Disulfide Cross-linking in Protein Microspheres

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Microencapsulation has diverse applications for drug delivery and targeting, medical imaging, and flavor and scent delivery\textsuperscript{1-2}. Many applications, especially the biomedical ones, use liposomes to form an encapsulating lipid bilayer, ranging in size from 10 nm up to microns in size to deliver some agent internally. Lipid vesicles, unfortunately, have limited stability since they are held together only by hydrophobic interactions. Microcapsules with polymeric shells are generally more robust, but are far less biocompatible. Often additional synthetic modifications are required for in vivo use to be possible. An encapsulation system that has the biocompatibility of lipids and the stability of polymeric microspheres would be ideal. Protein microspheres are such a system, in which both hydrophobic and covalent forces stabilize the vesicle.

High-intensity ultrasound can be employed to produce stable, near uniform sized vesicles from several proteins including human serum albumin (HSA), bovine serum albumin (BSA), pepsin, and lipase all of which contain the amino acid cysteine\textsuperscript{3-4}. The formation of these vesicles via high-intensity ultrasound occurs in a three-step mechanism. First, air or a nonaqueous liquid is emulsified within an aqueous protein solution. Second, protein molecules agglomerate at the amphiphilic boundary between the emulsified phase and the aqueous solution. The third step, essential to the formation of long-lived microspheres, is the formation of inter-protein disulfide bonds (i.e., oxidation of cysteine to cystine). During ultrasonic irradiation cavitation bubbles are generated\textsuperscript{5}. When these bubbles collapse, extreme temperatures and pressures are achieved that splits water molecules into hydrogen atoms and hydroxyl radicals\textsuperscript{6-9}. Hydroxyl radicals can combine to form peroxide, and hydrogen atoms can combine to form molecular hydrogen or in the presence of oxygen hydrogen atoms can form the hydroperoxy radical (HO\textsubscript{2}). Both peroxide and superoxide are known to oxidize thiols to disulfides\textsuperscript{10}. Experimental evidence (e.g., using superoxide dismutase to scavenge HO\textsubscript{2}) has indicated that the disulfide bonds are oxidized by HO\textsubscript{2}.

![Figure 1](image-url)

**Figure 1**
Microspheres composed of either serum albumin (HSA or BSA) or thiol-modified myoglobin have been extensively examined by a combination of techniques including particle size analysis, electron microscopy, polyacrylamide gel electrophoresis (SDS-PAGE), and size exclusion chromatography. Thiol modification of the cysteine deficient myoglobin was achieved by treating the native protein with varying molar equivalents (0-10) of 2-iminothiolane (Traut's reagent)\textsuperscript{11}. Particle size analysis reveals that the microspheres generated have a narrow size distribution (~1-4 μm), and are stable for over six months at 4 °C with minimal degradation (<10%). Scanning electron microscopy confirms the size distribution and clearly shows the morphology to be spherical. Transmission electron microscopy shows that the microspheres consists of encapsulated material surrounded by a protein shell 35-50 nm thick.

Examination of the protein microspheres via SDS-PAGE indicates that the protein shell is composed of various components consisting of monomeric protein as well as cross-linked protein dimers, trimers, tetramers, up to high molecular weight oligomers. In the myoglobin system, sonication of the native protein shows no high molecular weight aggregates forming. Increasing thiol conjugation shows a concomitant increase in the oligomeric protein fraction (Figure 1). Treatment of the protein microspheres with a disulfide reductant (dithiothreitol) degrades the cross-links reducing the oligomers to monomeric protein. Similar experiments analyzed via size exclusion chromatography confirm these results and have established that inter-protein disulfide bonds provide covalent stabilization. This extensive cross-linking and stabilization does not cause a loss of structure or activity. Circular dichroism spectra indicate that the protein has not been denatured and is highly α-helical. Oxygen binding experiments show that the myoglobin microspheres still reversibly bind oxygen (P_50= 0.8 torr) with an affinity similar to the soluble protein.

To further explore the disulfide cross-linking between protein molecules, MALDI-TOF mass spectrometry was utilized\textsuperscript{12-13}. Purified protein microsphere samples were digested with trypsin, chymotrypsin and the V8 proteases. These proteases provide site specific cleavage of the protein into smaller peptide units. None of the proteases are able to cleave disulfide bonds, allowing for their location to be selectively mapped. Comparison of mass spectra of digested sonicated myoglobin microspheres and digested soluble protein reveals new unique mass units. These new masses are peptides linked by disulfide bonds. Normalization of the intensity of the masses allows for the creation of a comparative coupling map (Figure 2). The map only contains intense disulfide linked peptides that are common to both the tryptic and chymotryptic digests. The specificity for certain disulphides to form can best be explained by examining the electrostatic surface using GRASP\textsuperscript{14}, a program that maps out charge distribution in proteins.

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![Figure 2](image_url)

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References
Binuclear Transition Metal Derivatives of 1,2-Bis(indenyl)ethane.

Ramin Khayatpoor  
Final Seminar  
June 1, 2000

The mononuclear ansa-metallocenes have been utilized effectively as stereoselective catalysts in a number of processes. Variations involving linked indenyl ligands have undergone considerable development in the context of providing chiral environments for such mononuclear transition metal catalysis; however, they have received scant attention as ligands for binuclear compounds. Our objective was to investigate the preparation of binuclear transition metal derivatives of 1,2-bis(indenyl)ethane and their subsequent chemistry.

The addition of doubly deprotonated 1,2-bis(indenyl)ethene to two equivalents of W(CO)5(THF), followed by reflux and subsequent oxidation by ferrocenium, leads to the di-tungsten complex ([η^5-C5H5]2(C2H4))W2(CO)6. The structure of this compound is shown in Figure 1. Another product, characterized principally by its 1H NMR spectrum, contained two linked ligands, each coordinated to one W(CO)5 moiety flanking a central metal-metal bond. The Mo analogues are also prepared in the same way. Isolation of the byproduct, which contains a M-M bond linking two different ligands, is an expected general feature of the synthesis, if a metal-metal bond can readily form. The W2 complex ([η^5-C5H5]2(C2H4))W2(CO)6 undergoes a facile transformation in chlorinated solvents. Cleavage of the metal-metal bond is concomitant with oxidative addition of one metal center to the C-H bond of the five-member ring attached to the adjacent tungsten center. The structure of the transformed product is shown in Figure 2. This transformation is not observed for the molybdenum analogue; however, it exhibits an interesting fluxional behavior.

**Figure 1.** A molecular structure diagram of ([η^5-C5H5]2(C2H4))W2(CO)6

**Figure 2.** A molecular structure diagram of W(CO)5([η^1-η^5-C5H5](C2H4)[η^5-C5H5])WCl(CO)5.