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[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[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 cavitating bubbles are generated[5]. When these bubbles collapse, extreme temperatures and pressures are achieved that splits water molecules into hydrogen atoms and hydroxyl radicals[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 hydroperoxyl radical (HO_2). Both peroxide and superoxide are known to oxidize thiols to disulfides[10]. Experimental evidence (e.g., using superoxide dismutatse to scavenge HO_2) has indicated that the disulfide bonds are oxidized by HO_2.

Lane 1 Marker
Lane 2 Soluble Mb
Lane 3 Sonicated Mb
Lane 4 1eq. 10% SDS
Lane 5 1eq. 30mM DTT
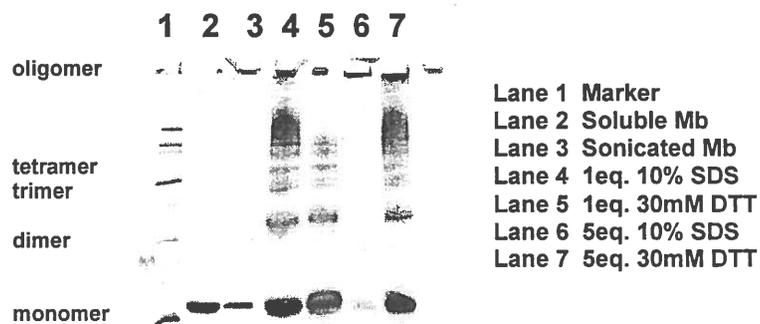Lane 6 5eq. 10% SDS
Lane 7 5eq. 30mM DTT

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)[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 (N=1, P_{50}= 0.8 torr) with an affinity similar to the soluble protein.
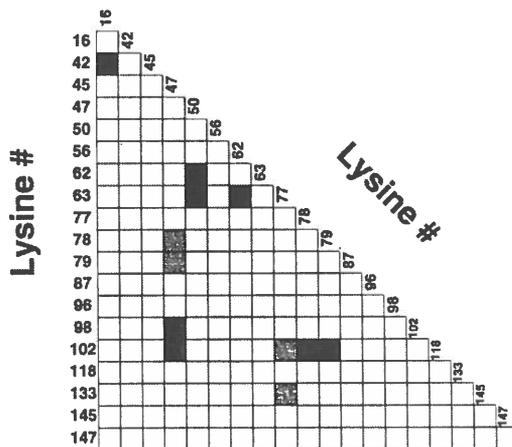

Figure 2

To further explore the disulfide cross-linking between protein molecules, MALDI-TOF mass spectrometry was utilized[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 disulfides to form can best be explained by examining the electrostatic surface using GRASP[14], a program that maps out charge distribution in proteins.

References

1. Uhrich, K.E.; Cannizzaro, S.M.; Langer, R.S.; Shakesheff, K.M. *Chem. Rev.* **1999**, *99*, 3181.

2. Lasic, D.D.; Vallner, J.J.; Working, P.K. *Curr. Opin. Mol. Ther.* **1999**, *1*, 177.

3. Grinstaff, M.W.; Suslick, K.S. Proc. *Natl. Acad. Sci.* **1991**, *88*, 7708.

4. Webb, A.G.; Wong, M.; Kolbeck, K.J.; Magin, R.L.; Suslick, K.S. *Magn. Res. Imag.* **1996**, *6*, 675.

5. Suslick, K.S.; et al. *Phil. Trans. R. Soc. Lond. A* **1999**, *357*, 335.

6. McNamara III, W.B.; Didenko, Y. Suslick, K.S. *Nature* **1999**, *401*, 772.

7. Flint, E.B.; Suslick, K.S. *Science* **1991**, *253*, 1397.

8. Riesz, P. *Adv. Sonochem.* **1991**, *2*, 23.

9. Misik, V.; Riesz, P. *Ultrason. Sonochem.* **1996**, *3*, S173.

10. Jocelyn, P.C. *The Biochemistry of the SH Group.* Academic Press, New York, 1972.

11. Traut, R.R.; Casiano, C.; Zecherle, N. in *Protein Function*, Creighton, T.E., Ed. Oxford University Press, Oxford, 1989.

12. Matsudaira, P. Ed. *A Practical Guide to Protein and Peptide Purification for Microsequencing.* Academic Press, San Diego, 1993.

13. Smith, D.L.; Zhou, Z. in *Methods in Enzymology* **1990**, *193*, Academic Press, San Diego, 374.

14. Honig, B.; Nicholls, A. *Science* **1995**, *268*, 1144.