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# Formulation Strategies for Proteins and Peptides

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## Present Status of Biotechnology Derived Pharmaceuticals

Biotechnology has broadened the scope of the Pharmaceutical Sciences causing a dramatic alteration in the approaches to drug discovery, research and development. Due to the pharmaceutical industry's slow adaptation to the "New" biotechnology the 1980s saw the emergence of numerous small Biopharmaceutical companies, principally fueled by venture capital and the entrepreneurial spirit. By 1993, 17 products were approved and over 120 are in clinical trials. The development of suitable formulations for clinical testing and the clinical evaluation phase have proven to be rate limiting steps in the development sequence. Formulation strategies over the next 5 to 6 years will continue to be in the solution, suspension and freeze-dried forms with the latter representing about half of these. Future strategies will include targeted delivery utilizing biodegradable particulate devices such as microspheres and liposomes and topical and implantable matrices for wound healing and bone growth. On the horizon, however, is the "newer" or "future" biotechnology such as gene therapy, DNA probes, antisense oligonucleotides, transgenic animals and plants and specific stereochemical synthesis. Economics will drive the industry to a less research-intensive posture and becoming more market oriented.

Since biotechnology derived products may exhibit multiple biological activities of great complexity, adequate non-clinical pharmacological studies are necessary before carefully designed clinical evaluations can be conducted. It is not surprising that the use of these products in humans has caused many regulatory concerns which led to the issuance of a number of new Guidelines and Points to Consider (PTCs) pursuant to the development and marketing of biopharmaceuticals. Most of the Guidelines for biopharmaceuticals are concerned with manufacturing and control of biopharmaceutical finished products, the manufacturing facility, clinical investigations and regulatory findings. These can be generalized as follows:

1. Description, characterization and use of the manufacturing cell line.
2. Viruses, adventitious agents and assessment of the risks of adventitious agents in clinical studies.
3. Preclinical evaluation and animal testing.
4. Formulation, potency, stability and production modes.
5. Process changes during clinical trials.
6. Product safety and efficacy.
7. Combinational uses of MoAbs and biological response modifiers.

The regulatory review process involves a case-by-case approach emphasizing sound scientific principles. While the principles and standards applied to biopharmaceuticals are not different from those applied to conventional drugs and biologicals they are much more multidisciplinary in nature. These basic principles deal with cellular and molecular biology of the

production system, physicochemical characteristics of the protein molecules and their pharmaceutical forms, *in vitro* and *in vivo* biological performance with respect to immunology and pharmacology, safe use in humans and assurance of quality manufacturing on a regular basis. The complexities involved in this technology will have significant economic ramifications and this will pressure the regulatory agencies to take the initiative in decreasing the time to review and approve biopharmaceuticals.

## The Development Process

Figure 1 illustrates the development process for biopharmaceuticals. The formulation process begins with the decision to undertake biological evaluation in animal models. If this phase is successful the development phase is accelerated as the emphasis is placed on a clincal trial. A final product package and a manufacturing procedure are required for the submission of an NDA/PLA. Then commercial batches can be prepared. This lecture will focus on those formulation activities which lead to preclinical and clinical investigations.

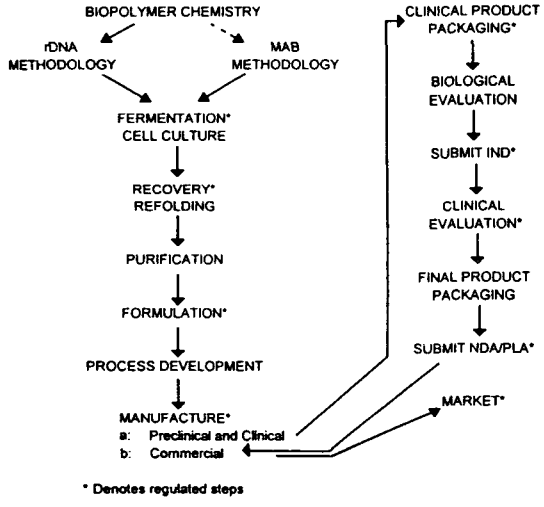



**Fig. 1.** The Development Process of Biopharmaceuticals.

The desirable characteristics for a protein formulation include:

- is sterilizable
- maintains stability and activity during storage
- in non toxic and has little or no side effects
- increases bioavailability of large molecules
- is cost-effective compared to alternatives
- enhances patient compliance
- drug sparing

The problems with protein/peptide delivery include the following:

- poor or no absorption via oral administration (MW>1000)
- degraded in the GIT
- short half-life
- conformational changes
- stability during processing and storage
- immunogenicity
- processing procedures (Mfg.)
- analytical methodology

Proteins can undergo covalent and non-covalent modifications. Each of these modifications can be of an intermolecular or intramolecular nature. The covalent and non-covalent modifications are listed below:

Covalent Modification of Proteins

| Intramolecular |
|---|
| Fragmentation/Hydrolysis |
| Transpeptidation |
| Deamidation |
| Disulfide Scission/Reduction |
| Disulfide Exchange |
| Deglycosylation |
| Oxidation |
| Photooxidation |

Intermolecular

Disulfide Crosslinking
Thio-esterification
Transpeptidation
Clipping-proteases
Concatenation
Medicated Crosslinking

Non-Covalent Modifications of Proteins

Intramolecular

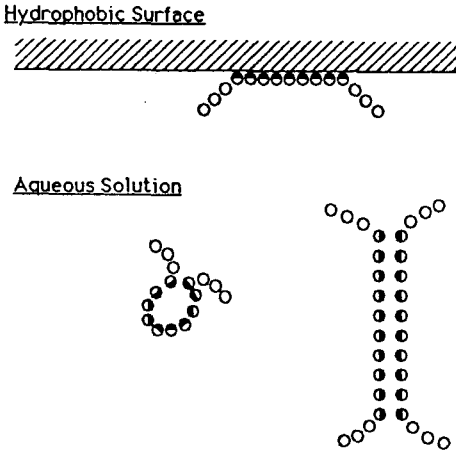
Misfolding
Adsorption

Intermolecular

Aggregation
- Molecular (oligomerization)
- microscopic (1-50 μm, turbidity)
- macroscopic (visible haze, ppt)

Adsorption

A serious problem is the non covalent interactions at hydrophobic surfaces which leads to aggregation and denaturation. This is illustrated in Figure 2.




HYDROPHOBIC AGGREGATION
AND
DENATURATION AT AN INTERFACE

**Fig. 2.**

*Analytical Methodology*

A multitude of analytical methods have evolved for characterizing proteins. These methods fall principally in the categories of spectroscopic, chromatographic and electrophoretic and are used to measure bioactivity, protein concentration, solubility, protein confirmation, molecular size and surface charge. Unfortunately, many of these attributes cannot be determined by a single method and generally several methods are required to adequately characterize a protein.

*Formulation Approaches*

The approved biopharmaceutical products are all injections packaged as either a conventional solution, suspension or lyophilized form. Other routes are being investigated such as nasal, inhalation and oral. It is becoming increasingly evident that the success of these proteins will depend on the method of delivery. Drug carriers such as liposomes and biodegradable polymers are being researched to effect controlled and/or site specific delivery. These carriers in addition to carrying the protein to specific sites can protect the protein prior to release thereby enhancing its therapeutic effectiveness. However, being immobilized in the carrier until administration, release will depend on dissolution within the carrier and diffusion from the carrier. Solution forms are the most desirable. The additives used in these solutions are those which are found only sparingly in conventional parenteral solutions. Protein solubility is generally influenced by ionic strength and pH. So sodium chloride is often present because it is part of the bulk protein solution and buffers range from the typical

phosphate system to various combinations of amino acids and their salts. The most popular being glycine, arginine and histidine.

Bufferes are also needed to prevent covalent modifications which lead to chemical degradation and loss of biological activity. Tris has become a popular system for this purpose. Additives are required to inhibit non covalent modifications such as aggregation and sticking of proteins to container surfaces which often leads to denaturation. Protective additives include human albumin and polysorbate 80. Solutions which are packaged in a multidose vial require a preservative. Instead of the widely used parenteral preservatives such as benzyl alcohol, the parabens or chlorobutanol, the phenols seem to be more common.

Several biopharmaceuticals have been approved in the suspension form. The vaccines for instance utilize an alum adjuvant and thiomerosal as a preservative. Human insulin which is available in several different formulations also includes a suspension of protamine-zinc crystals.

The most popular formulation approach for the approved products is a freeze dried form. About half of the approved products and those in clinical trials are freeze dried. In addition to the excipients previously mentioned, a cryprotectant such as mannitol or sucrose is added to prevent denaturation or aggregation of the protein as a result of removal of water. While these cryoprotectants stabilize the protein also present new challenges in the design of freeze drying cycles. Additionally, the freeze dried products are packaged with a diluent for reconstitution.

Therefore, the formulation task for a biopharmaceutical presents some serious challenges unlike those for conventional pharmaceuticals. The formulation strategies being investigated can be summarized as follows:

1. Solution form
2. Conjugation of protein/peptide
3. Suspension or gel
4. Freeze dried with a cryoprotectant
5. Incorporate in a matrix (carrier)

## Testing Formulation Strategies

The approaches over the next 5 or 6 years will continue to be in the solution, suspension and freeze dried forms, with the latter representing about half of these. Additionally, efforts will contiue to incorporate proteins into polymeric matrices for enhancing activity, site specificity and sustained release.

Examples of the following Formulation Strategies will be discussed:

1. Model protein-Ribonuclease A
2. Mass transfer accelerators in freeze drying
3. Formulation of a monoclonal antibody
4. Incorporation into a matrix

### *Model Protein*

Townsend, *et al.*, illustrated the effect of a cryoprotectant using Ribonuclease A as a model protein[1]. Figures 3A and B show the protection afforded by sucrose and Ficoll 70, a branched polymer of sucrose, in preventing loss of enzymatic activity. The extent of protection is illustrated in Figures 4 and 5. A rapid loss in activity occurred in freeze dried RNase dried at pH 3.0 and 10.0. The results suggest that buffer salts played a role in the degradation. Figure 5 shows the loss of activity of RNase A freeze dried at pH 10 without additives and sealed under a headspace of air and argon[2]. After 30 days storage at 45℃, samples stored in the light and dark had specific activities that were about 10% of initial activity. The major change was the large increase in

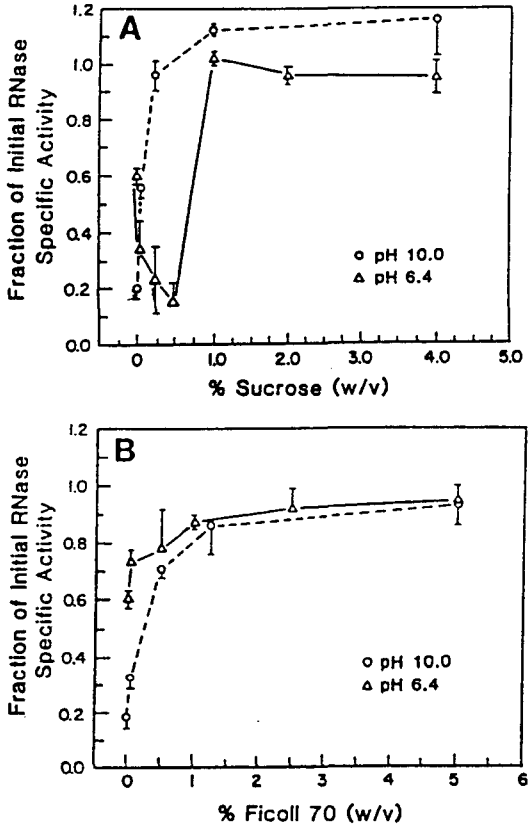



Fig. 3. Lyoprotectant effect of sucrose (A) and Ficoll (B) in freeze dried RNase stored at 45℃. Solutions containing 1.5 mg/ml RNase in 0.1 M phosphate buffer were freeze dried and stored for 30 days (pH 10.0) and 105 days (pH 6.4). N=4 for each point. (from reference 4).

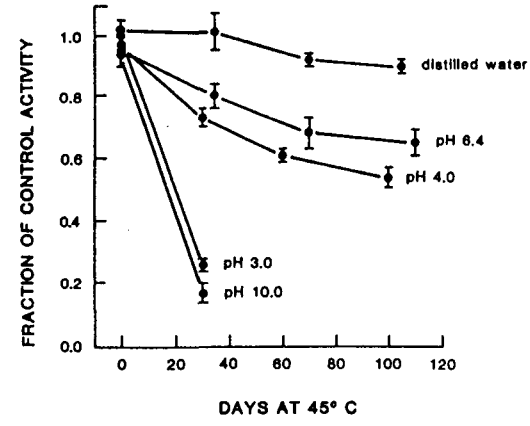



Fig. 4. Fraction of control specific activity for RNase freeze dried in distilled water and in phosphate buffer at various pH and stored at 45℃. (from reference 5).

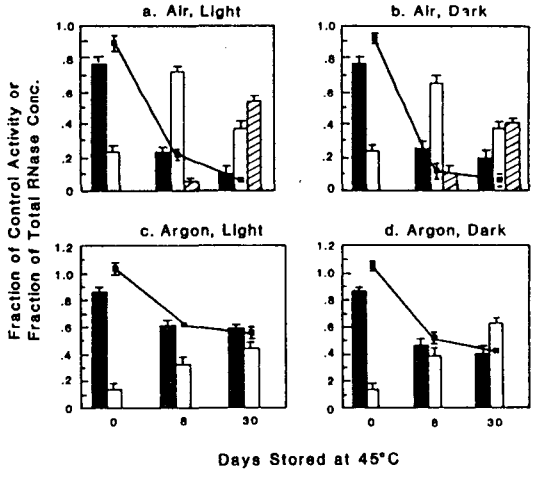



Fig. 5. RNase freeze dried in pH 10 phosphate buffer without additives. The line graph depicts the fraction of control activity and the bar graphs represent the fraction of total RNase concentration (from reference 5). ■=soluble monomeric RNase; ▥=soluble aggregate; ▨=insoluble aggregate.

insoluble aggregated protein. Removal of air prevented the formation of insoluble aggregates and reduced the soluble aggregates. The cryoprotectants sucrose and Ficoll 70, prevented aggregation during freeze drying and upon storage in the solid state. Aggregation was found to occur via an autooxidative mechanism and SDS-PAGE confirmed the aggregates as covalently bonded dimers of RNase A devoid of any enzymatic activity[3].

*Mass Transfer Acceleration in Freeze Drying*

Sugars and other polyhydroxy compounds have been used as stabilizers for proteins and biological materials. Sugars are also added in solutions to be freeze dried to protect certain protein compounds from freeze, freeze-thaw and freeze-drying damage. It has been proposed that the cryoprotectant action of these compounds may be primarily mediated through the solvent properties or maintenance of a re-

quisite water structure around the macromolecules. However, the freeze drying cycles of such solutions are excessively long and impractical. This is because sugar solutions exhibit collapse at very low temperatures and consequently, low shelf temperatures must be maintained throughout the drying stage.

During freeze drying of solutions in containers, mass transfer of water vapor across the partially dried layer is the rate limiting process. By reducing this resistance, a faster drying rate should result and the temperature of the frozen product will be maintained at a sufficiently low value thereby preventing melt back or collapse.

Table 1 shows preliminary observations on the freeze drying of sugar solutions. The solutions containing 2% sugars were successfully freeze dried at a shelf temperature of −30℃, but collapsed when the temperature was 0℃. In the case of 10% solutions, the temperature of frozen products rose above the respective collapse temperature and the solutions failed to dry.

Table 2 shows the effect of addition of 5% co-solvents on the freeze drying behavior. Except in the case of tertiary butyl alcohol (tBA), all other solutions failed to freeze-dry. Severe bubbling of the co-solvents and collapse occurred during the early drying phase. Solutions containing tBA, however, resulted in complete drying and yielded good cakes. All the co-solvents, except tBA, have freezing points well below the temperatures encountered during the freeze drying process. Visual observations of solutions containing tBA during the freezing stage indicated that tBA froze in characteristic needle-shaped crystals much before ice was formed. Phase diagrams of tBA-water and tBA-water-sucrose also indicated that tBA was frozen at the drying temperature during the cycle. Presence of such solidified tBA in the frozen solutions and its sublimination without passing

**Table 1.** Preliminary Studies with Freeze-Drying of Sugar Solutions. (chamber pressure = 100 mTorr)

| Solute | Conc. (%w/w) | Shelf temp.(℃) | Product temp.(℃) | Remarks |
|---|---|---|---|---|
| Sucrose | 2 | −30.0 | < −30.0 | good(100%) |
| | 10 | −30.0 | > −30.0 | collapse(100%) |
| | 2 | 0.0 | > −30.0 | collapse(50%) |
| | 10 | 0.0 | > −30.0 | collapse(100%) |
| Lactose | 2 | −30.0 | < −25.0 | good(100%) |
| | 10 | −30.0 | > −25.0 | collapse(20%) |
| | 2 | 0.0 | > −25.0 | collapse(20%) |
| | 10 | 0.0 | > −25.0 | collapse(50%) |
| Sorbitol | 2 | −30.0 | > −30.0 | collapse(100%) |
| | 10 | −10.0 | > −30.0 | collapse(100%) |

**Table 2.** Effect of Solvents on Freeze Drying Sugar Solutions

| System | Shelf temp.(℃) | Remarks |
|---|---|---|
| Sucrose alone | 0 | Collapse |
| Sucrose + methanol, ethanol, IPA, acetone nBA, dioxane | 0 | Boiling of solvent, Collapse |
| Sucrose + tBA | 30, 45 | Good |
| Lactose alone | 0 | Collapse |
| Lactose + methanol ethanol, IPA, acetone nBA, dioxane | 0 | Boiling of solvent Collapse |
| Lactose + tBA | 30, 45 | Good |

**Table 3.** Observations During Freeze Drying Sugar Solutions (10%w/w)

| | Freezing pattern | Collapse temp.℃ | Max shelf temp.℃ | Drying rate, g/hr |
|---|---|---|---|---|
| Sucrose alone | Spont. | −28 | −30 | 0.21 |
| Sucrose + 5% tBA | Slow Neddles | −21 | +30 | 0.60 |
| Sucrose + 10% tBA | Slow Needles | −20 | +30 | 0.67 |
| Lactose alone | Spont. | −23 | −15 | 0.19 |
| Lactose + 5% tBA | Slow Needles | −22 | +30 | 0.70 |
| Lactose + 10% tBA | Slow | −21 | +30 | 0.79 |

**Table 4.** Effect of tBA on Properties of Dried sucrose

| | Without tBA | With tBA |
|---|---|---|
| Initial drying | slow | fast |
| Texture | coarse | smooth, soft |
| Particle shape | irregular | irregular |
| SEM | plate-like | plates but porous |
| Polarized light | non-birefringent | partial birefringence |
| Crystallinity | amorphous | partial crystallinity |
| Residual moisture | 1-2% | 1-2% |
| Surface area | 0.9 $m^2/g$ | 1%-1.13 $m^2/g$<br>5%-2.25 $m^2/g$<br>10%-2.80 $m^2/g$ |
| Residual tBA | | <0.2% |
| Total drying time | 40 hours | 22 hours |

through the liquid phase may have strengthened the matrix and thereby prevented collapse.

Table 3 shows the freeze drying behavior of sugar solutions at various temperatures. In the presence of tBA, the initial drying rate was 3 times faster at 30℃, however only the solutions which contained tBA survived at this temperature. With lactose the corresponding increase in drying was almost 3.5 times faster.

Table 4 shows the comparison between the two methods of freeze drying. The smooth and soft surface morphology of the cake, porous crystal habits as seen by SEM and the associated high surface area indicates that the cake dried from a solution containing tBA was very porous. This porous nature may be responsible for reduced resistance to water vapor transfer during the sublimation resulting in faster drying.

*Hemoglobin*

The physical characteristics of freeze-dried hemoglobin cakes are summarized in Table 5. Freeze-drying of hemoglobin solutions alone, or in presence of sodium chloride or tBA, resulted in a dark brown product. In all these cases,

**Table 5.** Physical Characteristics of Freeze-Dried Hemoglobin

| Formulation | Shelf temperature | Cake color | Reconstitution |
|---|---|---|---|
| Hb alone | 0℃ | Dark brown | Difficult |
| Hb+NaCl(0.9%) | 0℃ | Dark brown | Difficult |
| Hb+tBA(5%) | 0℃ | Dark brown | Difficult |
| Hb+sucrose(5%) | 0℃ | Bright red | Easy |
| Hb+sucrose(5%)+tBA(5%) | 0℃ | Bright pink | Easy |
| Hb+sucrose(5%)+tBA(5%) | 30℃ | Bright pink | Easy |

**Table 6.** Chemical Properties of Freeze-Dried Hemoglobin

| Formulation | Hb content (% w/w) | MetHb content (% w/w) | Moisture (% w/w) |
|---|---|---|---|
| Hb alone | 52 | 46 | 5.3 |
| Hb+NaCl(0.9%) | 39 | 60 | 5.2 |
| Hb+tBA(5%) | 65 | 32 | 3.1 |
| Hb+sucrose(5%) | 97 | <1 | 1.6 |
| Hb+sucrose(5%)+tBA(5%) | 97 | <1 | 5.1 |
| Hb+sucrose(5%)+tBA(5%)(30℃) | 98 | <1 | 3.0 |

the product was extremely difficult to reconstitute. However, the solutions dried in the presence of sucrose alone were bright red in color and were easy to reconstitute. The solutions containing sucrose along with tBA (1 to 10% w/w) resulted in a bright pink product which also readily reconstituted. The chemical properties of freeze-dried hemoglobin are summarized in Table 6. In the absence of sucrose, the formation of methemoglobin during the freeze-drying was very high which imparted a brownish color to the dried cakes. When the sucrose was present, the methemoglobin concentration was less than 1% w/w and the dried cakes were bright red in color. Electrophoretic patterns of the dried hemoglobin formulations containing sucrose exhibited a single spot, indicating mono-component migration of the applied

sample. Other solutions exhibited a smear, indicating a possibility of structural changes involving dissociation and/or degradation of hemoglobin molecules.

Lactose and sucrose solutions were successfully lyophilized in the presence of tBA, at much higher shelf temperatures, resulting in at least a 3-fold increase in the drying rate. The addition of tBA (up to 10% w/w) during the lyophilization is promising because a) tBA remains frozen at the drying temperatures b) sublimation of tBA results in the formation of a porous dry layer which reduces the resistance for the mass-transfer of water and c) it results in lower product temperature, higher input of heat and hence shorter drying cycles. The function of tBA was not to modify the solubility of the solutes but it appears to elevate the collapse temperature (at least in the case of sucrose) and more importantly alter the amount of crystalline ice in the cake which creates channels during drying.

*Formulation of an IgM Monoclonal Antibody*

$T_{10}B_9$ is a murine monoclonal antibody (MoAb), directed against T-lymphocytes and which is presently being tested for its therapeutic efficacy in the treatment of renal allograft rejection in human clinical trials. This lecture will describe the development of the methodology for assessing the stability of the MoAb. Suitable solution and freeze dried forms were developed for clinical trials with the freeze dried form showing superior stability. Sucrose and maltose were found to be effective cryoprotectants and lyoprotectants for the IgM MoAb as illustrated by the data in Table 7. IgM binding activity as assessed by flow cytometry showed retention in antibody titre for over 1.5 years while a reduction occurred in the solution form in 14 months at 4℃. There was no evidence of particulate matter in the freeze dried form upon reconstitution.

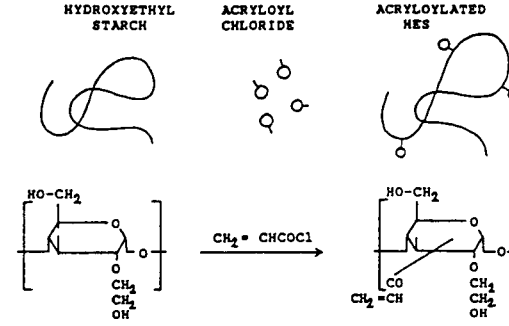
**Table 7.** Effect of Cryoprotectants and EDTA during Freeze-Drying in PBS* and Storage

| Additive | Percentage of Starting Material as Insoluble Aggregate | |
|---|---|---|
| | Zero time | After 60 days at 35℃ |
| None | 12-14 | 99-100 |
| None-vented to argon | 13-16 | 99-100 |
| 0.05% EDTA | 10-14 | 98-99 |
| 0.05% EDTA -vented to argon | 20-25 | 99-100 |
| 0.1 M sucrose | 4-6 | 13-14 |
| 0.1 M maltose | 7-10 | 19-22 |
| 0.4 M maltose | 5-7 | 9-12 |
| 0.1 M sucrose and 0.4 maltose | 0 | 0 |

*PBS=Phosphate buffered saline pH 7.4.

*Matrix Delivery System*

The benefits of many of the biopharmaceuticals will come to be realized through biodegradable polymeric matrices. Current methodologies are not suitable for preparation of protein containing microspheres as a result of denaturation of most proteins in organic solvents. A method for preparing hydrophilic microspheres from acryloyl esters of hydroxyethyl starch (HES) has been developed[4,5]. A soluble linear polymer like HES is derivatized with a low molecular weight monovinyl compound by the following scheme:



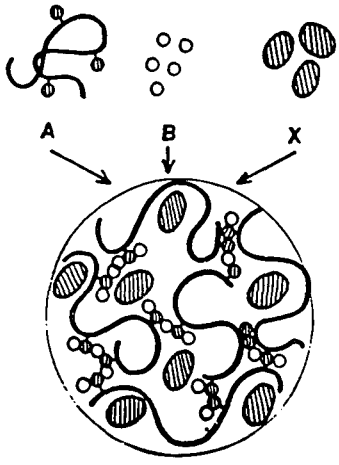



**Fig. 6.** Schematic illustration of the polymerization step in the preparation of HES microspheres containing a protein.
A=derivatized biodegradable polymer
B=low MW monovinyl crosslinking agent
X=protein molecule (from reference 7)

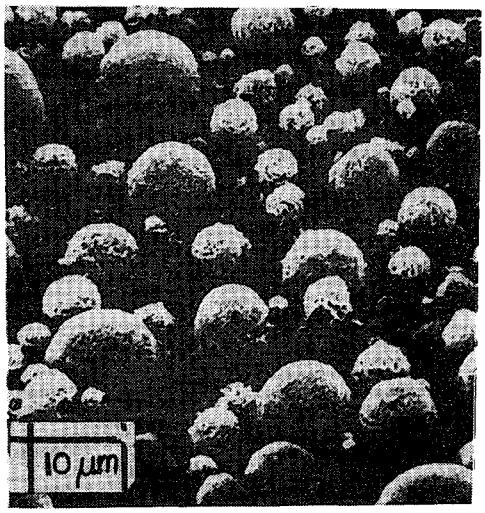



**Fig. 7.** SEM photograph of HES microspheres containing AAT which have been washed to remove surface protein. (from reference 7).

Then via a free radical polymerization of the acryloyl groups in an aqueous dispersed phase of a water-in-oil emulsion (Figure 6) spherical particles of crosslinked polysaccharide gel were formed containing free protein entrapped in the polymeric network (Figures 6 and 7). During the procedure the protein remains in an aqueous environment of appropriate pH and ionic strength and the temperature is kept close to 0℃ to ensure minimum inactivation of the protein. Figure 8 shows release profiles for 2 proteins alpha-1-antitrypsin (AAT), a protease inhibitor, and porcine somototropin (PST), a growth hormone.

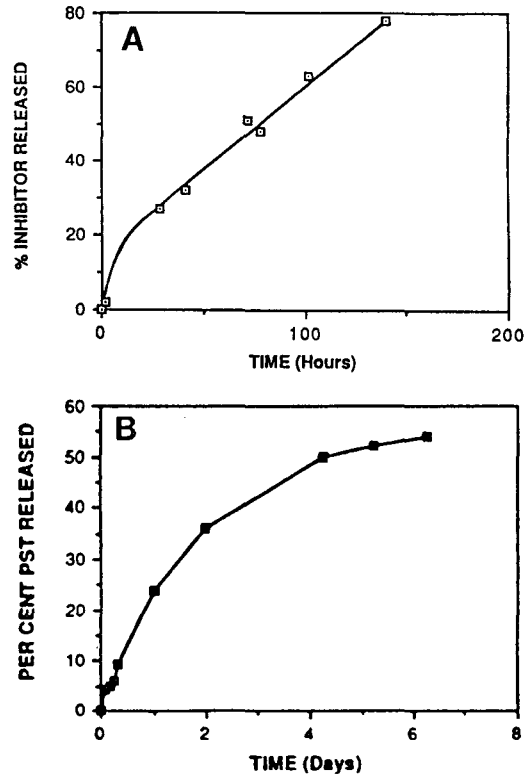



**Fig. 8.** *In Vitro* release of AAT (A) and PST (B) from HES microspheres (from reference 7).

## References

1. Townsend, M.W. and DeLuca, P.P., Use of lyoprotectants in the freeze-drying of a model protein, ribonuclease A, *J. Parent. Sci. & Technol.*, **42**, No.6, 190-199 (1988).
2. Townsend, M.W., Byron, P.R. and DeLuca, P.P., The effect of formulation additives on the degradation of freeze-dried ribonuclease A, *Pharm. Res.*, 7, 1086-1091 (1990).

3. Townsend, M.W. and DeLuca, P.P., Nature of aggregates formed during the storage of freeze-dried ribonuclease A, *J. Pharm. Sci.*, **80**, 63-66 (1991).
4. DeLuca, P.P., Hickey, A.J., Hazrati, A.M., Wedlund, P., Rypacek, F. and Kanke, M., Porous biodegradable microspheres for parenteral administration, Topics in Pharmaceutical Sci., p.429, Elsevier Science Publ., Amsterdam, Dec. 1987.
5. DeLuca, P.P. and Rypacek, F., Biodegradable microspheres as a carrier for macromolecules, U.S. Patent #4,741,872, May 3, 1988; European #0245820A2; Australian #600723.