Overview of Protein Formulations for Animal Health Applications†

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BACKGROUND

Over the past decade, important advances have been made in genetic engineering and biotechnology that have led to the practical production of a variety of polypeptides, including the somatotropins and cytokines. However, these large molecules represent extremely difficult challenges from a delivery standpoint because of their large size and their susceptibility to degradation. Such molecules are very difficult to deliver through oral or dermal routes, two common formulation types in the animal health market. While the possibility of parenteral administration exists, the short lifetimes of these molecules in vivo in many cases necessitates the need for a controlled release system that can both protect the molecule of interest from harm and release the molecule at a controlled rate in an unaltered form.

A variety of controlled release systems exist in human clinical medicine for low molecular weight drugs, such as transdermal patches for nitroglycerine, scopolamine, and estradiol and Norplant, an intramuscular 5-year contraceptive implant for birth control drug (Langer, 1990). Two polymeric peptide pharmaceutical controlled release systems have been approved in the United States, Lupron Depot (Takeda Abbott Pharmaceuticals) and Zoladex (ICI Pharmaceuticals). It has been far more difficult to release large molecules, like proteins, through polymers and in commercially viable dosage forms for either the human pharmaceutical or the animal health industries. This is because large molecules such as proteins were for many years not available in commercial quantities and were not considered feasible candidates for controlled release systems because they were too large to slowly diffuse through most polymeric materials, even after swelling of the polymer. Large molecules could diffuse through highly porous membranes such as Millipore filters or certain gels such as polyacrylamide. However, in these cases, diffusion was often too rapid to be useful, and biocompatibility problems often occurred. The discovery that matrices of solid hydrophobic polymers containing powdered macromolecules enabled molecules of nearly any size to be released for over 100 days permitted controlled delivery of a variety of proteins, polysaccharides, and polynucleotides (Langer et al., 1976). Examples of polymers that perform in this manner include nondegradable ethylene–vinyl acetate copolymers or silicone rubber, as well degradable lactic–glycolic acid copolymers. The release mechanism generally involves movement of a polypeptide through a complex porous path in the polymer matrix. If the polymer erodes, this will affect the porous structure and accelerate the release. This coupled with advances in recombinant technology leading to economically viable protein manufacture has improved the potential for commercial products in the animal health markets.

In this paper we discuss some of the major challenges in protein delivery in agricultural medicine, including protein stabilization and mechanisms of release. We conclude by speculating on future trends in this field.

PROTEIN STABILIZATION

One of the most significant challenges in protein delivery is stabilization of the protein. In particular, when proteins are in an animal for a long time, they may denature or aggregate as a result of exposure to moisture at 37 °C. This can cause loss of biological activity as well as potential changes in immunogeneity. To address these problems, it is critical to understand the molecular mechanisms by which proteins lose their activity. In this regard, the approaches outlined by Klibanov (1983) may be useful. Three basic questions are asked: (1) What is the cause (e.g., external agent) of inactivation? (2) What is the molecular mechanism of inactivation? (3) What approaches can be taken to prevent bypass or at least minimize this mechanism? To address this problem, it is important to recognize that numerous molecular mechanisms may cause loss of protein activity. These include both chemical (such as deamidation, oxidation, disulfide exchange, β elimination, hydrolysis, and racemization) and physical (aggregation, denaturation, precipitation, and adsorption) degradation pathways (Manning et al., 1989). We suggest that a critical step in devising rational approaches to stabilizing proteins is to understand which of these steps is rate limiting and most important for protein aggregation. Once this mechanism is known, rational approaches to stabilization of proteins can be devised. In one recent example, using solid proteins as a model, small amounts of water induced rapid aggregation of albumin, ovalalbumin, β-lactoglobulin, and glucose oxidase. For albumin, the aggregation as a function of water content went through a maximum with just 3 μL of water causing 97% aggregation of 10 mg of albumin in 24 h. While at higher and lower water concentrations, aggregation was reduced, it still occurred to a significant degree in a 1-day period. A variety of mechanisms were examined by simple experiments, such as adding guanidine hydrochloride (which could prevent noncovalent aggregation) and adding oxygen (which would induce oxidation). The absence of effects on aggregation via these experiments suggested these mechanisms were not responsible for aggregation. It was discovered, however, that the addition of dithiothreitol could prevent aggregation, suggesting that the mechanism was a thiol–disulfide exchange. This was further confirmed by specifically S-alkylating the one thiol group in albumin. When this was done, the aggregation was completely eliminated. While alkylation could be adopted as a strategy to specifically prevent albumin aggregation, it would mean creation of a new chemical entity. Understanding of the mechanism of aggregation, however, can suggest rational strategies for approaching stabilization without even employing chemical modification. For example, if a thiol–disulfide exchange occurs,

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this means that the active species is the thiolate ion. Therefore, approaches which minimize the number of thiolate ions, such as lyophilizing acidic solutions of albumin or employing specific polymer matrix compositions that would create a local acidic environment (e.g., lactic–glycolic acid copolymers, polyanhydrides), could be used. In addition, the mechanism of albumin aggregation was further studied, and it was recognized that when significant amounts of water were added, the effects of polymer aggregation were minimized due to a dilution effect. Once again, this result suggests specific strategies for protein stabilization. These include use of appropriate additives such as water-soluble polymers (e.g., dextrans, polyethylene glycol) that would simulate the dilution effect (Volkin et al., 1991; Liu et al., 1991). The mechanisms of aggregation will vary, of course, depending on the protein being studied. For example, in a study of ribonuclease, oxygen was found to be responsible for protein aggregation (Hageman, 1988).

Researchers working in the animal health field have extensively studied the aggregation tendencies of the animal somatotropins. Bovine and porcine somatotropin are 191 amino acid proteins containing two disulfide linkages with high degrees of homology. Crystallographic data have indicated that the porcine somatotropin tertiary structure consists of four anti-parallel α helices (Abdel-Meguid et al., 1987). Brems and co-workers have concluded that the bovine somatotropin aggregate forms as a result of a complex multistep process initiated by hydrophobic bonding at the interior surface of the third helix (Brems et al., 1985, 1986, 1988; Brems and Havel, 1989; Havel et al., 1986; Holzman et al., 1986). Buckwalter and co-workers have indicated that a disulfide-exchange reaction is involved in the aggregate formation of porcine somatotropin. Researchers have used chemical modification of the small loop cysteines to enhance the solution stability of this molecule (Buckwalter et al., 1992; Cady et al., 1990; Randawa et al., 1990). Several groups have used site-directed mutagenesis to make changes in the primary sequence of the somatotropins to improve the stability of these proteins. Researchers have replaced cysteines with other amino acids (Cady et al., 1990; Parcells et al., 1990). Lehrman and co-workers have used site-directed mutagenesis to prepare analogues with less α helicity between residues 109 and 127, thereby reducing the potential of this region of the molecule for hydrophobic interactions (Lehrman et al., 1991).

Lindsey and co-workers have stabilized porcine somatotropin by preparing a leupeptin (a tripeptide aldehyde protease inhibitor) complex and incorporated the complex into implants. These gave elevated porcine somatotropin plasma levels for 8–10 days when implanted into pigs, resulting in improved feed-to-gain and average daily gain over that of the untreated control pigs (Lindsey et al., 1991).

DELIVERY SYSTEM MECHANISMS

A variety of delivery system mechanisms have been used by researchers to achieve release of proteins from formulations. The approaches that have been explored can be classified into diffusion-controlled systems, chemically-controlled systems, solvent-activated systems, and pulsatile release systems.

Diffusion-Controlled Systems. Diffusion-controlled systems are the most widely used controlled release systems. They have been formulated in two basic configurations: reservoirs and matrices.

In reservoir systems, a core of drug is surrounded by a polymer film, and diffusion of the drug through the polymer is the rate-limiting step. These systems include membranes, capsules, microcapsules, liposomes, and hollow fibers. A critical problem, from a pharmaceutical standpoint, is the ability to achieve zero-order release rates; the principal advantage of reservoir systems is the ease with which they can be designed to achieve these kinetics. To accomplish this, powdered drug can be loaded at a level far above the solubility of the drug. As long as powdered drug is available, the drug concentration inside the reservoir will always be the saturation concentration of the drug, and zero-order release will occur.

While the great advantage of reservoir systems is the ease with which they can be engineered to produce near zero-order release kinetics, they also have several disadvantages. For example, these systems are generally nonbiodegradable; therefore, subcutaneous implants must be surgically removed. These systems are also generally not useful for the long-term delivery of high molecular weight drugs (e.g., insulin). In addition, should leaks occur, they could be potentially more dangerous in reservoir systems because all of the incorporated drug could be rapidly released.

There are two general types of membranes used in reservoir systems: nonporous homogeneous polymer film and microporous membrane. In the nonporous film, drug transport occurs via a solution–diffusion mechanism. In the microporous membrane, the drug diffuses through pores in the polymer structure (Langer et al., 1981). Researchers at Syntex have prepared reservoir devices using hydrogels as the rate-limiting membrane wall for the release of polypeptides and somatotropins (Sanders et al., 1990).

Researchers have reported sustained release of bovine somatotropin from several types of liposomes. Egg phosphatidylcholine, ethanolamine, and α-tocopherol hemisuccinate Tris salt vesicles released bovine somatotropin over more than a week period (Janoff et al., 1989) in hypophysectomized rats while hydrogenated soy phosphatidylcholine, cholesterol, bovine somatotropin liposomes injected into dairy cows resulted in increased weekly milk production (Weiner et al., 1989).

In matrix systems, the drug is uniformly distributed, throughout a solid polymer. As in reservoir systems, drug diffusion through the polymer matrix is the rate-limiting step. From the standpoint of fabrication cost, the ease of accomplishing this distribution pattern represents a significant cost decrease compared to reservoir systems. However, because of the different ways in which drug is distributed, release characteristics are not generally zero-order.

The reason drug release rates decrease with time from matrix slabs is because drug is released from the surface layer first and has only a short distance to travel; at later times drug from deeper within the matrix diffuses out and has a further distance and therefore longer time to travel. One approach to achieve zero-order kinetics in matrices is to compensate for the increasing diffusional distance with an increasing area of drug. Several shapes have been tested with this objective in mind. The best results have been from a cylinder sector that releases drug only from the inside surface and a hemisphere that is laminated with an impermeable coating in all places except for a small cavity in the center face (Hsieh et al., 1983). A variation of this system with multiple cavities placed in a drug-embedded slab has recently been introduced in veterinary medicine to release antihelmetic agents in cattle.

Several research groups have utilized microparticulate formulations for injectable bovine somatotropin delivery

systems. Oil suspensions have been prepared giving 14–28-day controlled release of the protein. These nonaqueous formulations form a semisolid depot at the injection site which is believed to slowly erode during contact with biological fluids. Variations in the hydrophobicity of the oil system influence the protein release rate. Researchers have used glyceride derivatives in an oil vehicle to effect bovine somatotropin release (Ferguson et al., 1988; Thakkar et al., 1988). Oil gels have been prepared using the antihydration agent aluminum monostearate (Mitchell et al., 1991). Fat microspheres have been prepared using triglycerides (Cady et al., 1989; Steber et al., 1989).

Chemically-Controlled Systems. In one type of chemically-controlled system, the biodegradable system, the drug is distributed, ideally uniformly, throughout a polymer in the same way as in matrix systems. The difference, however, is that while the polymer phase in matrix systems remains unchanged with time and drug is released by diffusion, the polymer phase in biodegradable systems decreases with time. Consequently, as the polymer surrounding the drug is eroded, the drug escapes. This property offers a significant advantage over nonerodible systems in many applications because biodegradable polymers are eventually absorbed by the body, obviating the need for surgical removal. However, this advantage must be weighed against the potential disadvantage that the absorption products may be toxic, immunogenic, or carcinogenic.

From a chemical standpoint, three structures can be utilized, each degraded by different mechanisms (Heller, 1984). These structures are (type 1) water-soluble polymers insolubilized by degradable cross-links, (type 2) water-insoluble polymers solubilized by hydrolysis, ionization, or protonation of pendant side groups, and (type 3) water-insoluble polymers solubilized by backbone-chain cleavage to small water molecules. These mechanisms represent extreme cases, and erosion by a combination of mechanisms is possible. The most commonly used biodegradable polymer is poly(lactic acid) or lactic–glycolic acid copolymers (type 3). Others include polyanhydrides (type 3), poly(vinylpyrrolidine) (type I), polyorthoesters (type 3), poly(ϵ-caprolactone) (type 3), and poly(amino acids) (type 3).

From a physical standpoint, polymers can display either surface erosion or bulk erosion. Bulk erosion involves dissolution of the polymer throughout the entire system. This leads to a progressive loosening of the matrix and can lead to several phases of release: release from the surface, release through pores in the matrix, followed by release during complete breakup of the matrix. Surface erosion involves dissolution of the system layer by layer—analogous to the way a bar of soap dissolves. This leads to one phase of release; constant release can be achieved by designing a system that does not change its surface area as a function of time. Sivaramakrishnan and co-workers have achieved sustained release of bovine somatotropin from a poly(lactic acid) implant enclosed in a microporous polyethylene sleeve (Sivaramakrishnan et al., 1989).

Oppenheim and co-workers have used a combination of drug diffusion and polymer hydration to achieve release of luteinizing hormone releasing hormone, epidermal growth factor, and luteinizing hormone from acrylic polymer coated polypeptide–hydrophilic excipient implants. Release periods from 1 week to several months were achieved depending on the implant type and coating thickness (Oppenheim et al., 1988).

In pendant chain systems, the second type of chemically-controlled system, a drug is chemically bound to a polymer backbone and the drug is released by hydrolytic or enzymatic cleavage. The polymer system can be either soluble or insoluble. Soluble backbone chains are generally used for transport functions such as cell-targeting; insoluble forms are more desirable for long-term controlled release implants. The backbone may also be biodegradable or nonbiodegradable. For in vivo use, it is important that the polymers do not cause immunological reactions and that drugs, when coupled to the polymers, do not function as haptens and induce allergic reactions. The drug itself can be attached directly to the polymer, or it can be attached via a spacer group. The spacer group may be used to affect the rate of release and hydrophilicity of the system.

Solvent-Activated Systems. Solvent activation can be accomplished by either swelling or osmosis. Swelling-controlled release of potent drugs may be achieved by employing the glassy/rubbery transition of polymers in the presence of a penetrant and the macromolecular relaxations associated with this transition.

In these systems the drug is originally dissolved or dispersed in a polymer solution; the solvent is then evaporated, leaving the drug dispersed in a glassy (solvent-free) polymer matrix. There is no drug diffusion in the solid phase. As the dissolution medium (e.g., water) penetrates the matrix, the polymer swells and its glass transition temperature is lowered below the temperature of the surroundings. Therefore, the swollen polymer is in a rubbery state, and it allows the drug contained in it to diffuse outward (Langer et al., 1981).

In osmotic systems, water may permeate a drug–polymer system as a result of osmotic pressure, causing pores to form and bringing about drug release. An attractive osmotic system that can provide constant release rates exists in the form of a pill that has a laser-drilled hole in the surface of a polymer coating (Theeuwes, 1975).

Eckenhoff and co-workers have prepared an osmotic implant device containing porcine somatotropin in a glycerol, gelatin, L-histidine gel. This device has an exit passageway for the release of the protein and a separated compartment for the gel and the osmotic driving excipients. Controlled release in vitro was demonstrated for over a 2-month period (Eckenhoff et al., 1990).

Pulsatile Polymeric Controlled Release Systems. It would be desirable if polymeric systems could be designed to release increased levels of drug when needed; this would mimic the body's physiologic processes. Both open-loop and closed-loop approaches are being studied with the major utility projected for human pharmaceuticals. One open-loop system contains drug and small magnetic beads embedded in a polymer matrix. Release rates are enhanced when desired by an oscillating external magnetic field. Parameters that affect the release rate include the magnetic field frequency and strength, the polymer composition, and the strength and orientation of the polymer-embedded magnets. Application of the magnetic field causes up to 30-fold increases in release rates (Edelman et al., 1989). Ultrasound can also be used to enhance drug release rates from polymers (Kost et al., 1989). Several closed-loop polymeric systems are being developed. Many of these are intended for the increased release of insulin in the presence of excess glucose. These systems often involve placing enzymatic biosensors inside the polymeric system (Kost, 1990).

Researchers at Alza have reported a patterned drug delivery device for porcine somatotropin. The device

consists of a semipermeable wall with osmotic tablets separated from porcine somatotropin tablets (Wong et al., 1991).

CONCLUSIONS AND FUTURE DIRECTIONS

There are numerous challenges ahead. Feed additives remain an important method for drug delivery to large animals. One challenge is the creation of bioadhesive polymers that could alter a drug's location when given orally. This could be particularly important for drugs that are adsorbed only in certain segments of the gastrointestinal tract. Even more significant, but more complex, is delivery of large molecules such as proteins orally. Research on novel anatomical delivery pathways such as the nose or lung may also permit the delivery of a broader spectrum of drugs and new dosage forms for large and small animals.

Furthermore, continuous advances in biotechnology will have at least several major effects on drug delivery. First, novel complex drugs will be created that will be difficult to administer by conventional means. Second, advances in materials science and chemical engineering should permit improved polymers and other substances to be created and effectively used in drug delivery. Third, manufacturing plant design will rely on engineering advances in the scale-up and sterilization of these formulations. These advances continue to create new challenges for formulation scientists in the manufacture of economically viable "biotech" formulations for the animal health industry.

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