LEHIGH UNIVERSITY

INSPIRING GREAT IMAGINATIONS

Protein Renaturation and Fertilization:
Effects on protein renaturation and sperm functions by novel organic compounds

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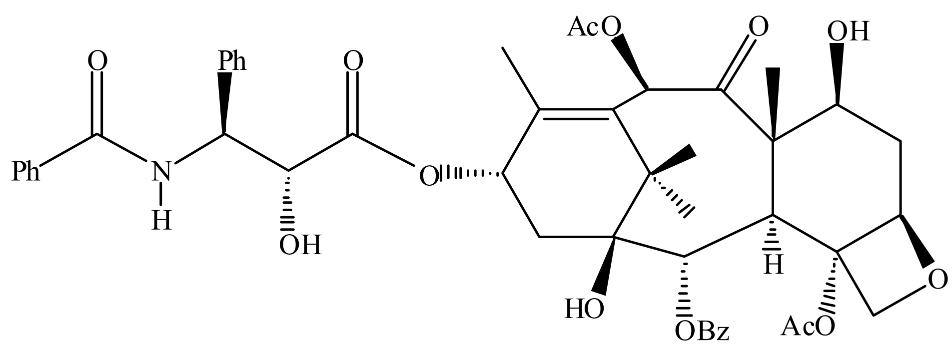


Taxol

Outline

I. Organic Synthesis

A. Valency and trivalent carbon

B. Nucleophilic Substitution

C. Bond Line Structures

II. Protein Structure and Renaturation

A. Current Protocols

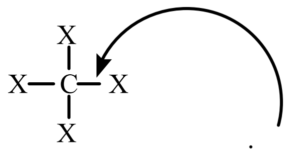
B. Organic Salts

C. Hybrid Fluorous Surfactants

III. Use of Hybrid Fluorous Surfactants as Novel Contraceptives

Organic Synthesis - Where to begin?

Organic chemistry is based on Carbon and related elements.

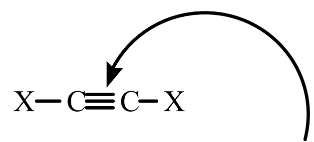


single bond, represents
two electrons

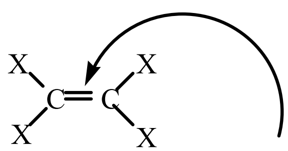
X = any other atom

Carbon is most stable when it has four bonds, the number of bonds is referred to as valency

Carbon is tetravalent



triple bond, three separate bonds, each
containing two electrons

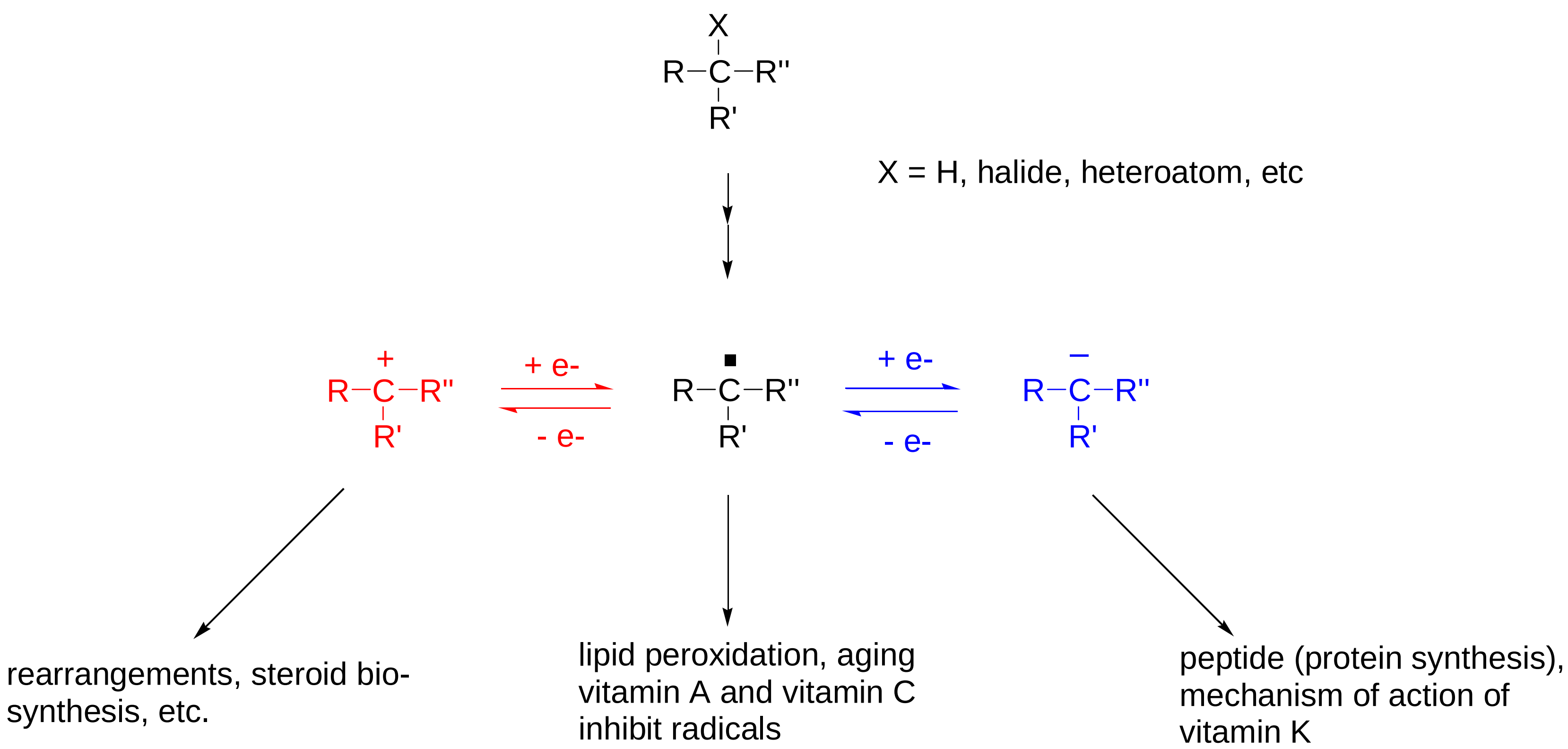


double bond, two different bonds, each
containing two electrons

Is Carbon tetravalent in all of these cases?

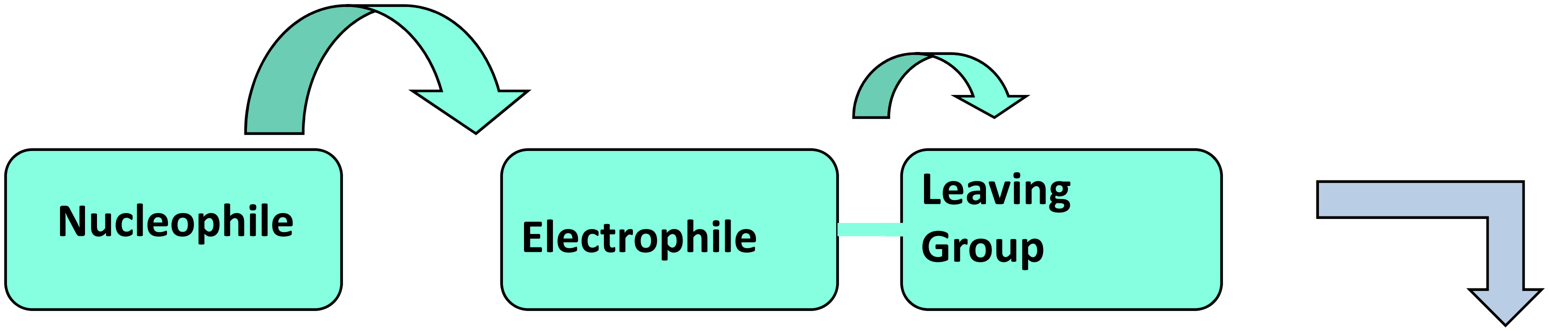
What happens when carbon is trivalent? Is it stable?

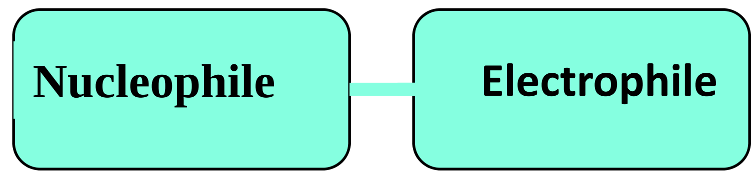
Oxidation States of Trivalent Carbon



R = H, alkyl, aryl; R' = H, alkyl, aryl, R'' = H, alkyl, aryl

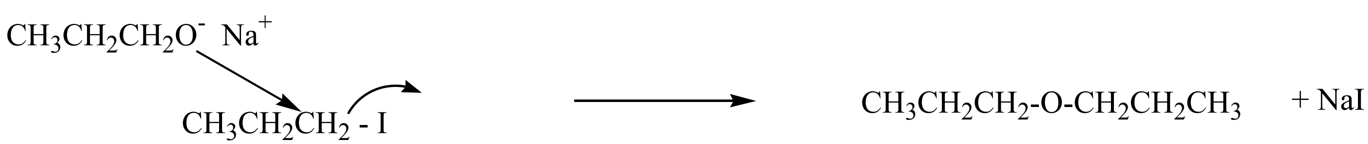
Nucleophilic Substitution





Nucleophile= electron-rich atom

Electrophile= electron-deficient atom



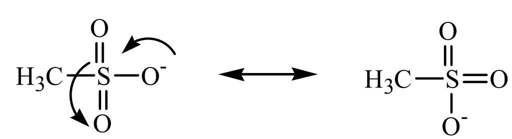
Good Nucleophiles are electron Rich

alkoxides (deprotonated alcohols)
amines

Good Leaving Groups stabilize negative charge well

halides (chloride, bromide, iodide)

molecules that stabilize charge through resonance

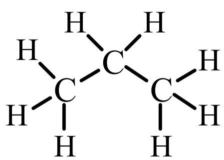


Bond Line Structures - The Language of Organic Chemistry

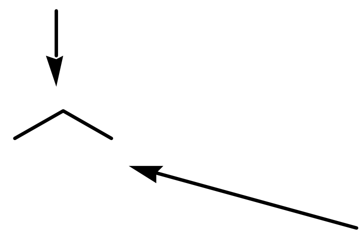
-Bond line structures are the shorthand of organic chemistry

-communication of complex structures

Carbon is located where lines intersect



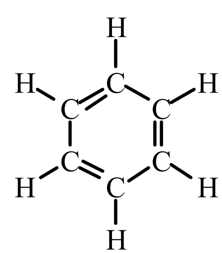
Each carbon is tetravalent



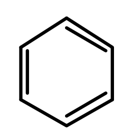
Bond-line structure

carbon is located at the end of a line

If no other type of atom is present, valency is filled out with hydrogen

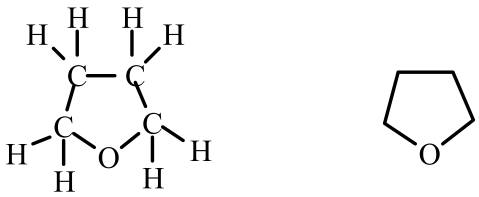


Benzene

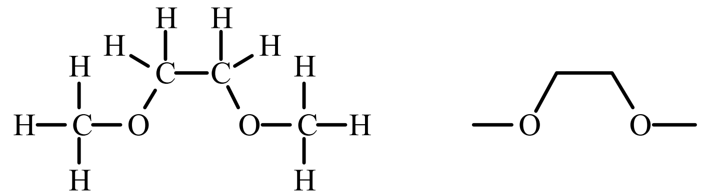


bond line structure of benzene

Bond line structures (continued)

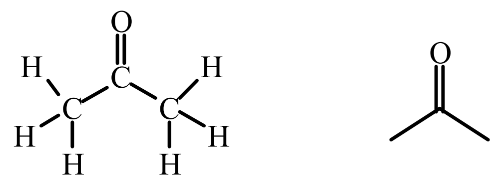


Tetrahydrofuran (THF)

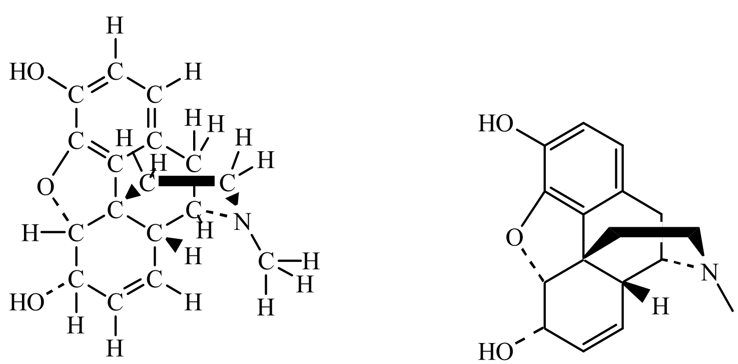


Dimethoxyethane (DME)

Acetonitrile

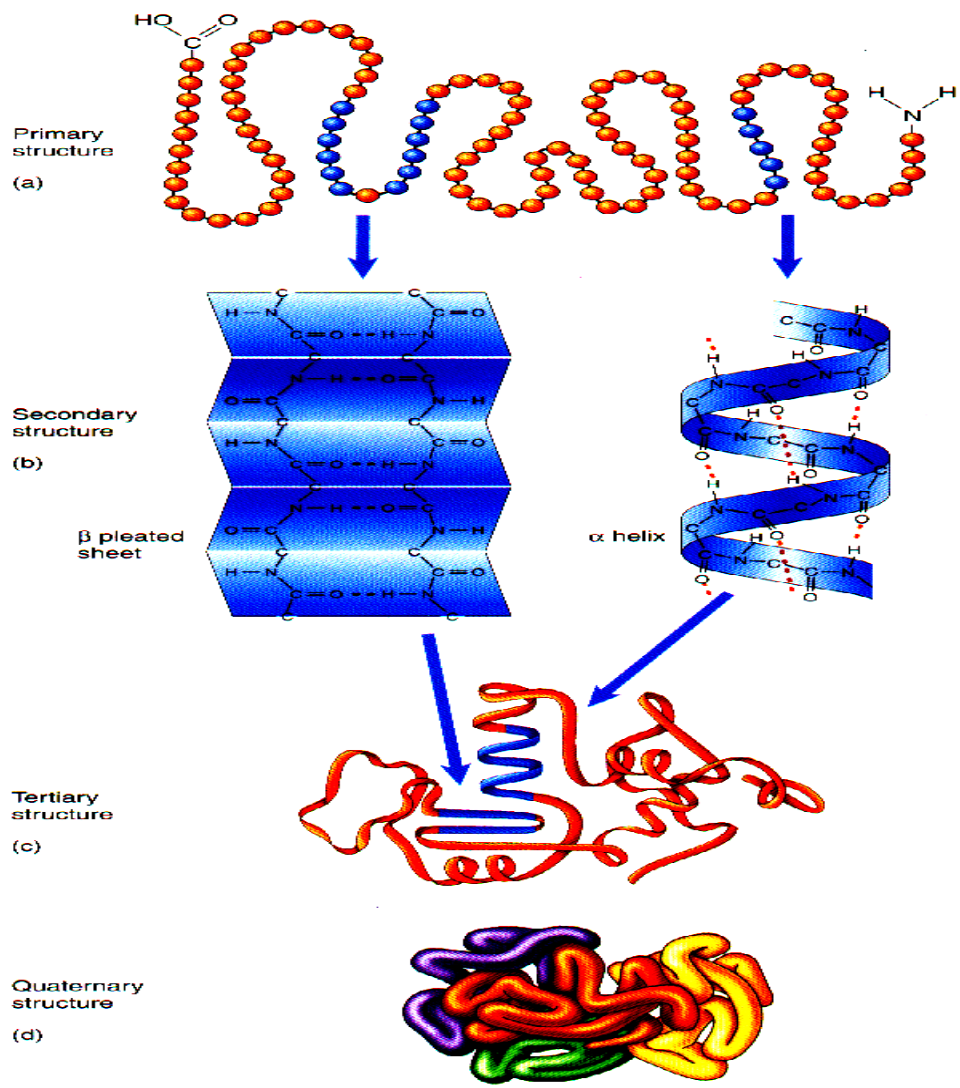


Acetone



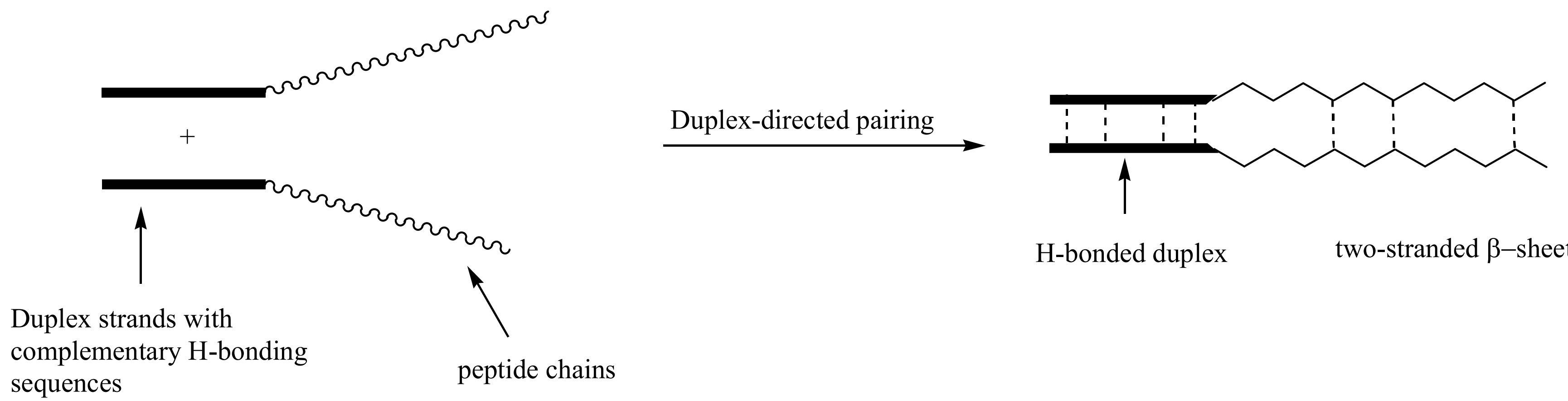
Morphine

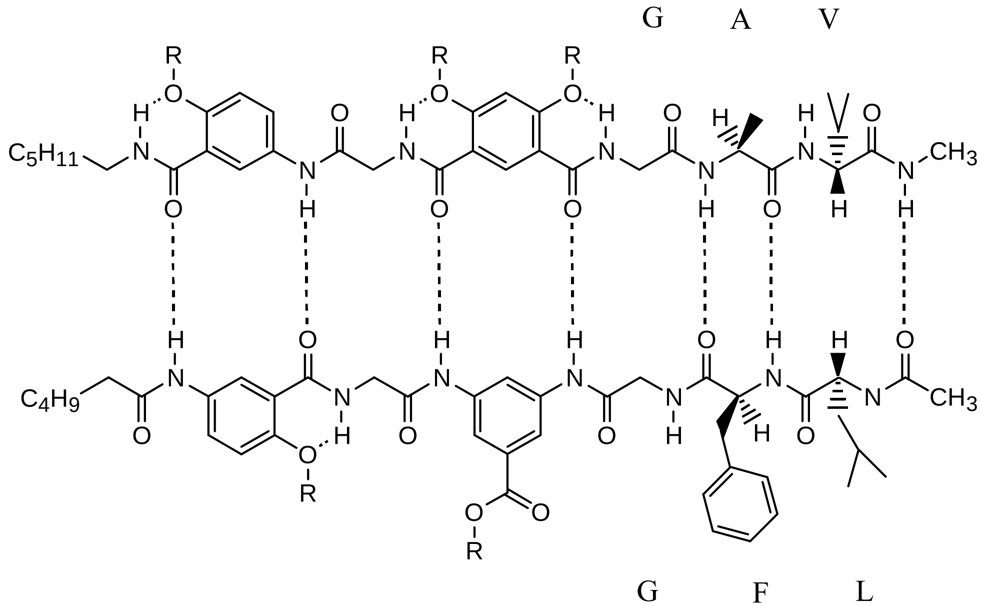
Protein Structure



faculty.uca.edu/~johnc/mole1440.htm

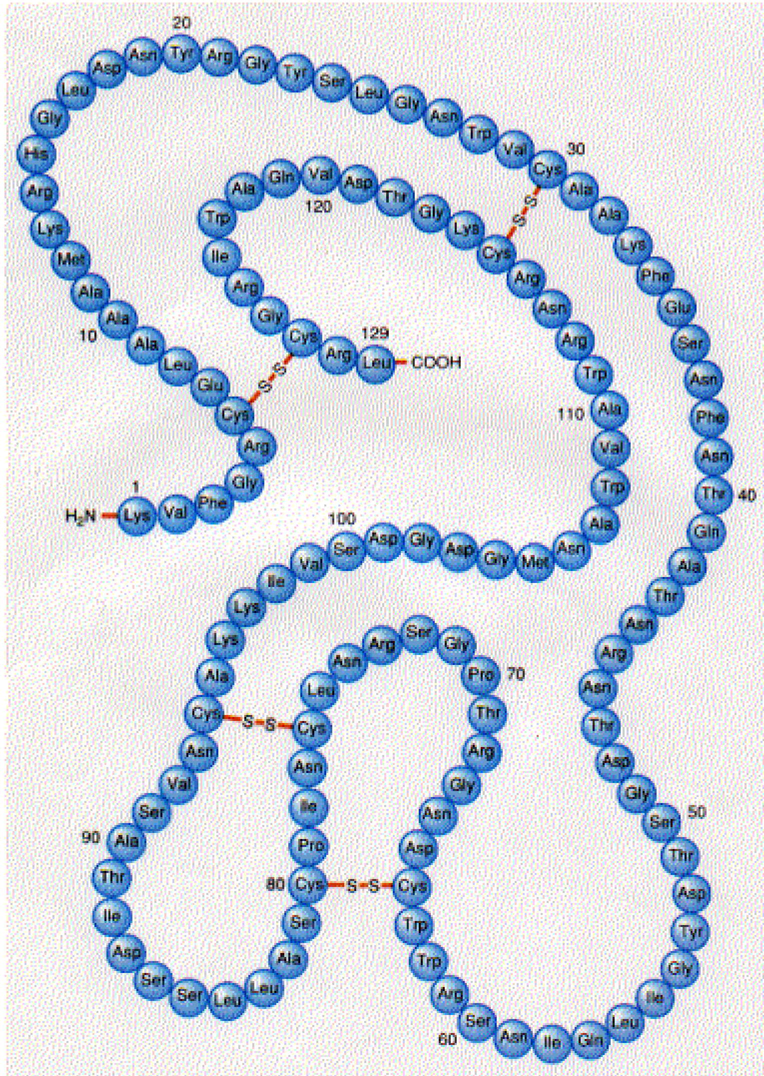
Nucleation of antiparallel β–sheet using a noncovalent, self-assembling approach



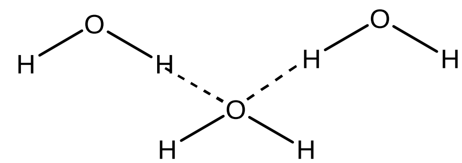


R=C_8H_{17}

Zeng, H.; Yang, X.; Flowers, R.A., II; Gong, B. *J. Am. Chem. Soc.* **2002**, *124*, 2903


H2N
1
Lys Val Phe Gly Arg Cys Glu Leu Ala
10
Ala Ala Ala Met Lys Arg His Gly Leu Asp Asn Tyr
20
Arg Gly Tyr Ser Leu Gly Asn Trp Val Cys
30
Ala Ala Lys Phe Glu Ser Asn Phe Asn Thr
40
Gln Ala Thr Asn Arg Asn Thr Asp Gly Ser
50
Thr Asp Tyr Gly Ile Leu Gln Ile Asn Ser
60
Arg Trp Trp Cys Asn Asp Gly Arg Thr Pro
70
Gly Ser Arg Asn Leu Cys Asn Ile Pro Cys
80
Ser Ala Leu Leu Ser Ser Asp Ile Thr Ala
90
Ser Val Asn Cys Ala Lys Lys Ile Val Ser
100
Asp Gly Asn Gly Met Asn Ala Trp Val Ala
110
Trp Arg Asn Arg Cys Lys Gly Thr Asp Val
120
Gln Ala Trp Ile Arg Gly Cys Arg Leu
129
COOH
S-S
S-S
S-S
S-S

Factors Involved in Protein Stability



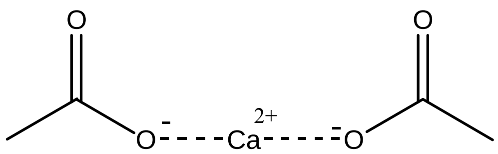
hydrogen bond

-Hydrophobic Core

-Disulfide Bonds

-Hydrogen Bonds

-Electrostatic Interactions



electrostatic interaction

Disruption of one or more of the stabilizing interactions can lead to unfolding

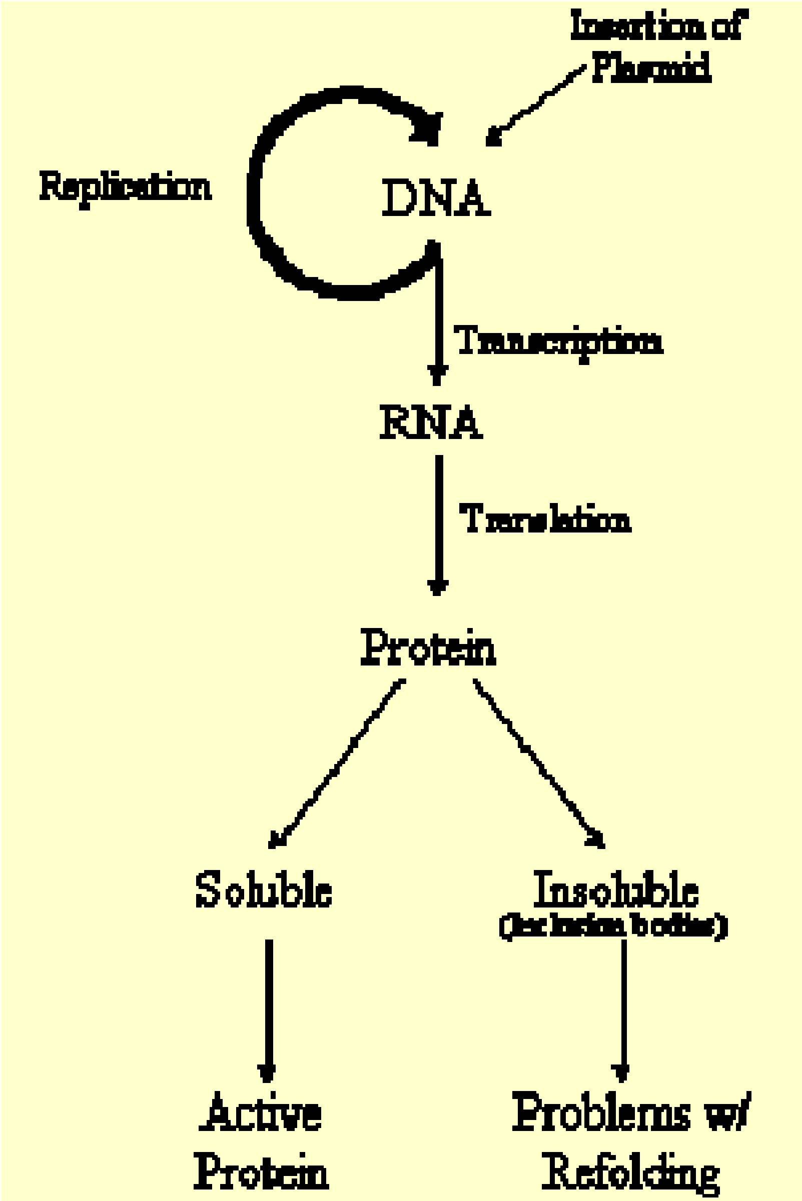
-pH extremes

-heat

-addition of denaturants

When denaturation conditions are removed, some proteins spontaneously refold

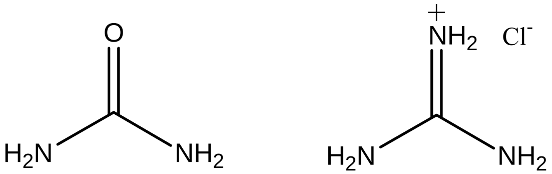
The Problem


Insertion of Plasmid
Replication
DNA
Transcription
RNA
Translation
Protein
Soluble
Insoluble
(inclusion bodies)
Active Protein
Problems w/ Refolding

Methods for Refolding Aggregates

Common steps in the renaturation process

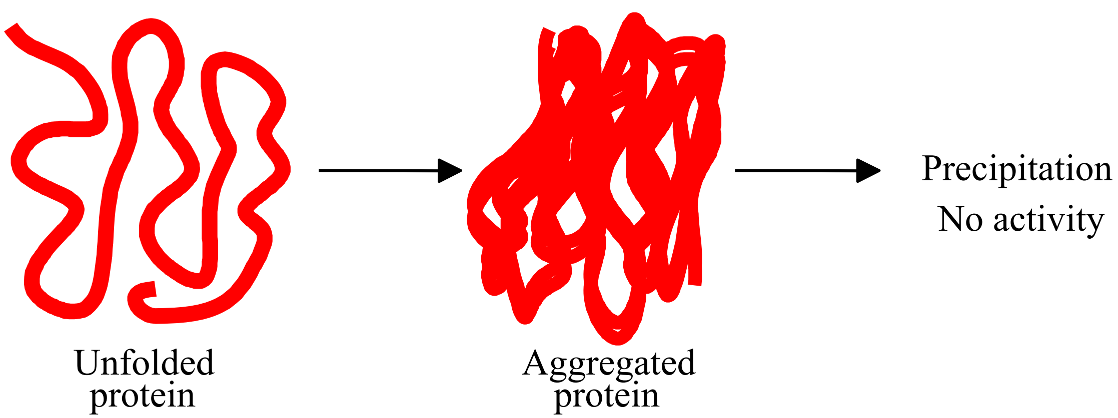
- Isolation of inclusion bodies
- Dissolution of the proteins by a denaturant
  Either urea or guanidinium hydrochloride
- Removal of the denaturant by dialysis or dilution
- Optimize the conditions to minimize aggregation



urea **guanidinum hydrochloride**

The problem with this method:

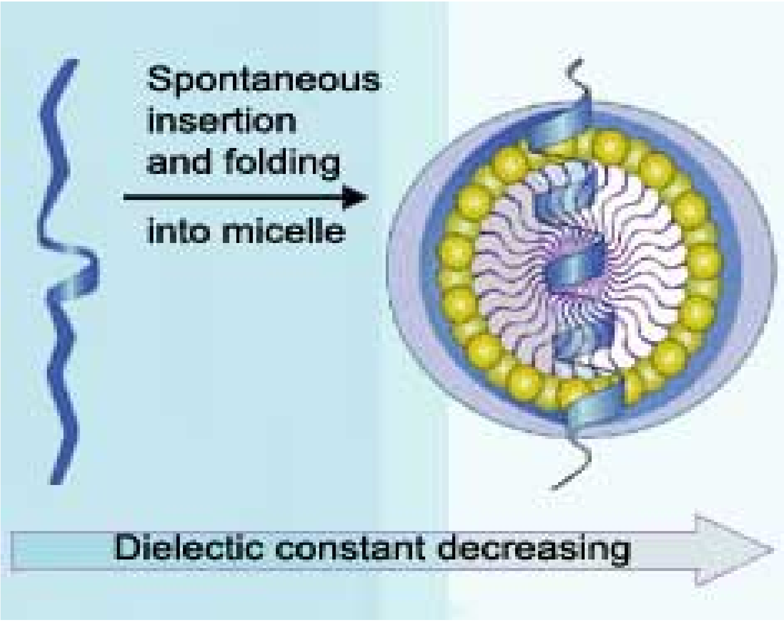
- Formation of inter- and intramolecular bonds
- Only works at low concentrations
- Low refolding yields



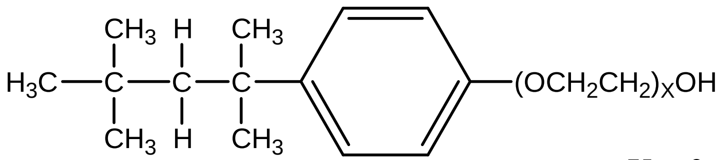
Detergent-Assisted Refolding

"Micelle Assisted Refolding"

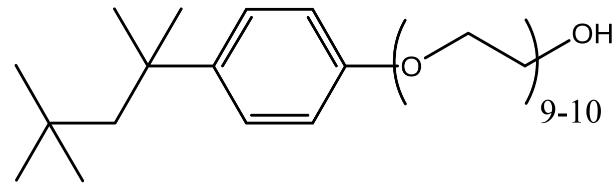
- Horowitz and Tandon found that the addition of detergents helped to refold Rhodanese.
- Intermediate with exposed hydrophobic surfaces can partition into native or inactive species.
- Detergent prevented self-association.
- Individual detergents has a specific concentration range that provided reasonable refolding yields.
  - Zardeneta, G.; Horowitz, PM *J. Biol. Chem.* **1992** *167*, 5811.



Some Detergents

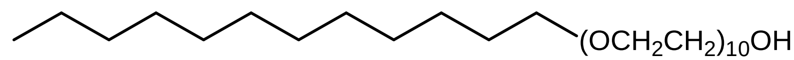


X = 9-10



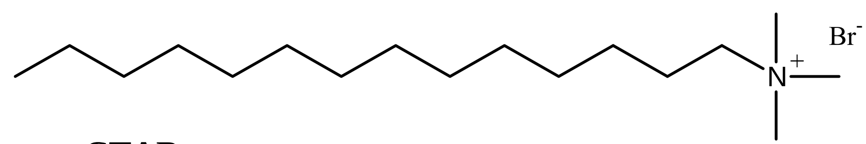
Triton X

$CH_3(CH_2)_{11}(OCH_2CH_2)_{10}$-OH



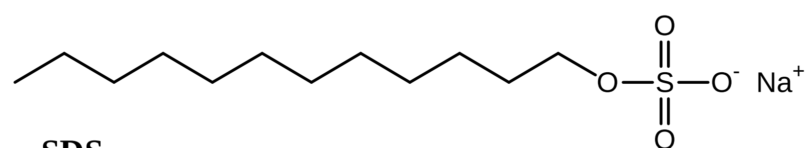
POE(10)L

$CH_3(CH_2)_{14}\overset{+}{N}(CH_3)_3\ Br^-$



CTAB

$CH_3(CH_2)_{11}OSO_3^-\ Na^+$



SDS

Solution?
Synthesize Compounds with shorter hydrocarbon chains

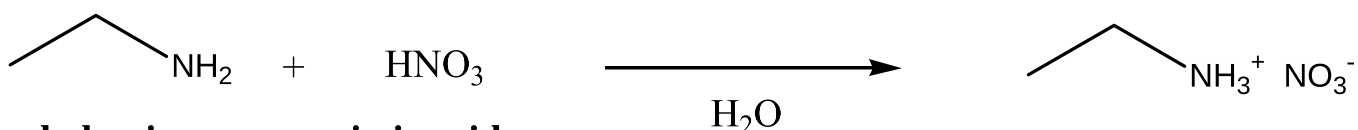
-acid base chemistry

$NaOH\,(aq) + HCl\,(aq) \longrightarrow NaCl + H_2O$

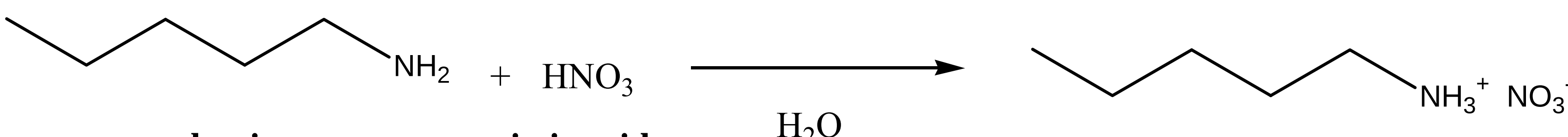
sodium hydroxide + **hydrochloric acid** → **sodium chloride**

$NH_3 + HCl \longrightarrow NH_4^+\,Cl^-$

ammonia + **hydrochloric acid** → **ammonium chloride**



ethylamine **nitric acid** **ethylammonium nitrate**



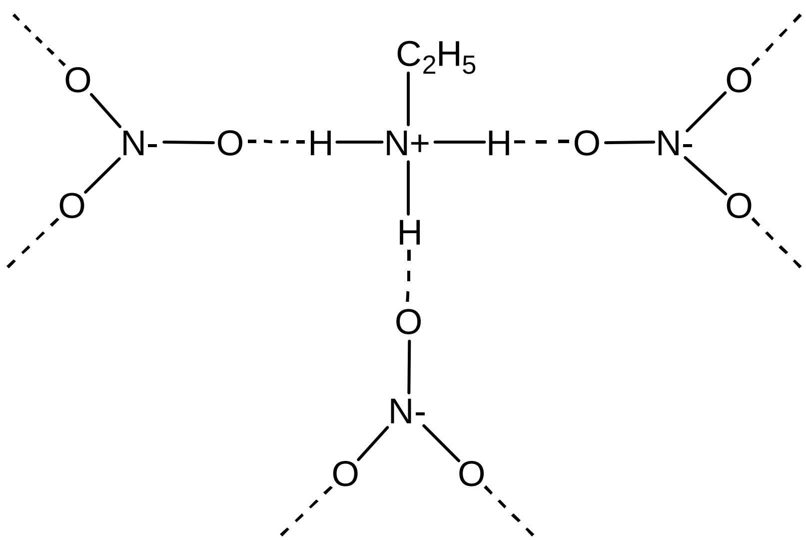
pentylamine **nitric acid** **pentylammonium nitrate**

Organic Salts prepared

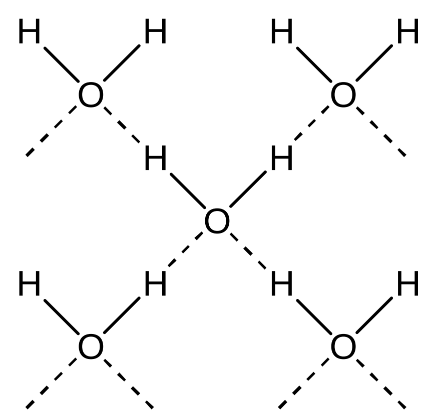
ethylamine	methylamine	propylamine	n-butylamine	n-hexylamine
sulfate SO_4^{2-}	sulfate SO_4^{2-}	sulfate SO_4^{2-}	sulfate SO_4^{2-}	sulfate SO_4^{2-}
nitrate NO_3^-	nitrate NO_3^-	nitrate NO_3^-	nitrate NO_3^-	nitrate NO_3^-
chloride Cl^-	chloride Cl^-	chloride Cl^-	chloride Cl^-	chloride Cl^-
bromide Br^-	bromide Br^-	bromide Br^-	bromide Br^-	bromide Br^-
fluoroborate BF_4^-	fluoroborate BF_4^-	fluoroborate BF_4^-	fluoroborate BF_4^-	fluoroborate BF_4^-
Acetate	Acetate	Acetate	Acetate	Acetate
Trifluoroacetate	Trifluoroacetate	Trifluoroacetate	Trifluoroacetate	Trifluoroacetate
Trichloroacetate	Trichloroacetate	Trichloroacetate	Trichloroacetate	Trichloroacetate
pentafluoropropionate	pentafluoropropionate	pentafluoro-propionate	Pentafluoro--propionate	Pentafluoro-propionate
phosphate				
$H_2PO_4^-$ / PO_4^{3-}				

Ethylammonium Nitrate

- Ethylammonium nitrate (EAN) is a clear, viscous, colorless, ionic liquid that freezes below room temperature.
- EAN resembles a detergent with a very small hydrophobic tail.



Ethylammonium nitrate



Water

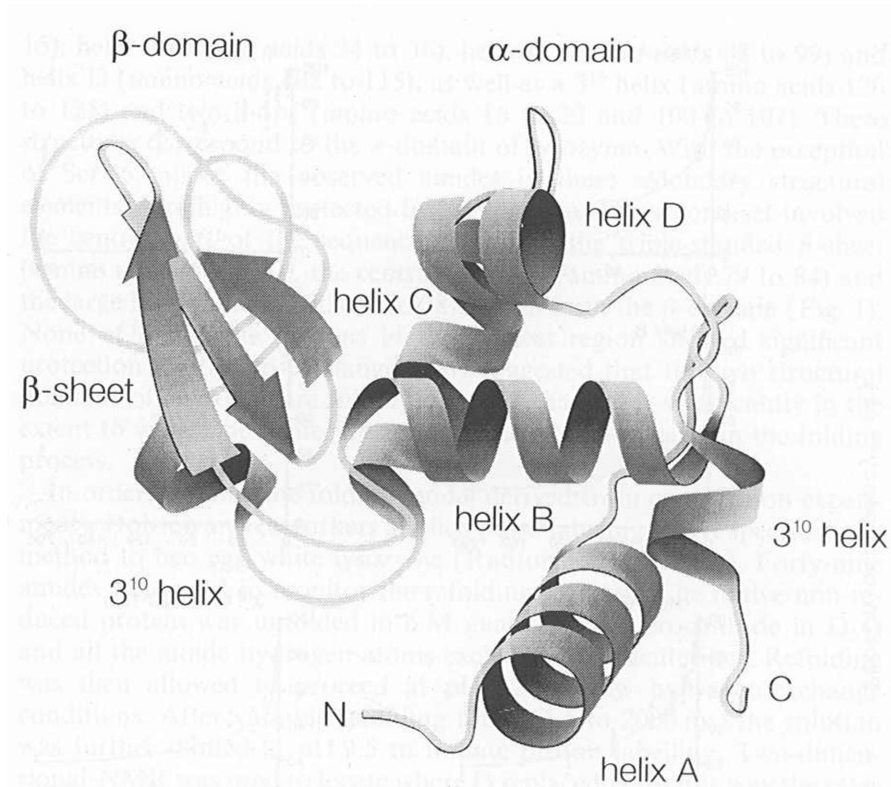
Hen Egg White Lysozyme

Damages bacterial cell walls by catalyzing hydrolysis of structural carbohydrates

Competition between aggregation and refolding is the major obstacle for the production of this enzyme.

Contains all the typical elements found in most proteins.

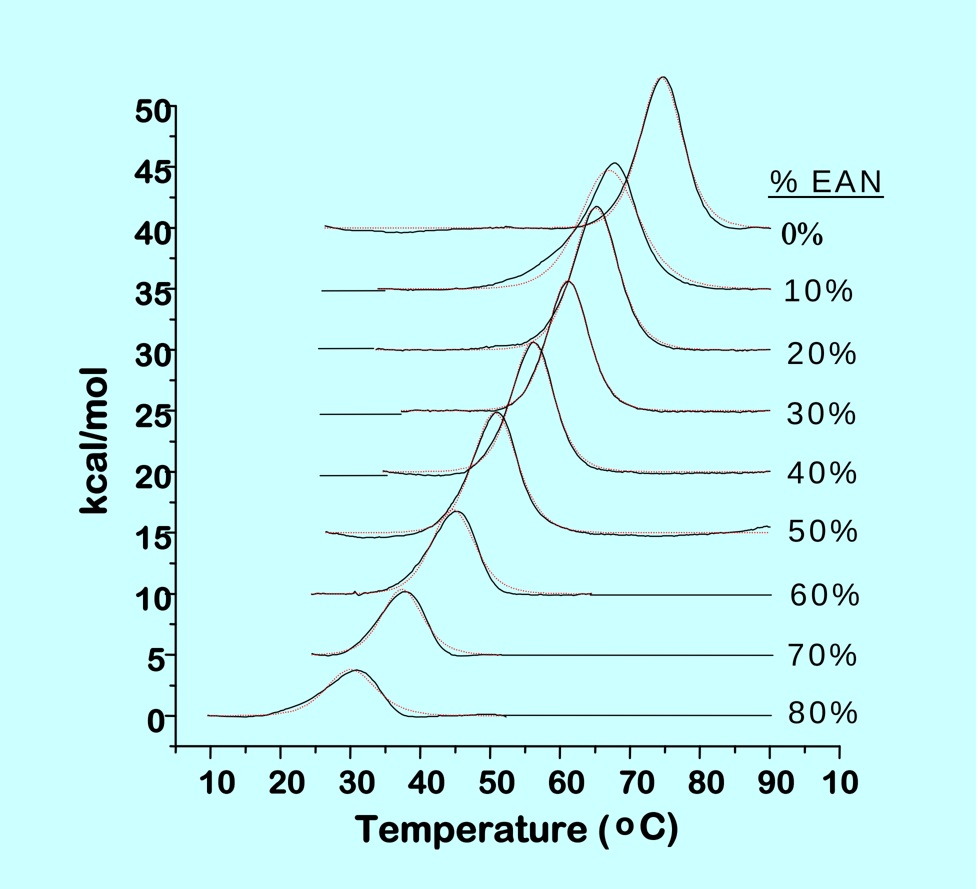
Renatures successfully at low concentrations, but precipitates at higher concentrations.



Increasing [EAN]

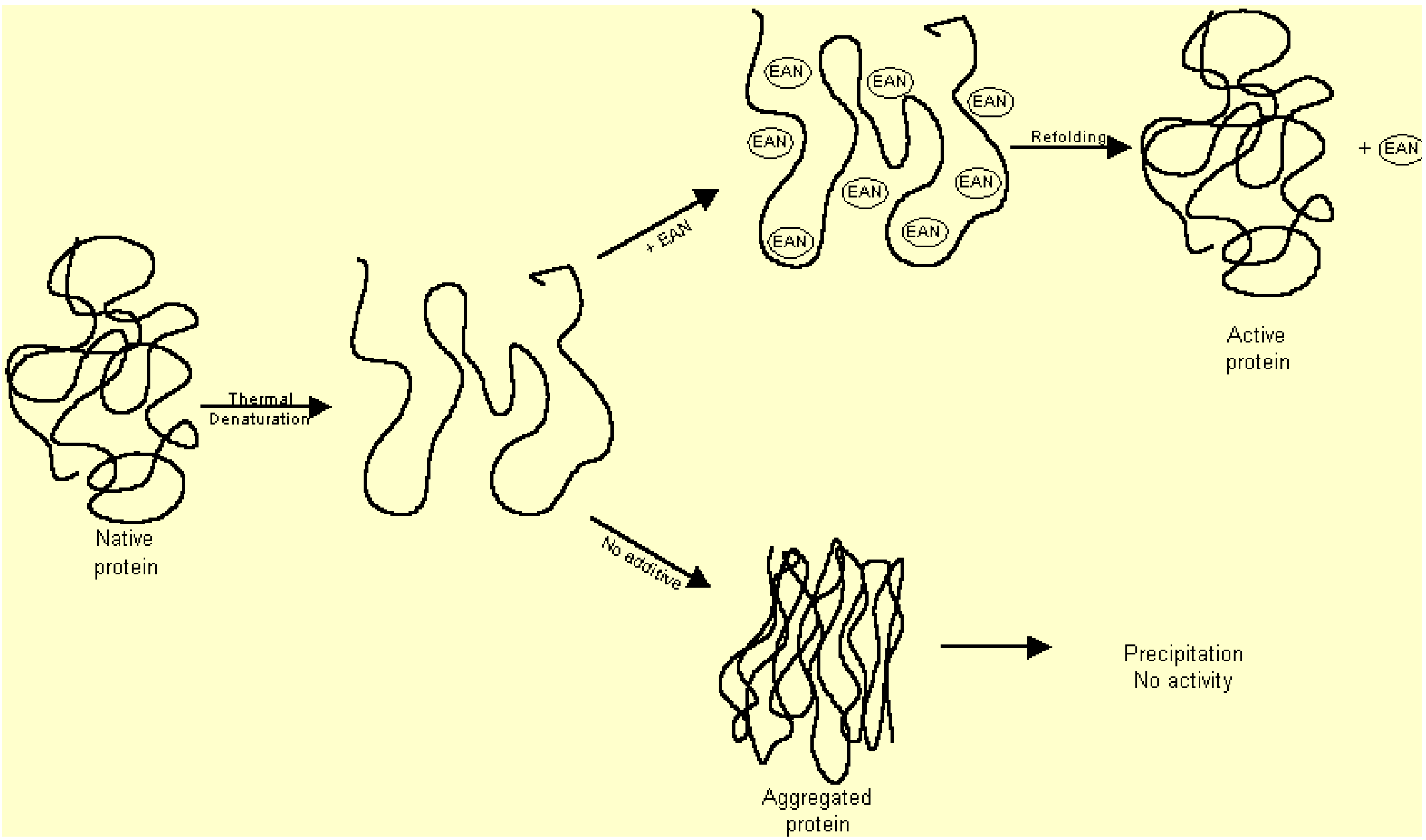
Thermodynamic Results

- Examination of HEWL in the presence of EAN solution
- The T_m steadily decreases as the amount of EAN increases
- There is still evidence of folding in an 80% EAN solution



What may be happening

-During thermal denatauration the hydrophobic core is exposed and the disulfide bonds remain intact



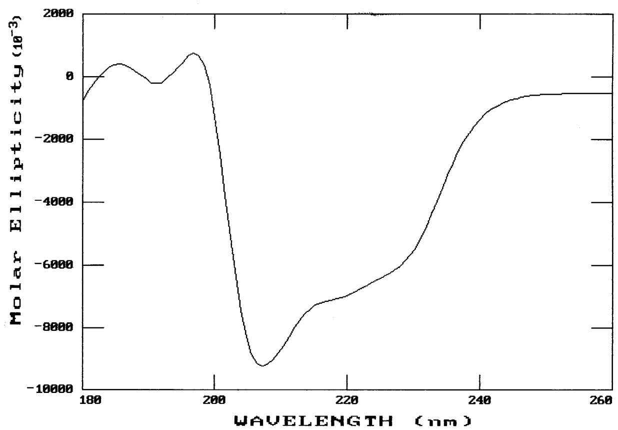
Increasing [EAN]

Sample[a]	$M_{additive}$	% EAN	Aggregation	Activity (x10[4] U/mg protein)	% Activity
control[b]	none	0%	no	1.5 ± 0.1	100 ± 10%
2	none	0%	yes	0.008 ± 0.001	1.0 ± 0.1%
3	0.05 M EAN	0.5%	yes	0.25 ± 0.09	16 ± 9%
4	0.16 M EAN	1%	yes	0.34 ± 0.03	22 ± 3%
5	0.54 M EAN	5%	yes	1.15 ± 0.03	75 ± 3%
6	1.01 M EAN	11%	yes	0.33 ± 0.09	22 ± 9%
7	3.07 M EAN	33%	yes	0.19 ± 0.03	12 ± 3%
8	5.09 M EAN	55%	yes	0.42 ± 0.03	27 ± 3%
9	0.018 M CTAB	0%	yes	0.08 ± 0.02	5 ± 2%

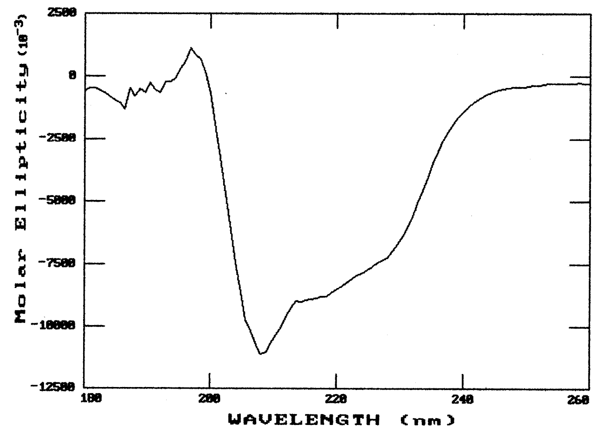
[a]Denaturation Conditions: 14 μL of a solution containing 8.7 M GdmHCl, 143 mM Tris buffer (pH 8.5) and 43 mM DTT, to which 6 μL of 83.5 mg/mL HEWL stock solution was added. The solution of denatured reduced HEWL was diluted in a 1:1 GSH:GSSG solution with Tris buffer (pH 8.5) and the concentration of EAN indicated above. [b]Control solution was diluted with buffer only and not denatured or renatured with any of the respective solutions.

Summers, C.A.; Flowers, R.A. *Protein Science* **2000**, *9*, 2001-2008.

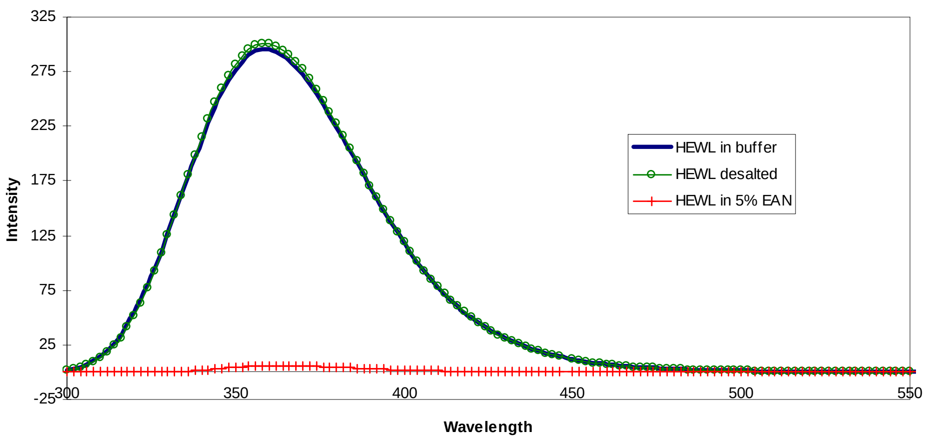
Desalting EAN after renaturation (dialysis)



Far-UV spectrum of native HEWL



Far-UV CD spectrum of HEWL after renaturation and removal of EAN



Fluorescence spectrum of native HEWL and renatured HEWL in the presence and absence of EAN

What about other proteins?

MMP13 (Collagenase 3)

-secreted 452 residue protein

-important in fetal bone development

-plays a role in osteo- and rheumatoid arthritis

-expression leads to intractable inclusion bodies

Johnson, A.R. and coworkers *J. Biol. Chem.* **2007**, *282*, 27781-27791.

Renaturation of MMP13 inclusion bodies

Sample[b]	Additive	Aggregation	Absorbance at 278 nm	Refolded percentage (%)
Control	None	No	0.754	
1	None	Yes	0.023	3
2	0.2M EAN	Yes	0.093	12
3	0.5M EAN	Yes	0.069	9
4	0.2M TFEAN	Yes	0.080	11
5	0.5M TFEAN	Yes	0.070	9

A solution was prepared as 10 mg/ml inclusion bodies of MMP13, 6 M GdmHCl, 25 mM Tris-sulfate, pH 7.6, and was allowed to stay at room temperature for 24 hours. The solution was then diluted with Tris-sulfate buffer containing $CaCl_2$, $ZnCl_2$ and a varying concentration of ammonium salts to give a solution of 0.25 mg/ml MMP13, 25 mM Tris-sulfate, pH 7.6, 10 mM $CaCl_2$, 1 mM $ZnCl_2$ with salt additives. After allowing the sample to stand for 24 hours, salt additives were separated out by dialysis with 0.1 M Tris-sulfate, pH 7.6. The concentration was then determined by reading the absorbance at 278 nm using UV-vis spectroscopy. The folded state of the enzyme was compared to the native one using CD measurements.

Advantages and Disadvantages of the Present Approach

Advantages

All renaturation studies were carried out at relatively high protein concentration.

Dialysis provides a efficient and straightforward way to separate salt additives from protein.

Disadvantages

Does not work well with larger proteins or those containing a significant amount of β-sheet secondary structure

Fluorous Surfactants: A Novel Approach to Protein Renaturation?

-It is known that fluorous solvents are immiscible in aqueous and organic solutions. Combination of a fluorous solvent with water and ether produces a three-phase system.

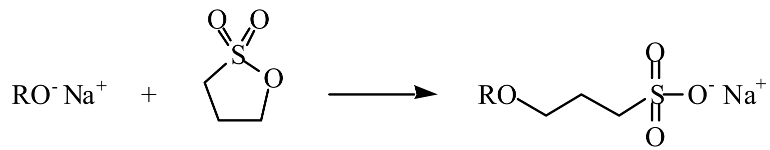
-Presence of a fluorous solvent was shown to have no effect on the activity of HEWL, CAB, etc.

-Design and synthesis of a series of fluorous surfactants.

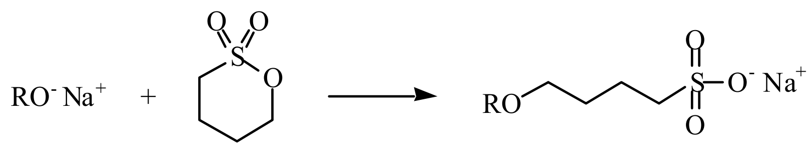
-Can low affinity fluorous surfactants decrease aggregation and promote refolding?

-Can these novel surfactants be removed through dialysis of fluorous biphasic separation?

Synthesis of Fluorous Surfactants.



propane sultone



butane sultone

$CF_3(CF_2)_5CH_2O(CH_2)_3SO_3Na$ **F13PS**

$CF_3(CF_2)_7CH_2O(CH_2)_4SO_3Na$ **F17BS**

$$R_fCH_2OH + ClSO_2C_6H_4CH_3 \xrightarrow{\text{NaOH}} R_fCH_2OSO_2C_6H_4CH_3$$

$$R_fCH_2OSO_2C_6H_4CH_3 + HO(C_2H_4O)_NH \xrightarrow[\text{THF}]{\text{KOH}} R_fCH_2O(C_2H_4O)_NH$$

$CF_3(CF_2)_5CH_2O(C_2H_4O)_3H$ **F13TEG**

Physical Characterization of Fluorous Surfactants

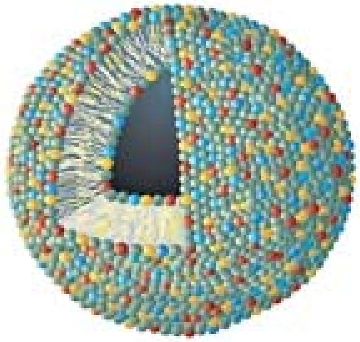
-Spectroscopic characterization

-Determination of critical micelle concentration.

-All fluorous surfactants have a lower CMC than the corresponding hydrocarbon surfactant.

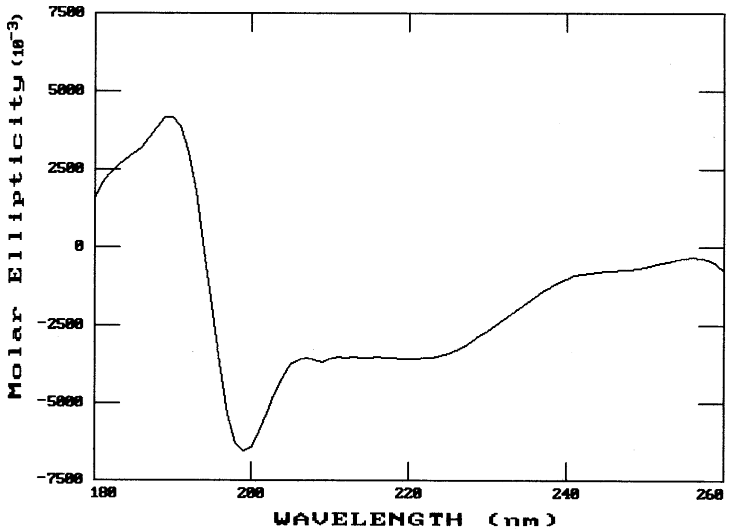
Sample	CMC (mM)[a]	Average Hydrodynamic Radius, $R_{h,0}$ (nm)[b]
$CF_3(CF_2)_2CH_2CH_2CH_2O(CH_2)_3SO_3Na$ (F7PS)	19	110.1
$CH_3(CH_2)_2CH_2CH_2CH_2O(CH_2)_3SO_3Na$ (C7PS)	32	176.1
$CF_3(CF_2)_5CH_2O(CH_2)_3SO_3Na$ (F13PS)	6	137.3
$CH_3(CH_2)_5CH_2O(CH_2)_3SO_3Na$ (C13PS)	15	225.6
$CF_3(CF_2)_5CH_2O(C_2H_4O)_3H$ (F13TEG)	0.01	118.9

[a]determined by surface tension measurements. [b]determined by dynamic light scattering.

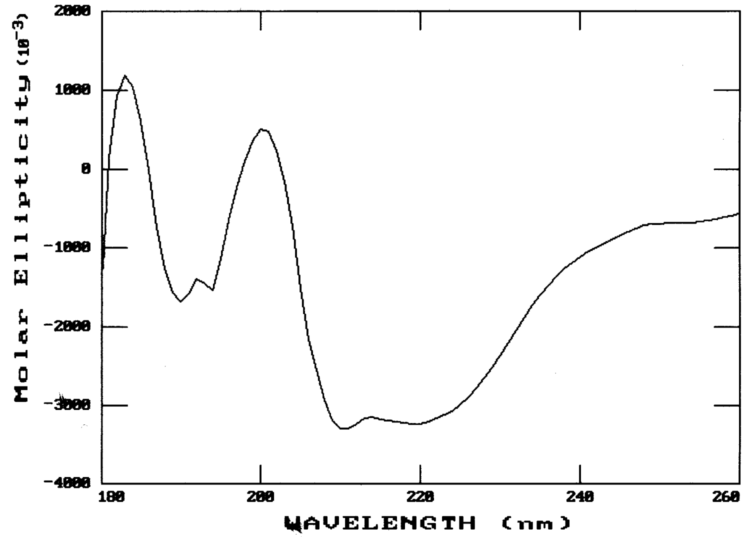


Dilution in the Presence and Absence of F13PS

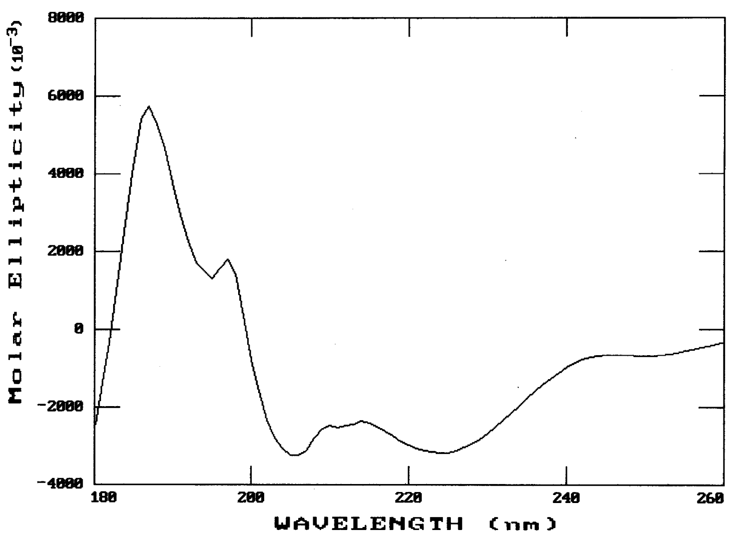
Time Dependent Renaturation of MMP13 in the presence of F-13



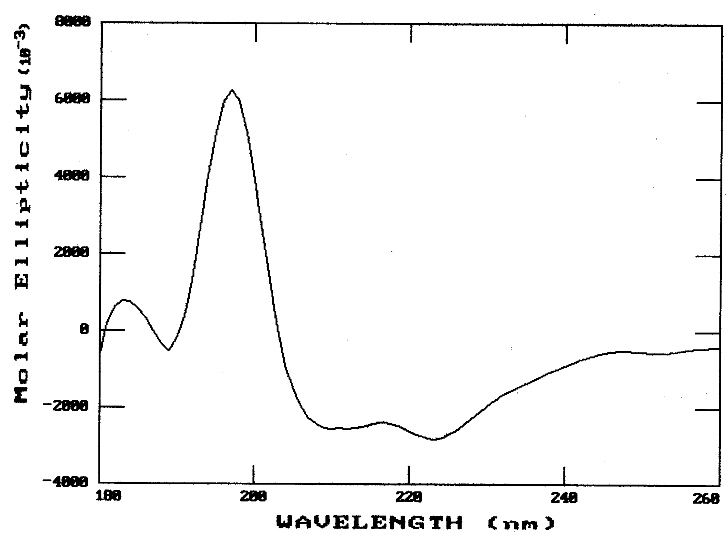
One hour after dilution



Three hours after dilution



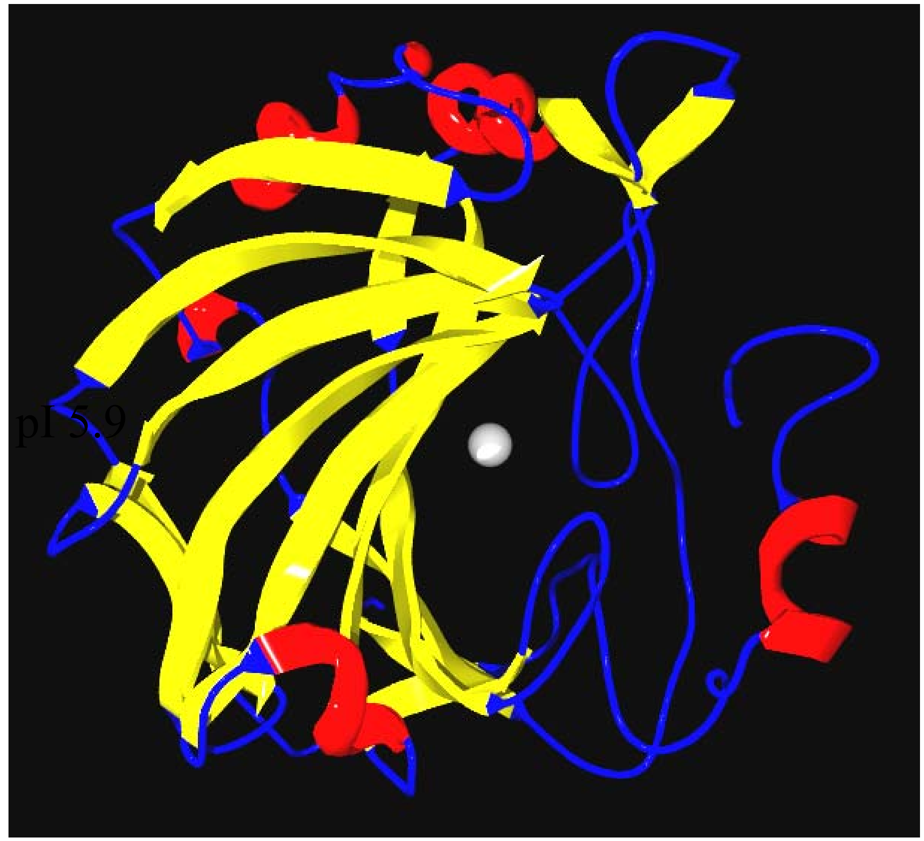
Five hours after dilution



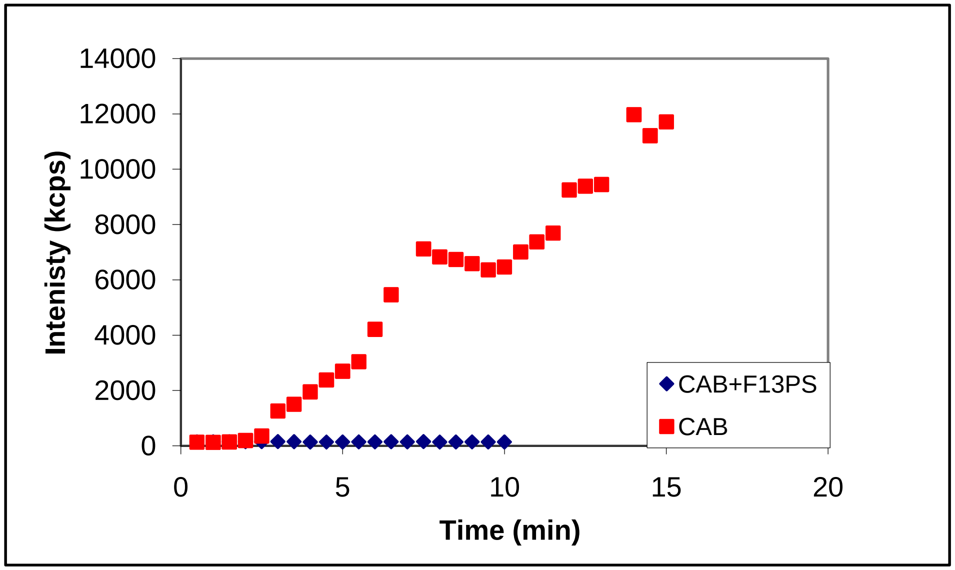
Twenty-four hours after dilution

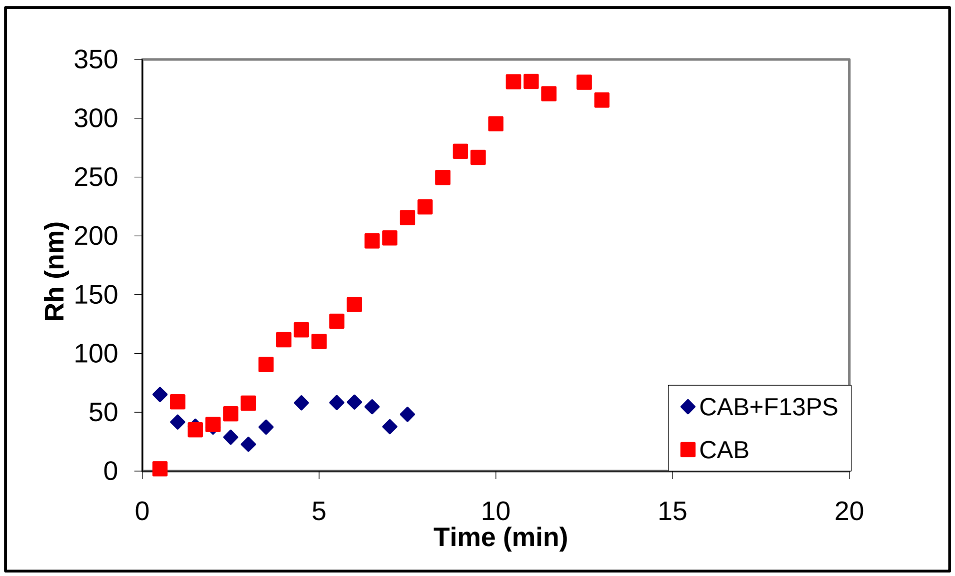
Bovine Carbonic Anhydrase (CAB)

- Catalyzes reaction of CO_2 and H_2O to HCO_3^- and H^+
- MW: 30 kDa; pI 5.9
- No disulfide bonds.
- Mainly contains β-sheet secondary structure.
- Due to the presence of the hydrophobic clusters during unfolding, CAB has a high tendency to aggregate during refolding.



Kinetics of CAB aggregation studied by Dynamic Laser Light Scattering (DLS): Thermal denaturation studies





DLS technique allows measuring the light scattering intensity and the size of particles in the course of protein aggregation.

Thermal denaturation of CAB was studied by DLS at 50 ^{o}C in 0.1M Tris-sulfate buffer pH 7.75 in the presence and absence of anionic fluorous surfactant F13PS

The scattering light was collected at 90^{o} scattering angle for approx. 15 min.

Comparison of refolding yields of CAB (0.03 mg/ml) using F13PS and F17PS as additives below their CMC.

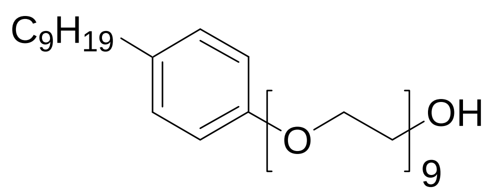
$CF_3(CF_2)_5CH_2CH_2CH_2OCH_2CH_2CH_2SO_3Na$ (F13PS)

$CF_3(CF_2)_7CH_2CH_2CH_2OCH_2CH_2CH_2SO_3Na$ (F17PS)

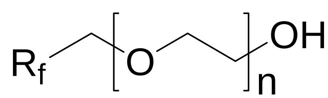
Additive	% RY* after thermal denaturation-renturation	% RY* after chemical denaturation-renturation
None	10	25
F13PS (3.1mM)	64	59
F17PS (0.5mM)	75	74

RY* - Refolding yield

What Does This Have to Do with the Development of Novel Spermicides and Microbicides?



Nonoxynol-9



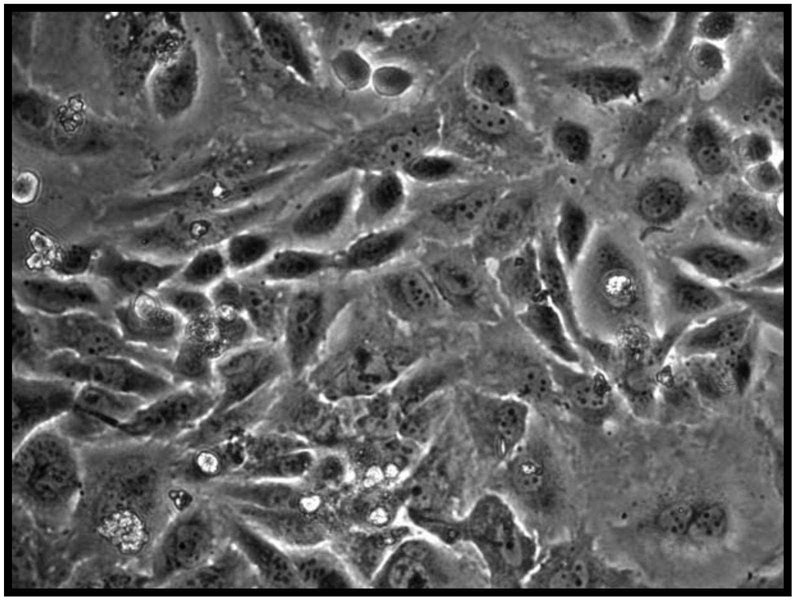
Fluorous Surfactant

$CF_3CF_2CH_2OCH_2CH_2OCH_2CH_2OCH_2CH_2OH$ **F5TEG**

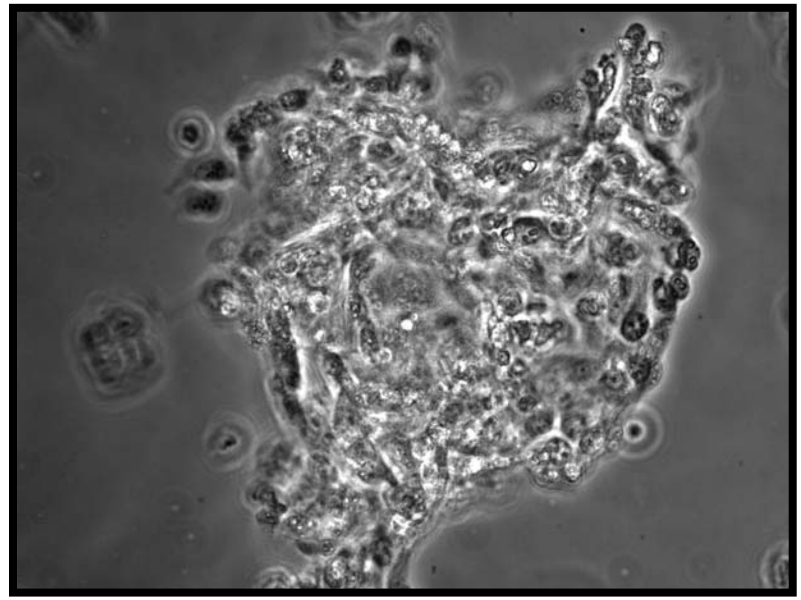
$CF_3CF_2CF_2CH_2OCH_2CH_2OCH_2CH_2OCH_2CH_2OH$ **F7TEG**

Effects of N-9 on Non-sperm Cells

WT HeLa Cells



0.1% N9 for 30 minutes



Results

Effects of N-9 on Sperm

Treatment	Motility	Percent Dead
Control	62%	28%
0.001% N-9	43%	38%
0.005% N-9	60%	32%
0.01% N-9	<1%	67%
0.1% N-9	0%	94%

Table 1: Averages of motility and PI stain data of N-9 treated semen for 5 minutes at 37°C with 5% CO_2

Effects of F5-TEG on Sperm

Treatment	Motility	Percent Dead
Control	62 %	28%
4% F5-TEG	0%	100%
5% F5-TEG	0%	96%
6% F5-TEG	0%	100%
7% F5-TEG	0%	98%
8% F5-TEG	0%	99%
9% F5-TEG	0%	100%
10% F5-TEG	0%	100%
10% F5-TEG (10min)	0%	100%
12% F5-TEG	0%	96%
15% F5-TEG	0%	94%
15% F5-TEG (10min)	0%	100%

Table 2: Averages of motility and PI stain data of F5-TEG treated semen for 5 or 10 minutes at 37°C with 5% CO_2

PI Staining

Control

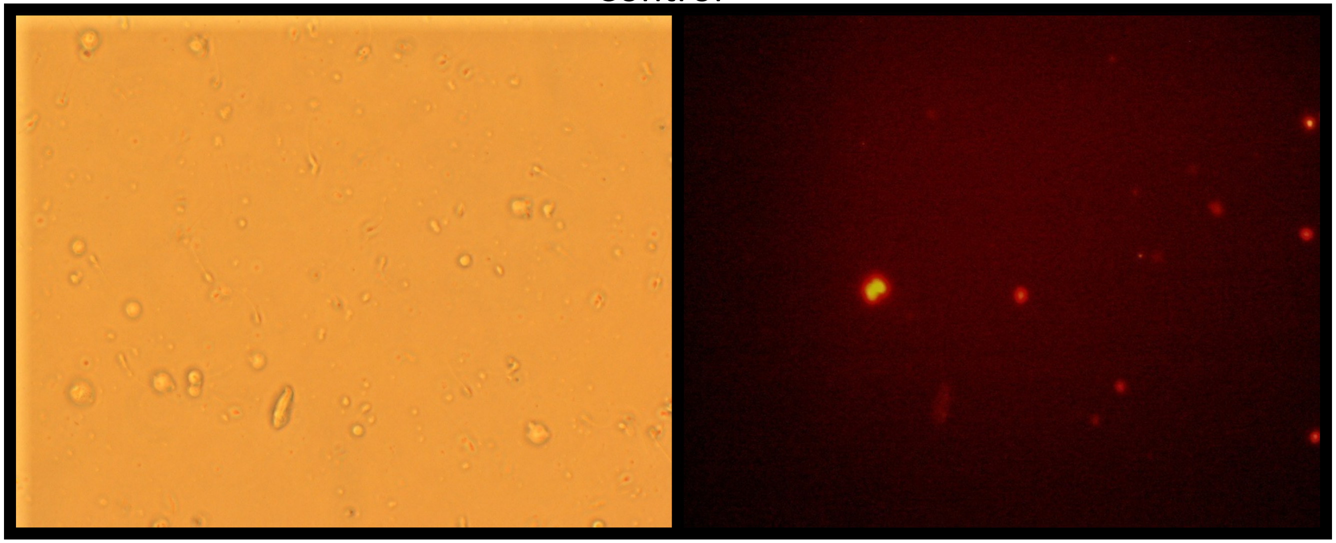


Figure 1: Control sample stained with PI. Bright field image is on the left and the right image is under fluorescence

Treated with 10% F5-TEG (5min)

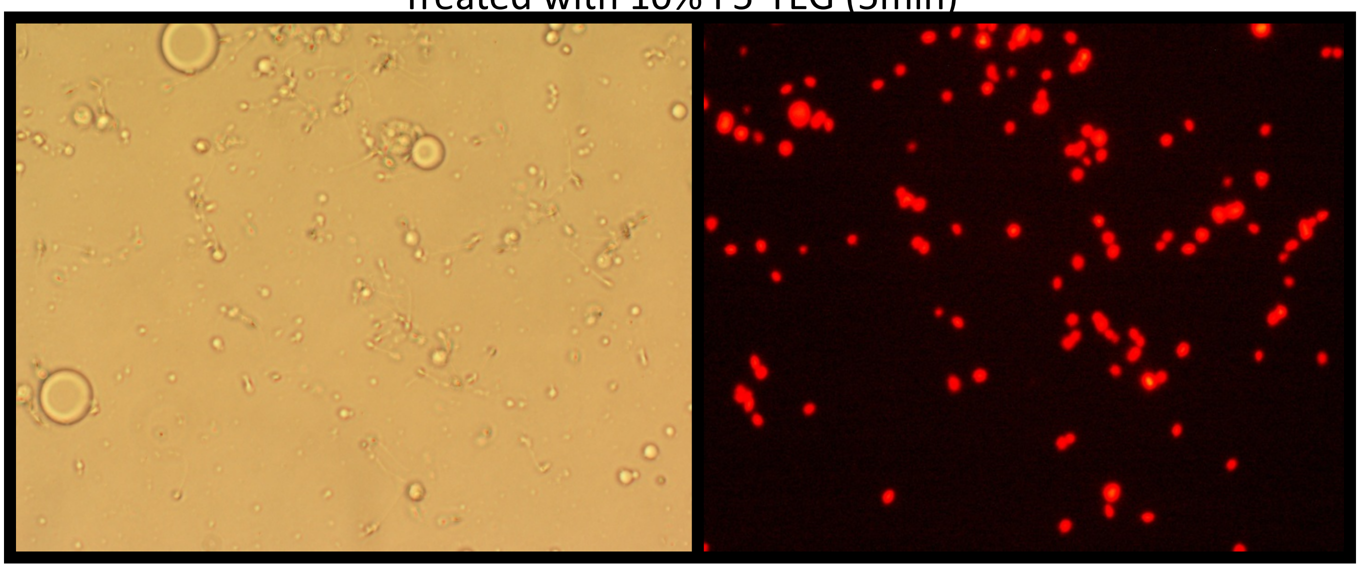


Figure 3: Sample treated with 10% F5-TEG and stained with PI. Bright field image is on the left and the right image is under fluorescence