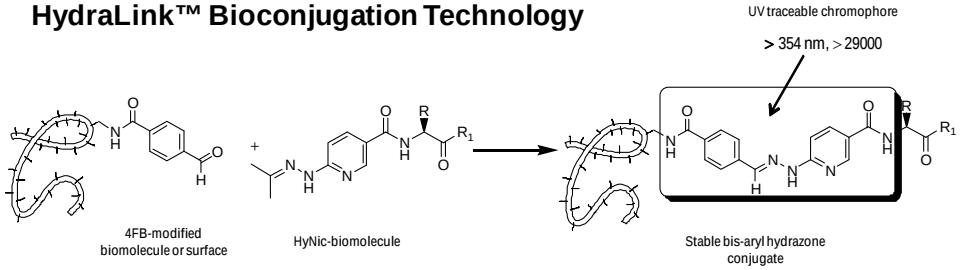
BIOL 052 - Conjugation and immobilization of proteins, peptides and oligonucleotides mediated by a stable bis-arylhydrazone based on 6-hydrazinonicotinic acid, David Schwartz*, Candice Miller, Jim Williams, Leopoldo Mendoza, and Jamie McDonald, Solulink Biosciences, 9853 Pacific Heights Blvd., Ste H, San Diego, CA 92121.

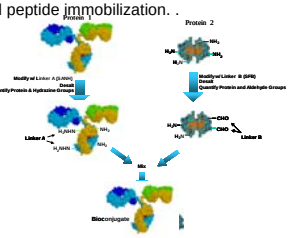
Abstract: Biomolecular conjugates are crucial components of both diagnostic assays and therapeutic modalities. Classical bioconjugation couples such as biotin/streptavidin and maleimido/thiol have been employed successfully for many years but both methods have significant deficiencies. Solulink (www.solulink.com) has discovered and engineered a bioconjugation chemistry to conjugate and immobilize all biomolecules that is based on the formation of a stable bis-aryl hydrazone formed by the reaction of HyNic (6-hydrazinonicotinamide) with 4FB (4-formylbenzamide). The hydrazone is stable to elevated temperature, >90^{O}C/2 h and pH 2-10. The bis-arylhydrazone that is formed is chromophoric, 354 nm, 29,000 extinction coefficient, that allows monitoring of conjugations in real time and direct quantification of molar substitution ratios of conjugates. Examples demonstrating the advantages of the conjugation and immobilization of biomolecules using this chemistry are presented below. Examples will include preparation of protein/protein conjugates, oligo/antibody conjugates for siRNA delivery and iPCR, peptide/oligo conjugates and peptide immobilization. .

HydraLink™ Bioconjugation Technology

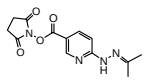


Ideal Conjugation/Immobilization Couple

- modified biomolecules stable over extended periods
- both moieties unreactive to all functional groups on biomolecules
  - no undesirable covalent modifications
  - no electrostatic/hydrophobic interactions
- formation of stable covalent bond
  - thermal stability
  - pH stability
- fast reaction kinetics
- amenable to oligonucleotide and peptide solid phase syntheses

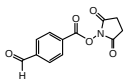


SoluLink's Bifunctional Linking Molecules



S-HyNic
Succinimidyl 6-hydrazinonicotinamide acetone hydrazone (SANH)

- *single step conversion: lysine → HyNic stable conjugates formed*
- *developed for bonding Tc(V)=O species to ligands (proteins, small molecules)*
  - *Phase III clinical trial for thrombus imaging*

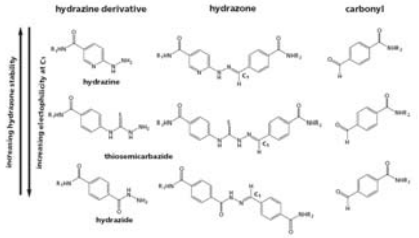


S-4FB
Succinimidyl 4-formylbenzoate (SFB)

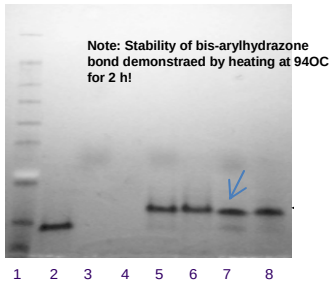
- *4FB bifunctional linker*
  - *amino reactive*
  - *4FB-modified biomolecues have extended stability in aqueous buffers*
  - *4FB do not interact with biomolecules*
  - *react readily with HyNic to form stable bis-aryl hydrazones*

Other HyNic and 4FB derivatives available including extended PEG4, disulfide and maleimido

Hydrazone Stability



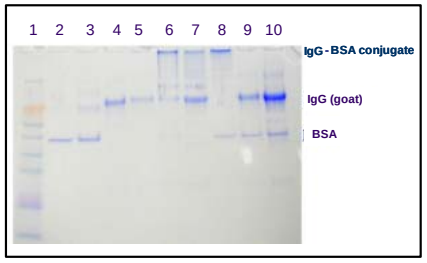
Peptide/Protein Conjugation



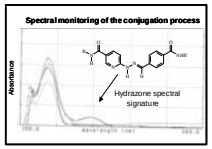
1) MW markers
2) 5'-4FB-18mer oligonucleotide
3) HyNic-peptde 1
4) HyNic-peptide 2
5) HyNic-peptide 1 + 5'-4FB-18mer oligonucleotide
6) HyNic-peptide 2 + 5'-4FB-18mer oligonucleotide
7) 5 heated at 94^{O}C for 2 h
8) 6 heated at 94^{O}C for 2 h

Demonstration of the preparation of a a peptide/oligo conjugate using HydraLink Bioconjugation Technology by the addition of 4 mol equiv HyNic-peptide to 1 mol equiv. 4FB-oligo. Both modified molecules were prepared by their respective solid phase synthesis methods.

Protein/Protein Conjugation

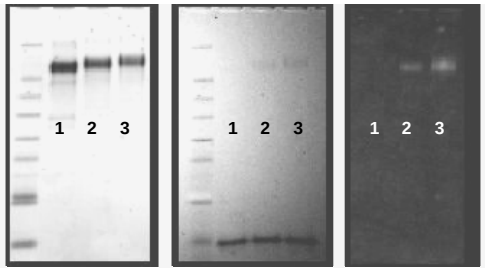


1) MW markers
2) BSA
3) BSA-(HyNic)$_7$
4) IgG
5) IgG-(4FB)$_8$
6) BSA-(HyNic)$_7$ + IgG-(4FB)$_8$ (1/1 stoichiometry)
7) BSA-(HyNic)$_7$ + IgG-(4FB)$_8$ (1/3 stoichiometry)
8) BSA-(HyNic)$_7$ + IgG-(4FB)$_8$ (3/1 stoichiometry)
9) BSA + IgG-(4FB)$_8$
10) BSA-(HyNic)$_7$ + IgG



Demonstration of the preparation of a a protein/protein conjugate using HydraLink Bioconjugation Technology. BSA is modified with S-HyNic to incorporate HyNic moieties and IgG is modified with S-4FB to incorporate 4FB moieties. Addition of HyNic-BSA to 4FB-IgG at in 100 mM phosphate, 150 mM NaCl, pH 6.0 yields the conjugate

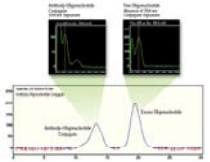
Antibody/Oligonucleotide Conjugation



Lane 1) Control unmodified protein + 5'-4FB-oligo

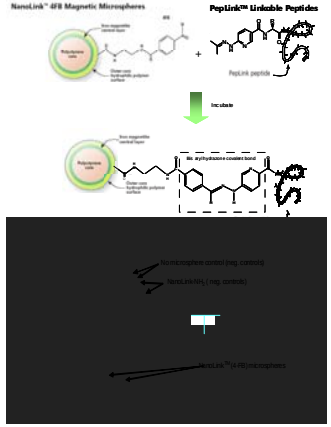
Lane 2) HyNic-modified IgG+ 5'-4FB-oligo (3 equiv)

Lane 3) HyNic-modified IgG+ 5'-4FB-oligo (6 equiv)



Demonstration of the preparation of oligo/protein conjugates by addition of 4FB-modified oligonucleotide to HyNic-modified IgG. The conjugation is very efficient as the ratio added oligo /conjugated oligo is 2/1 mol/equiv. The chromatogram on the lower left shows the separation of the conjugate from excess oligo by SE-HPLC. Note the A354 absorbance of the conjugate in its UV spectrum.

Peptide/Magnetic bead Immobilization



A 14mer N-terminus-HyNic-modified peptide was immobilized to Solulink's NanoLink 4FB-magnetic beads by incubation in 100 mM MES buffer, pH 5.0 for 1 h. Reaction was followed by monitoring loss of A280 absorbance from solution due to immbolization of HyNic-peptide on 4FB-beads.