATAATACAAAAGCATTAAGCTCAGAATTGGAGAAGGCTAAAGCTGACTTAGAAAATCAAAAAGCTAAA
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CCTCTTGAAGAACTTAAAAAATTAGAAGCTAGTGGTTATATTGGATCAGCTAGTTACAATAATTATTACAAAGAG
CATGCAGATCAAATTATTGCCAAAGCTAGTCCAGGTAATCAATTAAATCAATACCAAGATATTCCAGCAGATCGT
AATCGCTTTGTTGATCCCGATAATTTGACACCAGAAGTGCAAAATGAGCTAGCGCAGTTTGCAGCTCACATGAT
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ATTATGGTGTTGGGCCTCATGATAAAACTATTATTGAAGACTCTGCCGGAGCGTCAGGGCTCATTCGAAATGAT
GATAACATGTACGAGAATATCGGTGCTTTTAACGATGTGCATACTGTGAATGGTATTAAACGTGGTATTTATGAC
AGTATCAAGTATATGCTCTTTACAGATCATTTACACGGAAATACATACGGCCATGCTATTAACTTTTTACGTGTA
GATAAACATAACCCTAATGCGCCTGTTTACCTTGGATTTTCAACCAGCAATGTAGGATCTTTGAATGAACACTTT
GTAATGTTTCCAGAGTCTAACATTGCTAACCATCAACGCTTTAATAAGACCCCTATAAAAGCCGTTGGAAGTACA
AAAGATTATGCCCAAAGAGTAGGCACTGTATCTGATACTATTGCAGCGATCAAAGGAAAAGTAAGCTCATTAGA
AAATCGTTTGTCGGCTATTCATCAAGAAGCTGATATTATGGCAGCCCAAGCTAAAGTAAGTCAACTTCAAGGTA
AATTAGCAAGCACACTTAAGCAGTCAGACAGCTTAAATCTCCAAGTGAGACAATTAAATGATACTAAAGGTTCTT
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CTAGCATCGTTGAAAGCCGCACTGCACCAGACAGAAGCCTTAGCAGAGCAAGCCGCAGCCAGAGTGACAGCA
CTGGTGGCTAAAAAAGCTCATTTGCAATATCTAAGGGACTTTAAATTGAATCCTAACCGCCTTCAAGTGATACGT
GAGCGCATTGATAATACTAAGCAAGATTTGGCTAAAACTACCTCATCTTTGTTAAATGCACAAGAAGCTTTAGCA
GCCTTACAAGCTAAACAAAGCAGTCTAGAAGCTACTATTGCTACCACAGAACACCAGTTGACTTTGCTTAAAAC
CTTAGCTAACGAAAAGGAATATCGCCACTTAGACGAAGATATAGCTACTGTGCCTGATTTGCAAGTAGCTCCAC
CTCTTACGGGCGTAAAACCGCTATCATATAGTAAGATAGATACTACTCCGCTTGTTCAAGAAATGGTTAAAGAAA
CGAAACAACTATTAGAAGCTTCAGCAAGATTAGCTGCTGAAAATACAAGTCTTGTAGCAGAAGCGCTTGTTGGC
CAAACCTCTGAAATGGTAGCAAGTAATGCCATTGTGTCTAAAATCACATCTTCGATTACTCAGCCCTCATCTAAG
ACATCTTATGGCTCAGGATCTTCTACAACGAGCAATCTCATTTCTGATGTTGATGAAAGTACTCAAAGACTCGAG
GCG

- Amino Acid

DRASGETKASNTHDDSLPKPETIQEAKATIDAVEKTLSQQKAELTELATALTKTTAEINHLKEQQDNEQKALTSAQEIY
TNTLASSEETLLAQGAEHQRELTATETELHNAQADQHSKETALSEQKASISAETTRAQDLVEQVKTSEQNIAKLNAMI
SNPDAITKAAQTANDNTKALSSELEKAKADLENQKAKVKKQLTEELAAQKAALAEKEAELSRLKSSAPSTQDSIVGN
NTMKAPQGYPLEELKKLEASGYIGSASYNNYYKEHADQIIAKASPGNQLNQYQDIPADRNRFVDPDNLTPEVQNELA
QFAAHMINSVRRQLGLPPVTVTAGSQEFARLLSTSYKKTHGNTRPSFVYGQPGVSGHYGVGPHDKTIIEDSAGASG
LIRNDDNMYENIGAFNDVHTVNGIKRGIYDSIKYMLFTDHLHGNTYGHAINFLRVDKHNPNAPVYLGFSTSNVGSLNE
HFVMFPESNIANHQRFNKTPIKAVGSTKDYAQRVGTVSDTIAAIKGKVSSLENRLSAIHQEADIMAAQAKVSQLQGKL
ASTLKQSDSLNLQVRQLNDTKGSLRTELLAAKAKQAQLEATRDQSLAKLASLKAALHQTEALAEQAAARVTALVAKK
AHLQYLRDFKLNPNRLQVIRERIDNTKQDLAKTTSSLLNAQEALAALQAKQSSLEATIATTEHQLTLLKTLANEKEYRH
LDEDIATVPDLQVAPPLTGVKPLSYSKIDTTPLVQEMVKETKQLLEASARLAAENTSLVAEALVGQTSEMVASNAIVS
KITSSITQPSSKTSYGSGSSTTSNLISDVDESTQR

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

SpyCEP

- Nucleic Acid

GGAATTGCTAGCGCGGCGCAGGATGAGCTAAGCACAATGAGCGAACCAACAATCACGAATCACGCTCAACAA
CAAGCGCAACATCTCACCAATACAGAGTTGAGCTCAGCTGAATCAAAATCTCAAGACACATCACAAATCACTCT
CAAGACAAATCGTGAAAAAGAGCAATCACAAGATCTAGTCTCTGAGCCAACCACAACTGAGCTTGCTGACACA
GATGCAGCATCAATGGCTAATACAGGTTCTGATGCGACTCAAAAAAGCGCTTCTTTACCGCCAGTCAATACAGA
TGTTCACGATTGGGTAAAAACCAAAGGAGCTTGGGACAAGGGATACAAAGGACAAGGCAAGGTTGTCGCAGTT
ATTGACACAGGGATCGATCCGGCCCATCAAAGCATGCGCATCAGTGATGTATCAACTGCTAAAGTAAAATCAAA
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CACATAATTATGTGGAAAATAGCGATAATATCAAAGAAAATCAATTCGAGGATTTTGATGAGGACTGGGAAAACT
TTGAGTTTGATGCAGAGGCAGAGCCAAAAGCCATCAAAAAACACAAGATCTATCGTCCCCAATCAACCCAGGC
ACCGAAAGAAACTGTTATCAAAACAGAAGAAACAGATGGTTCACATGATATTGACTGGACACAAACAGACGATG
ACACCAAATACGAGTCACACGGTATGCATGTGACAGGTATTGTAGCCGGTAATAGCAAAGAAGCCGCTGCTAC
TGGAGAACGCTTTTTAGGAATTGCACCAGAGGCCCAAGTCATGTTCATGCGTGTTTTTGCCAACGACATCATGG
GATCAGCTGAATCACTCTTTATCAAAGCTATCGAAGATGCCGTGGCTTTAGGAGCAGATGTGATCAACCTGAGT
CTTGGAACCGCTAATGGGGCACAGCTTAGTGGCAGCAAGCCTCTAATGGAAGCAATTGAAAAAGCTAAAAAAG
CCGGTGTATCAGTTGTTGTAGCAGCAGGAAATGAGCGCGTCTATGGATCTGACCATGATGATCCATTGGCGAC
AAATCCAGACTATGGTTTGGTCGGTTCTCCCTCAACAGGTCGAACACCAACATCAGTGGCAGCTATAAACAGTA
AGTGGGTGATTCAACGTCTAATGACGGTCAAAGAATTAGAAAACCGTGCCGATTTAAATCATGGTAAAGCCATC
TATTCAGAGTCTGTCGACTTTAAAGACATAAAAGATAGCCTAGGTTATGATAAATCGCATCAATTTGCTTATGTC
AAAGAGTCAACTGATGCGGGTTATAACGCACAAGACGTTAAAGGTAAAATTGCTTTAATTGAACGTGATCCCAA
TAAAACCTATGACGAAATGATTGCTTTGGCTAAGAAACATGGAGCTCTGGGAGTACTTATTTTTAATAACAAGCC
TGGTCAATCAAACCGCTCAATGCGTCTAACAGCTAATGGGATGGGGATACCATCTGCTTTCATATCGCACGAAT
TTGGTAAGGCCATGTCCCAATTAAATGGCAATGGTACAGGAAGTTTAGAGTTTGACAGTGTGGTCTCAAAAGCA
CCGAGTCAAAAAGGCAATGAAATGAATCATTTTTCAAATTGGGGCCTAACTTCTGATCATATGTAG

- Amino Acid

GIASAAQDELSTMSEPTITNHAQQQAQHLTNTELSSAESKSQDTSQITLKTNREKEQSQDLVSEPTTTELADTDAAS
MANTGSDATQKSASLPPVNTDVHDWVKTKGAWDKGYKGQGKVVAVIDTGIDPAHQSMRISDVSTAKVKSKEDMLA
RQKAAGINYGSWINDKVVFAHNYVENSDNIKENQFEDFDEDWENFEFDAEAEPKAIKKHKIYRPQSTQAPKETVIKT
EETDGSHDIDWTQTDDDTKYESHGMHVTGIVAGNSKEAAATGERFLGIAPEAQVMFMRVFANDIMGSAESLFIKAIE
DAVALGADVINLSLGTANGAQLSGSKPLMEAIEKAKKAGVSVVVAAGNERVYGSDHDDPLATNPDYGLVGSPSTGR
TPTSVAAINSKWVIQRLMTVKELENRADLNHGKAIYSESVDFKDIKDSLGYDKSHQFAYVKESTDAGYNAQDVKGKI
ALIERDPNKTYDEMIALAKKHGALGVLIFNNKPGQSNRSMRLTANGMGIPSAFISHEFGKAMSQLNGNGTGSLEFDS
VVSKAPSQKGNEMNHFSNWGLTSDHM

MalE

- Nucleic Acid

GGAATTGCTAGCGCGGCGCAGGACTCAAAGACTATTAAACTTTGGGTACCGACTGGCTCTAAAAAATCATACG
CAGATACCATTGCAAAATTTGAAAAAGATTCTGGTTACACCGTTAAAGTGGTTGAATCAGAAGATCCAAAAGCA
CAAGAAAAAATCAAAAAAGATGCCTCAACTGCTGCCGATGTCTTTTCACTTCCTCATGATCAACTTGGTCAACTT
GTAGAATCTGGTACTATTCAAGAAGTTCCAGAAAAGTACAATAAAGAAATTGCTGCTACTTCTACTGATCAGGC
GCTCGTCGGTGCACAGTACAAAGGGAAAACATACGCCTTCCCATTTGGTATTGAATCACAAGTTCTTTTCTACA
ATAAATCAAAATTAGCTGCTGAAGACGTTACTTCATACGATACAATTACTACAAAAGCAACTTTTGGTGGTACCT
TTAAACAAGCTAACACTTACGCTACTGGTCCATTATTCATGTCAGTTGGTAACACTTTATTTGGGGAAAACGGTG
AAGATGTAAAAGGTACTAACTGGGGCAATGAAAAAGGCGCTGCTGTTCTTAAATGGATCGCAGACCAAGCTTC
AAATAAAGGTTTTGTTAGCTTAGATGCTAACAACGTTATGTCTAAATTTGGTGACGGTTCAGTAGCTTCCTTTGA
ATCAGGACCGTGGGACTATGAAGCTGCTCAAAAAGCTATCGGTAAAGAAAACCTTGGTGTTGCTATTTATCCAA
AAGTAACTATTGGAGGCGAAACGGTTCAACAAAAAGCATTCTTGGGTGTAAAACTTTACGCAGTCAACCAAGCA

CCAGCTAAAGGTGATACAAAACGTATCGCAGCAAGCTACAAATTGGCATCCTACTTGACTAATGCTGAAAGCCA
AGAAAACCAATTCAAAACTCGTAACATTGTCCCAGCCAACAAAGAAGTTCAATCTTCAGAAGCTGTTCAATCAAA
CGAACTTGCTAAAACCGTTATCACTATGGGTTCTTCTTCAGACTACACTGTCGTAATGCCAAAACTTAGCCAAAT
GGGCACATTCTGGACTGAAAGTGCTGCTATTCTTAGTGATGCCTTCAACGGTAAAATCAAAGAAAACGACTACC
TTACTAAGTTGCAACAATTCGACAAAGATATCGCTGCAACAAAACATATGTAG

- Amino Acid

GIASAAQDSKTIKLWVPTGSKKSYADTIAKFEKDSGYTVKVVESEDPKAQEKIKKDASTAADVFSLPHDQLGQLVES
GTIQEVPEKYNKEIAATSTDQALVGAQYKGKTYAFPFGIESQVLFYNKSKLAAEDVTSYDTITTKATFGGTFKQANTY
ATGPLFMSVGNTLFGENGEDVKGTNWGNEKGAAVLKWIADQASNKGFVSLDANNVMSKFGDGSVASFESGPWDY
EAAQKAIGKENLGVAIYPKVTIGGETVQQKAFLGVKLYAVNQAPAKGDTKRIAASYKLASYLTNAESQENQFKTRNIV
PANKEVQSSEAVQSNELAKTVITMGSSSDYTVVMPKLSQMGTFWTESAAILSDAFNGKIKENDYLTKLQQFDKDIAA
TKHM

SpyAD

- Nucleic Acid

GGAATTGCTAGCGCGGCGCAGGATGATAGAGCCTCAGGAGAAACGAAGGCGAGTAATACTCACGACGATAGT
TTACCAAAACCAGAAACAATTCAAGAGGCAAAGGCAACTATTGATGCAGTTGAAAAAACTCTCAGTCAACAAAA
AGCAGAACTGACAGAGCTTGCTACCGCTCTGACAAAAACTACTGCTGAAATCAACCACTTAAAAGAGCAGCAA
GATAATGAACAAAAAGCTTTAACCTCTGCACAAGAAATTTACACTAATACTCTTGCAAGTAGTGAGGAGACGCTA
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ACATTCAAAAGAGACTGCATTGTCAGAACAAAAAGCAAGCATTTCAGCAGAAACTACTCGAGCTCAAGATTTAG
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AAGCAGCTCAAACGGCTAATGATAATACAAAAGCATTAAGCTCAGAATTGGAGAAGGCTAAAGCTGACTTAGAA
AATCAAAAAGCTAAAGTTAAAAAGCAATTGACTGAAGAGTTGGCAGCTCAGAAAGCTGCTCTAGCAGAAAAAGA
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CACCGCAAGGCTATCCTCTTGAAGAACTTAAAAAATTAGAAGCTAGTGGTTATATTGGATCAGCTAGTTACAATA
ATTATTACAAAGAGCATGCAGATCAAATTATTGCCAAAGCTAGTCCAGGTAATCAATTAAATCAATACCAAGATA
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GCAGCTCACATGATTAATAGTGTAAGAAGACAATTAGGTCTACCACCAGTTACTGTTACAGCAGGATCACAAGA
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CGTGGTATTTATGACAGTATCAAGTATATGCTCTTTACAGATCATTTACACGGAAATACATACGGCCATGCTATT
AACTTTTTACGTGTAGATAAACATAACCCTAATGCGCCTGTTTACCTTGGATTTTCAACCAGCAATGTAGGATCT
TTGAATGAACACTTTGTAATGTTTCCAGAGTCTAACATTGCTAACCATCAACGCTTTAATAAGACCCCTATAAAA
GCCGTTGGAAGTACAAAAGATTATGCCCAAAGAGTAGGCACTGTATCTGATACTATTGCAGCGATCAAAGGAAA
AGTAAGCTCATTAGAAAATCGTTTGTCGGCTATTCATCAAGAAGCTGATATTATGGCAGCCCAAGCTAAAGTAA
GTCAACTTCAAGGTAAATTAGCAAGCACACTTAAGCAGTCAGACAGCTTAAATCTCCAAGTGAGACAATTAAAT
GATACTAAAGGTTCTTTGAGAACAGAATTACTAGCAGCTAAAGCAAAACAAGCACAACTCGAAGCTACTCGTGA
TCAATCATTAGCTAAGCTTGCATCGTTGAAAGCCGCACTGCACCAGACAGAAGCCTTAGCAGAGCAAGCCGCA
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ACAAGAAGCTTTAGCAGCCTTACAAGCTAAACAAAGCAGTCTAGAAGCTACTATTGCTACCACAGAACACCAGT
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GAAATGGTTAAAGAAACGAAACAACTATTAGAAGCTTCAGCAAGATTAGCTGCTGAAAATACAAGTCTTGTAGC
AGAAGCGCTTGTTGGCCAAACCTCTGAAATGGTAGCAAGTAATGCCATTGTGTCTAAAATCACATCTTCGATTA
CTCAGCCCTCATCTAAGACATCTTATGGCTCAGGATCTTCTACAACGAGCAATCTCATTTCTGATGTTGATGAAA
GTACTCAAAGACATATGTAG

- Amino Acid

GIASAAQDDRASGETKASNTHDDSLPKPETIQEAKATIDAVEKTLSQQKAELTELATALTKTTAEINHLKEQQDNEQK
ALTSAQEIYTNTLASSEETLLAQGAEHQRELTATETELHNAQADQHSKETALSEQKASISAETTRAQDLVEQVKTSE
QNIAKLNAMISNPDAITKAAQTANDNTKALSSELEKAKADLENQKAKVKKQLTEELAAQKAALAEKEAELSRLKSSAP
STQDSIVGNNTMKAPQGYPLEELKKLEASGYIGSASYNNYYKEHADQIIAKASPGNQLNQYQDIPADRNRFVDPDNL
TPEVQNELAQFAAHMINSVRRQLGLPPVTVTAGSQEFARLLSTSYKKTHGNTRPSFVYGQPGVSGHYGVGPHDKTI
IEDSAGASGLIRNDDNMYENIGAFNDVHTVNGIKRGIYDSIKYMLFTDHLHGNTYGHAINFLRVDKHNPNAPVYLGFS
TSNVGSLNEHFVMFPESNIANHQRFNKTPIKAVGSTKDYAQRVGTVSDTIAAIKGKVSSLENRLSAIHQEADIMAAQA
KVSQLQGKLASTLKQSDSLNLQVRQLNDTKGSLRTELLAAKAKQAQLEATRDQSLAKLASLKAALHQTEALAEQAA
ARVTALVAKKAHLQYLRDFKLNPNRLQVIRERIDNTKQDLAKTTSSLLNAQEALAALQAKQSSLEATIATTEHQLTLLK
TLANEKEYRHLDEDIATVPDLQVAPPLTGVKPLSYSKIDTTPLVQEMVKETKQLLEASARLAAENTSLVAEALVGQTS
EMVASNAIVSKITSSITQPSSKTSYGSGSSTTSNLISDVDESTQRHM

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).

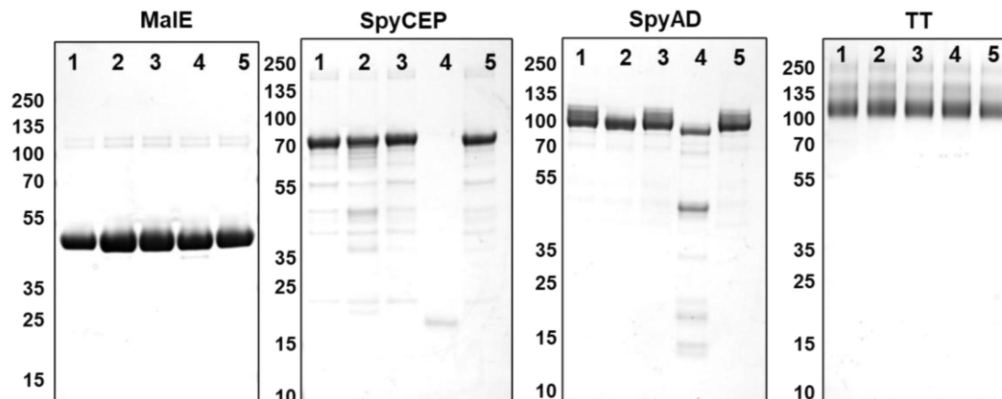

Figure 7.1: 10 ug of purified recombinant proteins separated by SDS-PAGE and Coomassie stained after storage in chemical conjugation reaction buffers for one month at various temperatures.

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_{600nm} reduction. Δ*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 Δ*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_{600nm} before incubation with PlyC.

Time (mins)	WT GAS	Δ*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

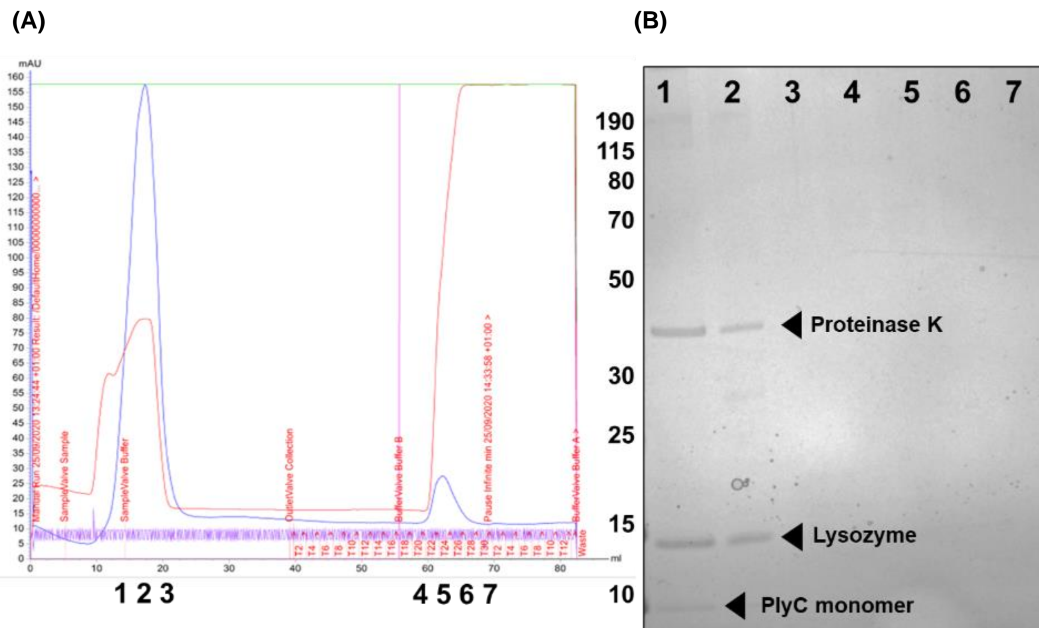

Figure 7.2: PlyC purification from GAC polysaccharide.
(A) Chromatograph of PlyC extracted GAC and resulting eluted fractions. UV_{280nm} absorbance shows removal of contaminating proteins (1 – 3) and elution of GAC (4 – 7).

(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 Δ*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 Δ*gacI* GAS cell walls (Figure 7.3b).

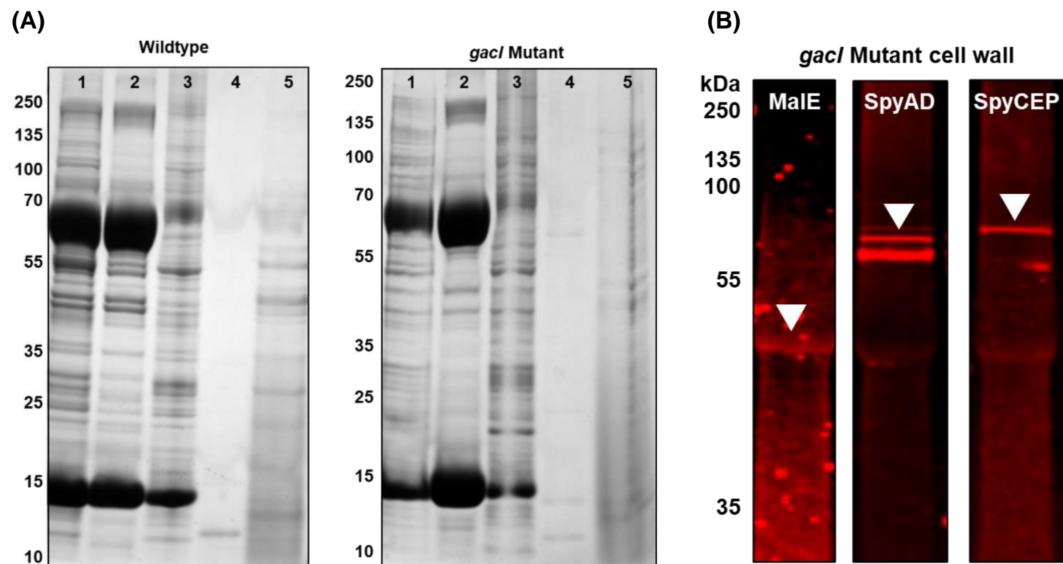

Figure 7.3: WT and Δ*gacI* GAS protein profiles and recognition by murine GAS protein antigen specific antisera.

(A) WT and Δ*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 Δ*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 MalE, 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.

Protein Carrier	Reactant Ratios	Conditions
MalE	14 : 1 *m/m* GAC : MalE 1 : 0.5 *w/w* GAC : MalE (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 *m/m* GAC : MalE 1 : 3.6 *w/w* GAC : MalE (repeated with 2-ME inactivation and $NaHCO_3$ 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
SpyCEP	1 : 9.2 *m/m* GAC : SpyCEP 1 : 1 *w/w* GAC : SpyCEP 1 : 2.7 *m/m* GAC : SpyCEP 1 : 24 *w/w* GAC : SpyCEP	Lyophilised GAC EDC 50 mM MES Protein buffer exc. into MES Protein incubated room temp overnight Hydrophobicity purification – C-18
	1 : 2 *m/m* GAC : SpyCEP 1 : 17.9 *w/w* GAC : SpyCEP 5 : 1 *m/m* GAC : SpyCEP 1 : 17.9 *w/w* 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
	8.75 : 1 *m/m* GAC : SpyCEP 1 : 1 *w/w* GAC : SpyCEP	As above pH increase with $NaHCO_3$ 2-ME inactivation Reaction left 2 hrs and overnight 4 °C Affinity purification – His tag
TT	2 : 1 *m/m* GAC : TT 6.4 : 1 *w/w* GAC : TT	Sulfo-NHS 2 step reaction pH increase with $NaHCO_3$ 2-ME inactivation Reaction left 3 hrs and overnight SEC purification
	12.8 : 1 *m/m* GAC : TT 1 : 1 *w/w* GAC : TT	Sulfo-NHS 2 step reaction pH increase with $NaHCO_3$ 2-ME inactivation Reaction left 3 hrs and overnight Spin filter buffer exc. PBS
SpyAD	23.3 : 1 *m/m* GAC : SpyAD 1 : 0.62 *w/w* GAC : SpyAD	Sulfo-NHS 2 step reaction 10 fold molar excess EDC in MES 2 hr GAC activation 2-ME inactivation pH increase with $NaHCO_3$ Reaction left 3 hrs and overnight 4 °C Spin filter buffer exc. PBS

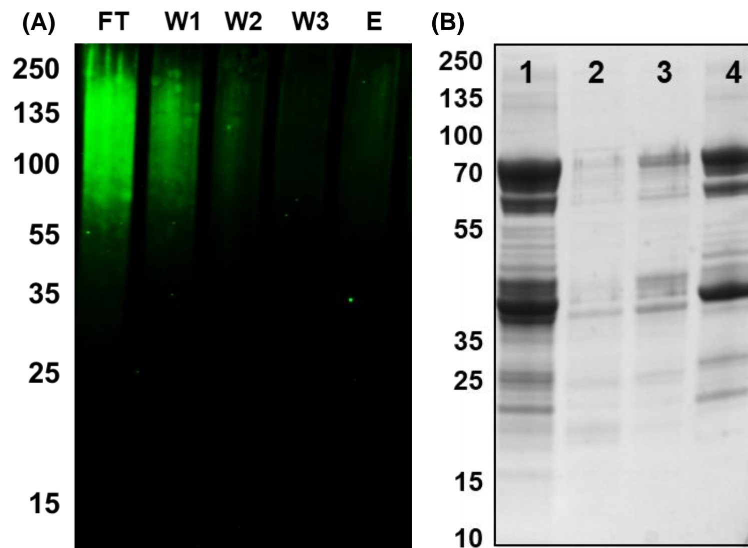

Figure 7.4: Analysis of chemical conjugation reactions and clean up approach.
(A) GAC interaction with 50 kDa MWCO spin filter. Flowthrough **(FT),** three washes **(W 1 – 3)** and final elution **(E)** separated by SDS-PAGE and stained with anti-GAS antibodies detected with Rabbit anti-Goat IgG IRDye® - 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.

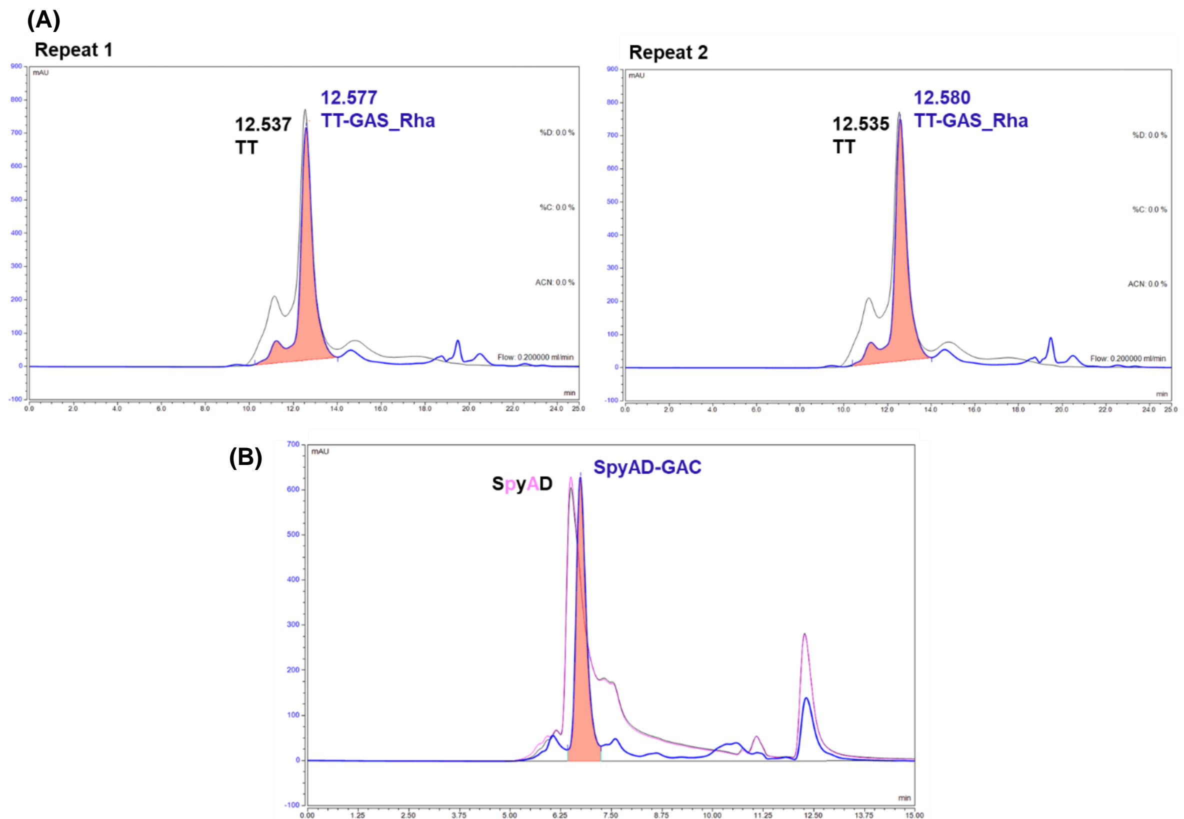

Figure 7.5: TT-GAS_Rha and SpyAD-GAC chemical glycoconjugate HPLC-SEC testing.
(A) TT-GAS_Rha (blue) showed consistent retention times (RT) between two independent runs (RT = 12.577 and 12.580 for repeat 1 and 2 respectively) with a minor shift from TT protein alone (black) (RT = 12.537 and 12.535 for 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).

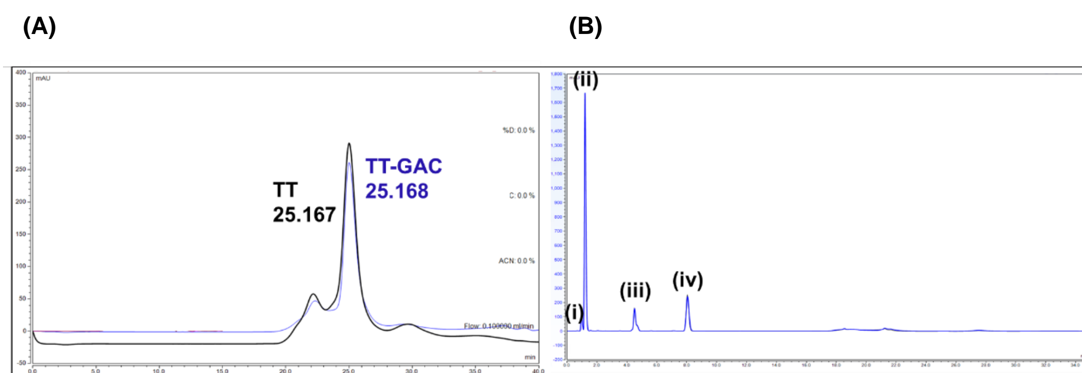

Figure 7.6: TT-GAC physicochemical testing using HPLC-SEC and RP-HPLC.
(A) TT-GAC (blue) did not show any difference in retention time compared to TT alone (black) using HPLC-SEC techniques with UV 280 nm as a detection method. RT = 25.167 (TT) and 25.168 (TT-GAC).

(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 heterogenicity (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	
	Affinity Chromatography	Separation of unconjugated PS based on presence of His tag on conjugated protein carrier – HisTrap / NiNTA	Small scale only
	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	Colourmetic 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	