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Review Paper

GLIMPSE ON PROTEIN DRUG DELIVERY: AN UTMOST RESEARCH AREA FOR BIOPHARMACEUTICALS

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ABSTRACT

A major challenge confronting pharmaceutical scientists in the future will be to design successful dosage forms for the next generation of drugs. Many of these drugs will be complex polymers of amino acids (e.g., peptides, proteins), nucleosides (e.g., antisense molecules), carbohydrates (e.g., polysaccharides), or complex lipids. Protein and peptide therapeutics currently represent eight of the top 100 prescription pharmaceuticals in the US, and biotechnology products are projected to account for 15% of the total US Prescription drug market by 2003. Conventional drug formulation has the same focus but, due to the unique structures of peptide and protein molecules, formulation of these compounds is more complex and challenging. Therapeutic peptides and proteins always enjoyed unique place in pharmaceutical biotechnology. Peptides and proteins are expected to mitigate suffering in coming years as anticancer, hormones, analgesic antihypertensive, thrombolytics, growth factors, and many others. This review represents outstanding contributions in the field of biopharmaceuticals.

Keywords: *Protein and Peptides, Protein Stability, Formulations, Future prospective, Regulatory issues*

Protein and Peptides: – A Gleam!

Proteins are biomolecules that are essential in determining the structure and carrying out most of the functions in living cells that make up all living organisms. They are made up of individual units called amino acids, which although similar in structure, have different characteristics. There are about 20 different amino acids in nature and these assemble in chains of varying lengths to form proteins. The order of amino acids determines the structure and function of the protein. Genes specify this order. [1]

Types of protein based drugs: [2]

Cytokines - these drugs regulate the immune system. That is, they are proteins that activate the immune system cells to carry out different immune functions.

Hormones - protein drugs that regulate functions in the body. As drugs, these proteins can be used to elevate levels of certain hormones, such as estrogen during menopause or growth deficiency. They can also be used to treat certain diseases such as diabetes.

Clotting factors - proteins that regulate the clotting of blood. These drugs are used to treat blood clotting disorders such as hemophilia. **Vaccines** - proteins that stimulate the immune system to produce specific antibodies. **Monoclonal Antibodies** - proteins that mark a specific foreign material (such as cancer cells, disease-causing bacteria and viruses), for removal or destruction by other components of the immune system. These are also used as effective diagnostic tools for many specific genetic diseases and other conditions such as pregnancy. Some of the US FDA approved

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protein and peptide based drugs along with its applications are given in Table I. [3]

Table I: List of Therapeutic protein and peptide drugs.

Product name	Indication	Approval Date
Granulocyte colony-stimulating factor	Bone marrow transplant	June 1994
Glucocerebrosidase	Gaucher's disease	May 1994
Somatotropin	Chronic renal insufficiency	November 1993
Interferon β-1b	Multiple sclerosis	July 1993
Interlukin-2	Renal cell carcinoma	May 1992
Granulocyte/macrophage colony stimulating factor	Bone marrow transplant	March 1991
Granulocyte colony- stimulating factor	Chemotherapy-induced neutropenia	February 1991
Hepatitis B vaccine	Hepatitis B	September 1989
Erythropoietin	Dialysis anemia	June 1989
Interferon α-2a and α-2b	Kaposi's sarcoma	November 1988
Somatotropin	Human GH deficiency in children	March 1987
Interferon α-2a and α-2b	Hairy cell leukemia	June 1986
Hepatitis B vaccine	Hepatitis B prevention	July 1986
Somatrem	Human GH deficiency in children	October 1985
Human insulin	Diabetes	October 1982

Stability of Protein and peptides:

A major challenge to the formulation of Proteins and peptides in to efficious dosage form is to ensure their stability over their shelf-life. Instability of Proteins and peptides may be broadly classified as

Physical instability

Physical instability refers to any change of the folded state that does not include bond cleavage or formation, i.e. changes in the spatial, three-dimensional conformation (secondary, tertiary and quaternary structure) of the protein.

1. Denaturation (Protein destabilization)

To assure protein stability in both liquid and solid formulations, the processes that cause physical destabilization of protein must be understood. Denaturation refers to an alteration of the global fold of a protein molecule, i.e. a disruption of the higher-order structure, such as tertiary and often also the secondary structure. An extreme description of this state is that only random non-covalent interactions are present although conformational preferences do exist. This situation is often referred to as a 'random coil' configuration. Unfolded states can vary considerably in the extent of residual structure, but some feature that relates to the structure of the native protein will remain and some hydrophobic interactions may persist even under extreme denaturation conditions. [4]

2. Aggregation and precipitation

Chemical reactions (polymerization) between different protein molecules result in the formation of covalently linked protein dimers or polymers.

Because the origin of protein aggregates or precipitates is frequently not known, the term 'aggregation' is unfortunately often used in protein literature to describe this process, which should rather be termed 'polymerization'. In practice, however, protein ensembles/precipitates often are mixtures of covalently and non-covalently linked protein molecules. [5] There are two basic types of protein non-covalent interaction, which for clarity here are termed association if the process involves protein molecules with native structure, and aggregation when denatured protein molecules are involved. Self-association of the native protein happens as a result of changes in solvent environment (solvent composition, pH, ionic strength, protein concentration, etc.) analogous to the conditions facilitating protein crystallization, isoelectric precipitation, salting out, etc. Thus, if a protein is placed in a high concentration of salt, the surface charges on the protein become masked such that charge-charge repulsion between different native protein molecules does not occur. A similar phenomenon of reduced charge repulsion can occur when the pH of the solution approaches the isoelectric point of the protein. In both cases the surface charge neutralization can result in association of protein molecules with native tertiary structure, and, if the protein concentration is high enough, precipitation of the protein oligomers occurs. [6]

3. Surface adsorption

The presence of an air-water or solid-water interface has an important influence on the normal forces stabilizing protein higher-ordered structure in solution. Partial unfolding of

the protein can occur at the interface which leads to adsorption to the surface through interaction of hydrophobic amino acid residues and a hydrophobic surface, or through binding of polar amino acid residues to charged surfaces. Therefore, proteins can be adsorbed both to non-polar solid (or air) surfaces and to surfaces with ion-exchange properties (such as glass), although greater adsorption normally occurs at hydrophobic than at hydrophilic interfaces. Normally a protein in solution reaches a surface by diffusion, and therefore the rate of adsorption is a function of the protein concentration. [7]

Stabilization strategies

From the previous sections, it is obvious that rational strategies to improve the practical

Physical stability of protein drugs includes the following main approaches:

1. Stabilize the native structure of the protein (i.e. promote self-association, improve native

hydrophobic interaction by adding co-solvents or by genetic engineering

2. Prevent aggregation of unfolded structures (polyethylene glycols, cyclodextrins)

3. Avoid or block unwanted hydrophobic interfaces (surfactants, avoid head space in Container)

4. Reduce shear forces (avoid head space in container).

Chemical instability

Chemical instability involves covalent modification in the amino acid sequence (primary structure),i.e. bond formation or cleavage, resulting in a new chemical entity. It is an outcome of reactions, such as

1. Deamidation

The deamidation reactions of asparagine (Asn) and glutamine (Gln) side chains are among the most widely studied nonenzymatic covalent modifications to proteins and peptides. The primary reaction mechanism for the deamidation of Asn in water-accessible regions of peptides and proteins at neutral pH. The key step is the formation of a deprotonated amide nitrogen, which carries out the rate-determining nucleophilic attack on the side chain carbonyl, resulting in the formation of the five-

membered ring succinimide intermediate. For such a reaction, the leaving group must be easily protonated, and in this case it is responsible for the formation of ammonia (NH3). The succinimide ring intermediates subject to hydrolysis, resulting in either the corresponding aspartic acid or the iso aspartic acid (B-aspartate). Often, the ratio of the products is3 : 1, isoaspartate to aspartate . The reaction also appears to be sensitive to racemization at the a carbon, resulting in mixtures of D- and L-isomers. [8]

2. Oxidation

Oxidation is one of the most commonly observed chemical degradation pathways of peptides and proteins. Among the amino acids most susceptible to oxidative modification are those that contain sulphur (e.g. Met and Cys) or an aromatic ring (e.g. His, Tyr, and Trp). There are a number of mechanisms that may result in oxidative modification of amino acids. [9]

Despite the variations in conditions used to induce oxidation, the underlying principle involves the activation of oxygen to generate a few key reactive oxygen species, which ultimately then react with the protein or peptide.

A) Autooxidation

Autooxidation refers to the direct reaction between ground state molecular oxygen and a

Compound in the absence of any catalytic processes. [10] For a hypothetical formulation that contained a standard protein of molecular weight 22,000 in an air-saturated and metal- and peroxide-free aqueous buffer. The rates of autooxidation of the most labile amino acid residues, Cys and Met, were calculated for a pseudo-first-order process. True autooxidation of peptides and proteins is a very slow process that poses no significant threat to the stability of these compounds.

B) Metal-catalysed oxidation

In the presence of a transition metal ion (i.e. Fe(III) and Cu(II)), oxygen, and an electron

donor (reducing agent), metal-catalysed oxidation of proteins and peptides can occur at significant rates. Normally, transition metal ions in their oxidized states (i.e. Cu(II) and Fe(III)) do not readily react with oxygen to generate a more reactive oxygen species. An electron donor or reducing agent known as a prooxidant is needed to reduce the transition metal ion; this reduced form may then interact with oxygen to generate reactive oxygen species. The prooxidant in pharmaceutical formulations may arise from contaminants in buffers or may have been added to the formulation, ironically, as. an antioxidant (i.e. ascorbic acid). [10]

C) Photooxidation

Some pharmaceutical proteins are photosensitive and, therefore, undergo oxidative modification when exposed to light. Light-induced oxidation of protein pharmaceuticalsmay occurs during protein processing and storage. Photooxidation is initiated when a compound absorbs a certain wavelength of light, which provides energy to raise the molecule to an excited state. The excited molecule can then transfer the energy to oxygen, converting it to singlet oxygen, while returning to ground state. Alternatively, the excited molecule can react directly with other molecules. This compound, which in essence initiates photooxidation reactions, is often referred to as a photosensitizer. [11]

3. ß-Elimination reactions

ß-Elimination is another pathway of peptide/protein degradation. Amino acid residues that undergo ß-elimination reactions include Cys, Ser, Thr, Phe, and Lys. ß-Elimination reactions result from the abstraction of a proton from the a-carbon of an amino acid residue in a polypeptide chain resulting in the formation of a carbanion intermediate. Addition of a proton to the opposite face of the

molecule can lead to racemization. Alternatively, the carbanion intermediate can undergo further reaction to forma dehydroalanine residue (R=H). ß-Elimination reactions have been observed in a number of proteins, i.e. lysozyme and bovine pancreatic ribonuclease A. [12]

4. Disulphide exchange reactions

Disulphide bonds are of great importance to the structural stability of many proteins, as

they are the most frequently encountered covalent crosslink's in proteins. The disulphide bond may serve to join two independent polypeptide chains in an inter molecular fashion or may form between two Cys residues of one polypeptide chain in an intramolecular fashion. Thus, the interchange and/or cleavage of disulphide bonds can lead to an altered protein three-dimensional structure, which may result in the loss of biological activity [13]

Challenges in Protein delivery:

Despite many features that proteins have as therapeutic agents, they have some serious limitations. Proteins are relatively large molecules with complex architecture. Unlike low-molecular weight drugs, they possess secondary, tertiary and in some cases, quaternary structure with labile bonds and side chains with chemically reactive groups. Disruption of these structures or the modification of side chains, which occurs readily with many proteins, can lead to loss of activity or immunogenicity. Protein purification, analytical characterization is also critical issues. Above all, the most challenging task is their delivery. The main barriers to successful delivery of proteins are enzymatic barriers and absorption barriers imposed by gastrointestinal tract. Various kinds of enzymes in gastrointestinal tract which results in degradation are proteases like pepsin, intestinal proteases like trypsin, elastases, brush border proteases like amino peptidases, carboxy peptidases, and systolic proteases like di-tripeptidases. Because of close correlation between protein efficacy and molecular three-dimensional structure it is essential to maintain the structural integrity through all formulation steps of delivery system and while the drug is released from the site of delivery; otherwise, the activity the protein may be reduced or lost entirely. Chemical degradation may also occur at many points during formulation delivery; the most common being oxidation, example, oxidation of methionine causes loss of bioactivity. [14] Since peptide/protein drugs are not absorbed orally, prolonged maintenance of therapeutically active drugs in the circulatory system is of primary clinical importance. Another major obstacle of injected polypeptide drugs is the elevated concentration of 100–1000 times above the therapeutically level that may be present in the circulatory system shortly after administration. Such overdosing may lead to undesirable down-regulation of receptor sites. In this review we describe two new strategies that overcome these two problems of systemically injected peptide/protein drugs. The first strategy includes derivatization of peptides and second strategy, reversible pegylation. Inactive pegylated peptide/protein drugs release the native active parental molecules at slow rates, and in homogeneous fashion under physiological conditions, thus facilitating prolonged therapeutic effects, following a single administration. [15]

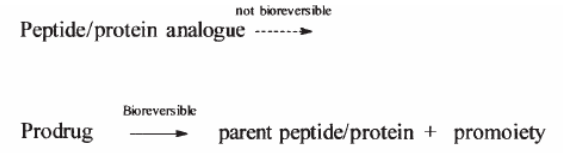
Researched Technology Area and Opportunities:

1. Chemical Modification Approach

Two approaches may be possible: either a permanent chemical change in the drug molecule (i.e. the analogue approach) or bioreversible derivatization of the bioactive peptide or protein (i.e. the prodrug approach). A prodrug is by definition a pharmacological inactive derivative of a drug molecule that is capable of releasing the parent

molecule quantitatively, either spontaneously or enzymatically, in the body. The chemical group used for derivatization of the drug molecule, called the pro-group or pro-moiety, should be non-toxic. In contrast to prodrugs, should possess improved absorption and/or stability characteristics over the parent drug molecule. The analogue should also have high receptor selectivity and affinity. [16]

In recent years several types of analogues of various bioactive peptides and proteins have been explored. Possible strategies used in the development of analogs include *N*-and-terminal modifications (e.g. conversion of the *C*-terminal carboxylic acid residue to an amide); amino acid manipulations (e.g. systematic replacement of L-amino acids with Daminoacids); peptide backbone modifications, where the use of amide isosters is common(e.g. *N*-methylation of the peptide amide bond); and replacement of larger structural moieties in a compound with dipeptide or tripeptide analogue structures or analogues of the secondary structure . [17]



List of an examples of Prodrugs and Analogue

1) 4-Imidazolidinone prodrugs
2) Derivatives of Insulin and Desmopressin
3) Prodrugs of Thyrotropin Releasing Harmone
4) Cyclic prodrugs

2. Conjugation with Polymers

The conjugation of peptides with water-soluble polymers slows down renal filtration, therefore increasing their residence time in circulation. One of the most commonly employed processes is polyethylene glycol (PEG)-ylation technology. This technology enlarges the size of an active molecule by attaching a web-like shield of hydrated PEG polymer chains around the molecule. One of the key benefits of this modification is to increase clearance half-life and provide the possibility of the drug staying in systemic circulation for longer. Furthermore, this technology increases molecular stability, changes the volume of distribution within the body and reduces immune reactions, making the drug more effective. Polymer molecules attached to the protein globule creates steric hindrances for the interaction of protected polypeptides with active sites of proteases, opsonins and antigen-processing cells. [18] The anticancer activity of PEGylated interferon (IFN),which was approved by the US Food and Drug Administration (FDA) for the treatment of chronic hepatitis C. In addition, certain polymers that are not large enough to prevent the renal clearance, but attach themselves, together with the conjugated drug. For example, conjugation with poly (styrene-co-maleic acid anhydride (SMA)) with a molecular weight (MW) as low as 1.5kDa increases the circulation time of anticancer polypeptides via binding to plasma albumin. [19] Neocarzinostatin-SMAconjugate is currently approved in Japan for the treatment of hematoma.

3. Pulmonary Delivery Approach

Research has shown that many molecules are absorbed through deep lungs into the blood with high bioavailability; this does not need enhancers used by other non-invasive routes. [20] The transportation occurs through transcytosis (through the cells) or by paracellular tranpsort (through cell junctions). The route taken depends upon the molecular weight of the peptide with transcytosis for large molecules and paracellular for small molecule transport. After crossing the membranes, the molecules are absorbed rapidly into the blood through the capillary endothelial cells or, in cases of large proteins, slowly drained through pulmonary lymphatics that eventually empty into the bloodstream. In this way, the high bioavailability

via deep lungs and the robustness of the organ make it natural port of entry into the human body for protein and peptide drugs including small molecules. Since low molecular weight drugs absorb faster, they can therefore be exploited for pain, hypertension and anaphylaxis management. [21]

4. Micro / Nano – Particulate System

The development of this formulation includes screening for the appropriate pH, buffer, and excipients. Each proteins likely to have a unique set of conditions (e.g., pH, excipients, etc.) that provide stability during microencapsulation. The development of a stable protein formulation for microencapsulation also includes consideration of the potential for protein-polymer interactions. For example, proteins that are very basic (high PI) may interact with the free acid groups generated by the degradation of polylactides. Inthis case, it may be necessary to add excipients such as polyionic compounds (anionic for protein binding, cationic for polymer binding) that prevent or reduce the interaction between the protein and the polymer. For polylactides,it may be unlikely that the protein will form a covalent adduct with the polymer under normal physiological conditions, but other polymers may react with surface moieties on the protein (e.g., lysines) during degradation. If an adduct of the protein and polymer is formed, the protein may become inactivated or immunogenic. Therefore, it is essential to assess the possible interactions (covalent and no covalent) between the polymer and protein. [22]

Although, a number of microencapsulation techniques have been developed and reported to date, the choice of the technique depends on the nature of the polymer, the drug, the intended use, and the duration of the therapy. The microencapsulation method employed must include the following requirements [23],[24]

(i) The stability and biological activity of the drug should not be adversely reacted during the encapsulation process or in the final microsphere product.

(ii) The yield of the microspheres having the required size range (up to 250 μm, ideally 125 μm) and the drug encapsulation efficiency should be high.

(iii) The microsphere quality and the drug release profile should be reproducible within specified limits.

(iv) The microspheres should be produced as a free flowing powder and should not exhibit aggregation or adherence.

As described in the previous sections, injectable microparticles from PLA and PLGA have been successfully prepared to deliver drugs like peptides, proteins, and vaccines over a period of days, weeks, or even months ata constant rate depending upon the degradation behavior of the polymer employed. [25] However due to their large size, it was impossible to direct the drug to target tissues via systemic circulation or across the mucosal membrane. Following oral administration, particles less than 500 nm can cross the M cells in the Payer'spatch and the mesentery on the surface of the gastrointestinal mucosa, delivering the drug to the systemic circulation. [26] Nanoparticles (nanospheres and nanocapsules) could be prepared by the same methods as those described for microparticles, except that manufacturing parameters are adjusted to obtain nanometer-size droplets. [27]

Manufacturing techniques of Micro / Nano – Particulate System

- Solvent evaporation and solvent extraction process
- Double (multiple) emulsion process.

- Phase separation (coacervation)
- Spray drying
- spray freeze drying (Gombotz et al. 1990**)** [28]
- Supercritical fluid extraction techniques (Randolph et al.1994) [29]

Table II lists various marketed formulations of protein based biodegradable microspheres. [30]

Table II: Marketed formulations of proteins based on biodegradable microsphere.

Drug	Trade Name	Company	Route	Application
Leuprolide acetate	Lupron Depot	Takeda-Abott	3-month depot suspension	Prostate cancer
Recombinant human growth Hormone	Nutropin Depot	Genentech Alkermes	Monthly s /c injection	GH deficiency
Goserelin acetate	Zoladex	I.C.I	S/c implant	Prostate cancer
Octreotide acetate	Sandostatin LAR Depot	Novartis	Injectable s /c suspension	GH suppression
Triptorelin	Decapeptyl	Decapeptyl	Injectable depot	LHRH agonist
Recombinant Bovine Somatropin	Posilac	Monsanto	Oil based injection	milk production in cattle

5. Diffusion-Controlled Delivery from Hydrogels

Hydrogel is diffusion-controlled delivery systems, where water is the main transporting

medium, the protein solution stability governs the type of device. Extended releasing times can be achieved with reservoir systems (Fig. 1) for highly stable proteins [31] Alternatively, dehydrated delivery systems can be created by direct compression of protein and hydrophilic polymer powder blends to form a matrix tablet (Fig. 2). To delay or prevent contact of biological fluid with the protein, additional measures must be taken such as laminating hydrophobic layers onto the hydrophilic polymer/protein core. [32] Such a device prevents contact of the protein with the biological fluids until diffusion and release occurs at the appropriate time, pH, or location and hence prolongs storage and shelf life. The reservoir, matrix, and biodegradable (initial release) systems are examples of diffusion-controlled delivery. Swelling-controlled delivery systems involve three processes: absorption of water into the polymer to form the hydrogel, dissolution of the protein, and subsequent release of the protein from the device. Hydrogels exhibit this type of release behavior when placed in an aqueous medium initially in the dehydrated state. Swellable hydrogel systems are produced by cross-linking water-soluble polymers, such as poly(2-hydroxyethyl methacrylate) (PHEMA) In addition to PHEMA, some of the other commonly used hydrogels include poly(acrylic acid)(PAA), polyacrylamide (PA), poly vinyl alcohol (PVA), poly (methacrylic acid)(PMAA),poly methyl methacrylate (PMMA), poly-(*N*-vinylpyrrolidone) (PVP), poly(ethyleneoxide) (PEO), poly ethylene glycol (PEG), and polysaccharides. [33]

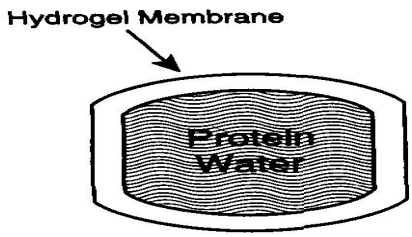


Fig. 1 The hydrated state of a protein reservoir system**.**

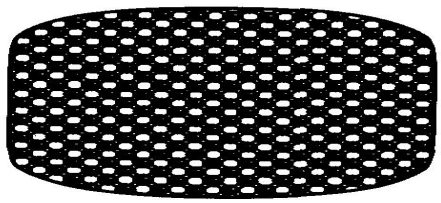


Fig. 2: The dehydrated delivery device produced by compression of Protein and hydrophilic polymer powder.

6. Injectable Implant

Biodegradable implants and microspheres for parenteral administration could extend the half-life of serum-labile proteins and provide an effective mechanism for localized as well as systemic delivery. Recently, a liquid polymer system (ATRIGEL™) has been developed which has both the simplicity and control of solid biodegradable implants and the injectability of microspheres for delivering drugs. [34] This drug delivery system combines a biodegradable polymer with a biocompatible solvent, resulting in a solution that can be injected using standard syringes and needles. When the system contacts physiologic fluid, the polymer precipitates as the solvent diffuses into the surrounding tissues. As a result, a biodegradable polymeric implant is formed. For controlled release applications, a drug can be incorporated into the delivery system. The incorporated drug is physically entrapped within the precipitated polymer matrix and is then slowly released. The polymer type, concentration, and molecular weight as well as the carrier solvent, drug load and formulation additives each influence the release kinetics. Manipulation of these formulation variables provides diverse drug delivery profiles as well as polymer biodegradation rates for specific applications. Candidate biodegradable polymers for use in the drug delivery system include homopolymers of poly (DL -lactide) (PLA) and copolymers of poly(DL -lactide-*co*-glycolide) (PLG) and poly(DL-lactide-*co*-caprolactone)(PLC). These polymers are similar in chemical composition to biodegradable sutures and have been well characterized in the literature [35] Biocompatible solvents utilized with the system include *N*-methyl-2-pyrrolidone (NMP) and dimethyl sulfoxide (DMSO). Safety studies conducted with pharmaceutical-grade solvents provide extensive toxicological profiles thatsupport substantial margins of safety for both the neat solvents and ATRIGEL™ formulations prepared with these solvents. [36] Proteins Studied in the ATRIGEL™ Drug Delivery System given in Table III.

Table III: List of Proteins Studied in the ATRIGEL™ Drug Delivery System.

Model proteins	Enzymes	Hormones	Cytokines
Ovalbumin	Trypsin	Somatotropin	Interleukin-2
Bovine serum albumin	Horseradish peroxidase	Growth hormone-releasing factor	Fibroblast growth factor
Cytochrome *c*	Lysozyme	Insulin	Interferon-b

7. Transdermal Peptide Delivery Using Electroporation

The feasibility of passive transdermal delivery, however, is limited by the size, charge, and dose of the drug to be administered. Owing to these limitations, viable candidates for passive transdermal delivery remain few in number and are restricted to small molecular-weight, lipophilic, uncharged, and potent drugs. Peptides and proteins, owing to their large size and ionic character, do not readily pass through the skin, and effective transport often requires enhancement techniques, Iontophoresis, the electromigrational movement of charged molecules through the skin under a low-voltage and continuous electrical driving force, is one such enhancement method. The iontophoretic delivery of large-molecular-weight compounds such as luteinizing hormone-releasinghormone (LHRH) or analogs, thyrotropin-releasing hormone (Burnetteand Marrero, 1986), and insulin [37] as well as smaller-molecular-weight compounds such as lidocaine [38] has been reported. However, the success of iontophoretic delivery of large-molecular-weight compounds such as peptides remains elusive, primarily owing to the impermeable nature of skin and the consequent

inability to deliver therapeutically meaningful doses in humans. Large-molecular-weight (or, more correctly, larger molecular-volume) compounds can be introduced into cells via a process known as electroporationor electropermeabilization [39] This technique involves the application of short, transient (microsecond–millisecond) electrical pulses of high magnitude (1 kV/cm) which induce a short-lived (up to seconds) and reversible, high-permeability state in the membrane lipids. The mechanism underlying electroporation has been studied using artificial lipid bilayer membranes and liposomes. It has been hypothesized that the lipid bilayers are reversibly permeabilized by the formation of transient pores, shown schematically in Fig. 3. Although tantalizing data exist indicating the formation of transient "pores" in cell membranes following electroporation, the definitive demonstration of pores remains elusive. [40]

Future Prospective and Regulatory issues:

The recombinant proteins listed in Table I which have been marketed as drugs at the time of writing. As can be seen, they represent a broad variety of biological substances from hormones and cytokines through enzymes and blood coagulation regulators to vaccines. According to various sources, there are at present around 250 new proteins in advanced clinical trials. However, around 100 represent truly novel pharmaceutical substances with no precedent in medical therapy. It is estimated that around 2000 gene technology-based drugs, comprising both proteins and non-proteins, are in early stages of development. As far as regulatory issues for macromolecules is concerned there is clinical demonstration. It includes test of safety and efficacy for a new pharmaceutical needed for registration, approval and marketing generally comes from 'pivotal' trials. A common requirement has been for the manufacturer to perform two randomized, double blind trials controlled with either a placebo group or a group treated with a pharmaceutical approved for the same indication. Such trials are expected to show a meaningful benefit to the patient with appropriates safety parameters. These issues are evaluated on a 'case-by-case' basis, with the agencies balancing their risks and benefits of the new treatment. These evaluations (and consequent requirements) are performed by the agencies in each country where the drug is to be marketed and, although 'harmonization' discussions have simplified the task of the manufacturers to meet the individual requirements of separate countries, it is not uncommon for the data from a trial in one country to be insufficient for approval in another country. 'Worldwide' approval to market anew drug therefore requires approval in each target country. [41]

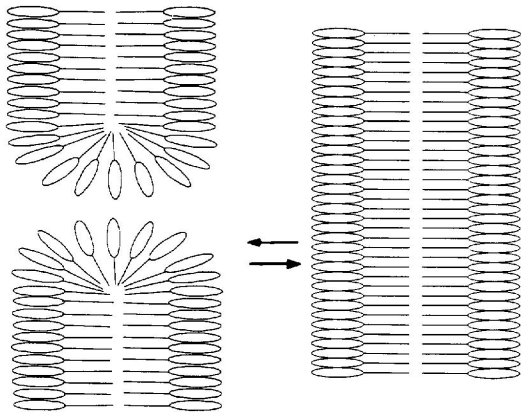


Fig. 3 Schematic representation of changes in lipid bilayer structure induced by Electroporation.

Legends:

µg	Micro Gram
µm	Micro Meter
Asn	Asparagine
Gln	Glutamine
Met	Methionine
Cys	Cystine
His	Histedine
Tyr	Tyrosine
Trp	Tryptophene
Lys	Lysine
kDa	Kilo Dalton
INF	Interferon
SMA	Poly Styrene-Co-Maleic Acid Anhydride
MW	Molecular Weight
PHEMA	Poly(2-Hydroxyethyl Methacrylate)
PAA	Poly(Acrylic Acid)
PA	Polyacrylamide
PVA	Poly Vinyl Alcohol
PMAA	Poly (Methacrylic Acid)
PMMA	Poly Methyl Methacrylate
PVP	Poly-(*N*-Vinylpyrrolidone)
PEO	Poly(Ethyleneoxide)
PEG	Poly Ethylene Glycol
PLA	Poly(DL -Lactide)
PLG	Poly(DL -Lactide-*Co*-Glycolide)
PLC	Poly(DL-Lactide-*Co*-Caprolactone)
DMSO	Dimethyl sulfoxide

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