Strategies for Delivery of Peptides and Proteins

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1990 – 1994, BS in Molecular and Chemical Physics

PhD in Pharmaceutical Sciences, OSU

1995

2000

P&G or Esperion?

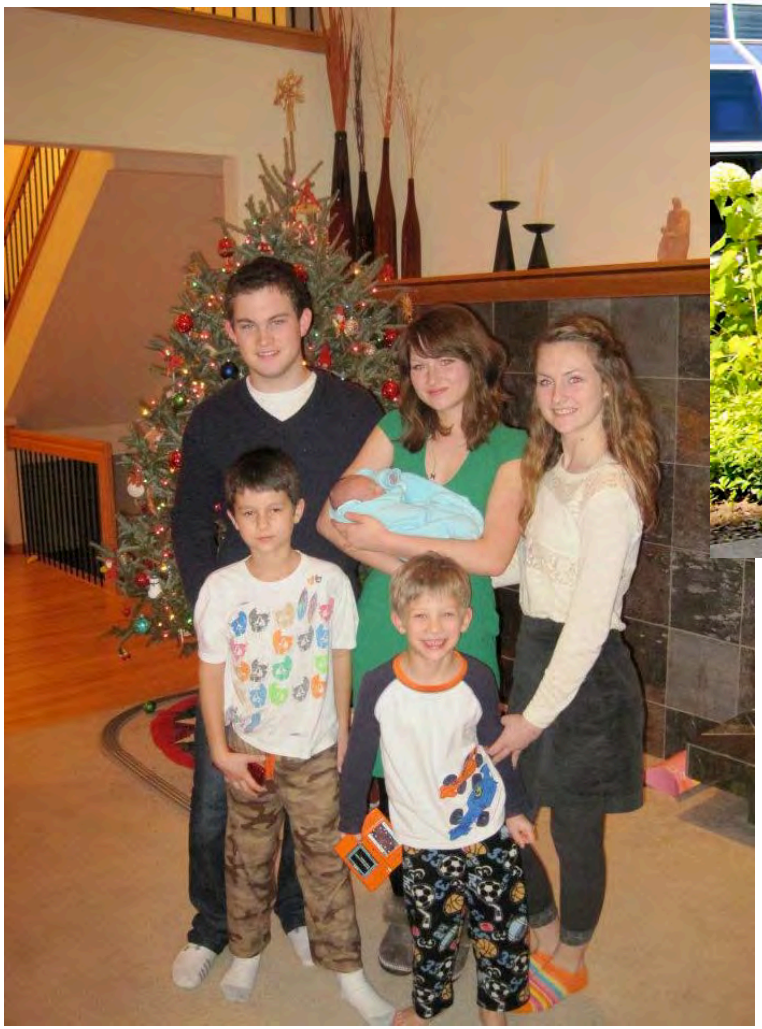
Esperion Therapeutics 2000 - 2005

Pfizer to buy Esperion for $1.3bn

Sunday, December 21, 2003

Cerenis Therapeutics 2006 – 2011

2012, Assistant Professor UM COP



December 2016

Top 20 products (2015)

	RANK	2015 SALES (US$ Mn)	2015 GROWTH (LC$ %)	2014 SALES (US$ Mn)
GLOBAL MARKET		954,116	9.5	943,934
HARVONI	1	18,144	1,159.0	1,565
HUMIRA	2	14,950	27.7	12,437
LANTUS	3	11,458	14.6	10,380
ENBREL	4	9,471	9.2	9,194
CRESTOR	5	8,608	5.8	8,511
REMICADE	6	8,195	6.2	8,299
SERETIDE	7	7,996	-2.6	8,684
SOVALDI	8	6,578	-28.2	9,388
MABTHERA	9	6,298	3.3	6,631
AVASTIN	10	6,183	9.6	6,134
LYRICA	11	6,035	7.7	6,022
ABILIFY	12	5,799	-36.3	9,299
NOVORAPID	13	5,612	23.5	4,736
HERCEPTIN	14	5,596	8.4	5,688
JANUVIA	15	5,440	14.3	4,965
SPIRIVA	16	5,364	3.3	5,521
XARELTO	17	5,144	38.9	3,996
NEXIUM	18	5,065	-32.2	7,682
COPAXONE	19	5,050	7.1	4,920
NEULASTA	20	4,737	5.1	4,654

IMS Health

Top 20 Most Valuable R&D Projects (Ranked by Net Present Value)

Rank	Product	Company	Phase (Current)	Pharmacological Class	WW Product Sales ($m) 2020		Today's NPV ($m)
1	**Nivolumab**	Bristol-Myers Squibb	Phase III	Anti-programmed death-1 (PD-1) MAb	6,012		**23,150**
2	**MK-3475**	Merck & Co	Filed	Anti-programmed death-1 (PD-1) MAb	4,063	new entry	**16,747**
3	**RG7446**	Roche	Phase III	Anti-programmed death-1 ligand-1 (PD-L1) MAb	2,837	new entry	**15,639**
4	**Obeticholic acid**	Intercept Pharmaceuticals	Phase III	Farnesoid X receptor (FXR) agonist	2,992	new entry	**11,426**
5	**Ledipasvir/Sofosbuvir**	Gilead Sciences	Filed	Hepatitis C nucleoside NS5A & NS5B polymerase inhibitor	2,818		**9,876**
6	**Palbociclib**	Pfizer	Phase III	Cyclin-dependent kinase (CDK) 4 & 6 inhibitor	2,950		**7,925**
7	**DCVax-L**	Northwest Biotherapeutics	Phase III	Cancer vaccine	2,046	new entry	**5,502**
8	**VX-809 + ivacaftor**	Vertex Pharmaceuticals	Phase III	Cystic fibrosis transmembrane conductance regulator (CFTR) corrector	1,900		**5,011**
9	**MEDI4736**	AstraZeneca	Phase III	Anti-programmed death-1 ligand-1 (PD-L1) MAb	967	new entry	**4,711**
10	**Lampalizumab**	Roche	Phase II	Anti-complement factor D MAb	1,122	new entry	**4,520**
11	**Revascor**	Mesoblast	Phase III	Mesenchymal stem cell	-	new entry	**4,332**
12	**Idelalisib**	Gilead Sciences	Filed	Phosphatidylinositol 3-kinase (PI3K) inhibitor	1,273	new entry	**3,615**
13	**Evolocumab**	Amgen	Phase III	Anti-proprotein convertase subtilisin-like kexin type 9 (PCSK9) MAb	1,093	new entry	**3,563**
14	**LCZ696**	Novartis	Phase III	AT1 receptor-neprilysin (ARNI) inhibitor	1,329	new entry	**3,005**
15	**Nivolumab**	Ono Pharmaceutical	Filed	Anti-programmed death-1 (PD-1) MAb	348		**2,996**
16	**Alirocumab**	Sanofi	Phase III	Anti-proprotein convertase subtilisin-like kexin type 9 (PCSK9) MAb	1,048		**2,950**
17	**Plegridy**	Biogen Idec	Filed	Interferon beta	1,047	new entry	**2,931**
18	**Abemaciclib**	Eli Lilly	Phase III	Cyclin-dependent kinase (CDK) 4 & 6 inhibitor	651	new entry	**2,922**
19	**Ocrelizumab**	Roche	Phase III	Anti-CD20 MAb	894	new entry	**2,777**
20	**Secukinumab**	Novartis	Filed	Anti-interleukin-17 (IL-17) MAb	1,030	new entry	**2,735**
	Top 20				36,520		**136,332**
	Other				111,649		282,192
	Total				148,169		**418,525 46%**

Source: EvaluatePharma® (1 JUN 2014)

NPV of R&D Pipeline June 2013: 286,367

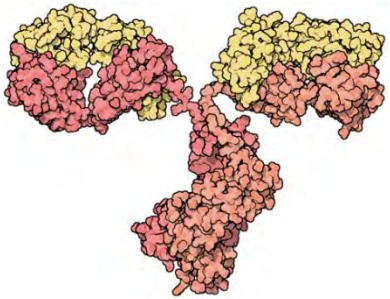
Protein Therapeutics

Functions of Proteins

Structural

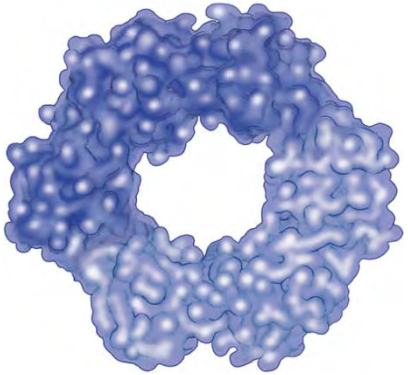
collagen

Immune



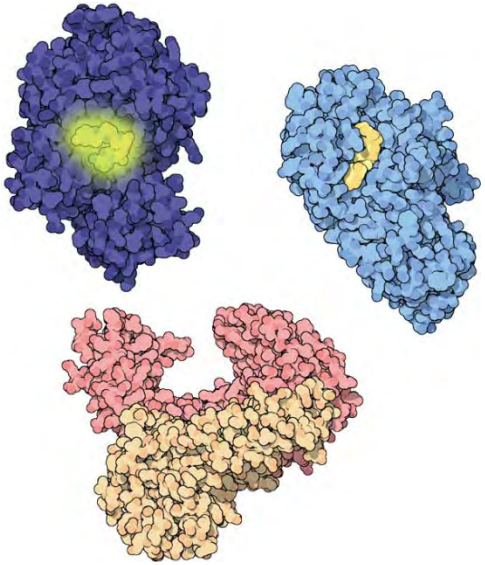
antibodies

DNA Regulation



DNA polymerase III

Enzymes



luciferase

amylase

reverse transcriptase

Protein Therapeutics

- First "protein vaccine" was cow-pox (Jenner, 1796)
- First protein pharmaceutical was insulin (Banting & Best, 1922)
- Now more than 200 approved peptide and protein pharmaceuticals on the FDA list
- Many different types…
- Many different sources…

Protein Therapeutics

- First "protein vaccine" was cow-pox (Jenner, 1796)
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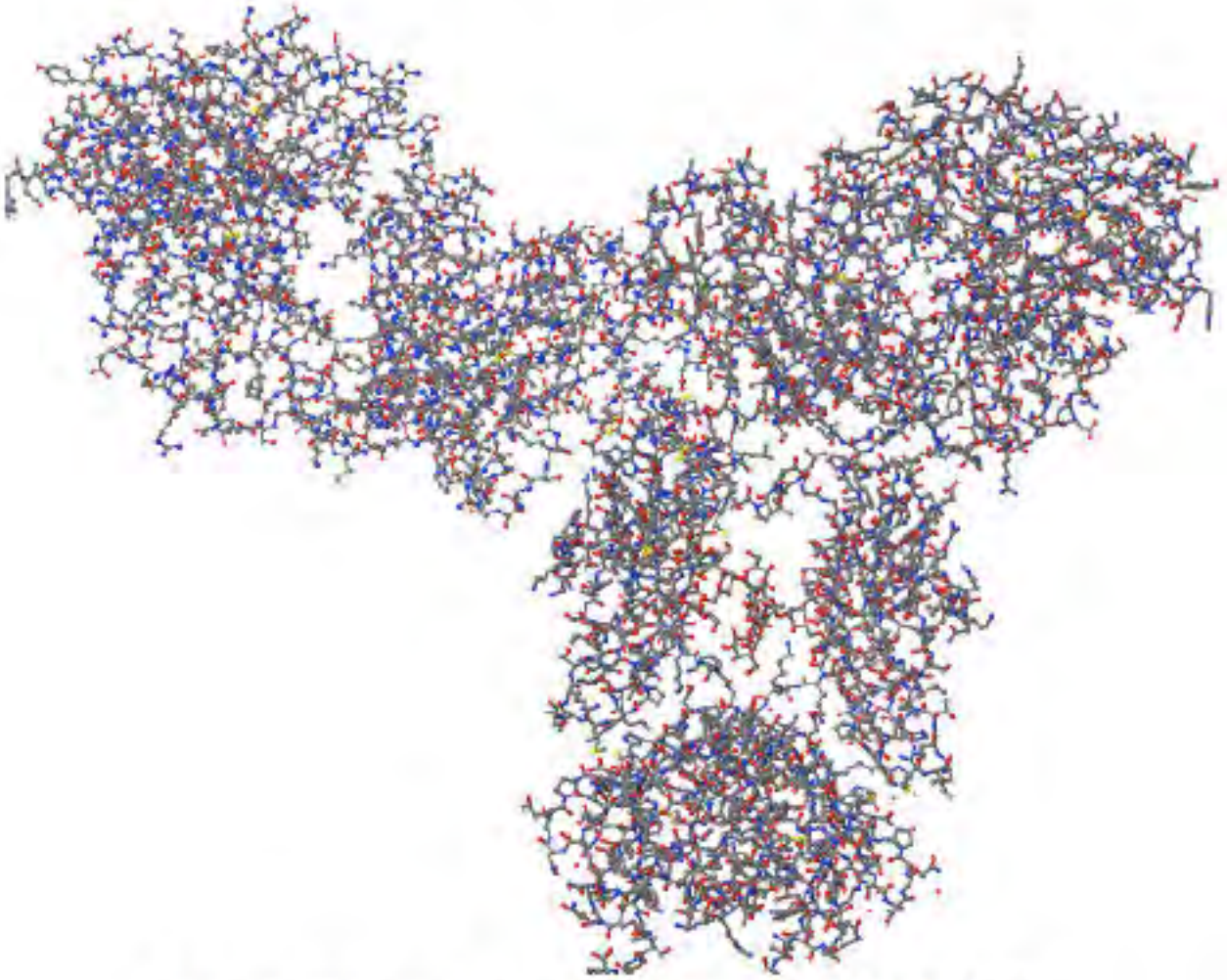
Size and Complexity of Proteins

Aspirin 180 Da



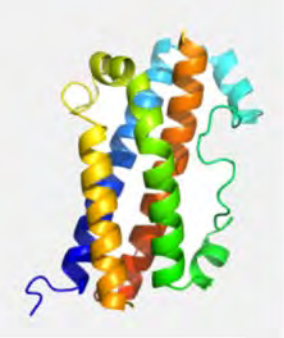
Monoclonal Antibody ~150,000 Da

How are Biological Products Different?

Small Molecule Drugs	Biological Products
Generally low molecular weight	Generally high molecular weight
Usually organic or chemical synthesis	Made with/from live cells/organisms → *inherent & contamination risk*
Fewer critical process steps	Many critical process steps
Well-characterized	Less easily characterized
Known structure	Structure may or may not be completely defined or known
Homogeneous drug substance	Heterogeneous mixtures → *May include variants*
Usually not immunogenic	Often Immunogenic

Lionel Andrés "Leo" Messi (born 24 June 1987) is an Argentine professional footballer plays as a forward for FC Barcelona

Born and raised in central Argentina, Messi was diagnosed with a **growth hormone deficiency** as a child. At age 13, he relocated to Spain to join Barcelona, who agreed to pay for his medical treatment.



History of hGH

1956 Purified from from human human pituitary gland by Li/Papkoff and Raben

1956-1960 Clinical trials show that GH-deficient children will benefit from pituitary GH

1960 National Pituitary Agency (NPA) formed to coordinate pituitary collection and HG extraction

1963-1985 7700 children in USA were treated for severe GH deficiency and 27,000 worldwide

1985 Reports of four people die from (prion-mediated) Creuzfeldt Jacob Disease (CJD) who had GH in the 1960s. FDA suspends extraction of hGH.

1985 Recombinant Protropin (Genentech) is approved ($10,000-30,000/yr)

1986 – now Other pharmaceutical companies Eli Lilly, Kabi, Novo Nordisk, Serono to follow with their versions of rhGH

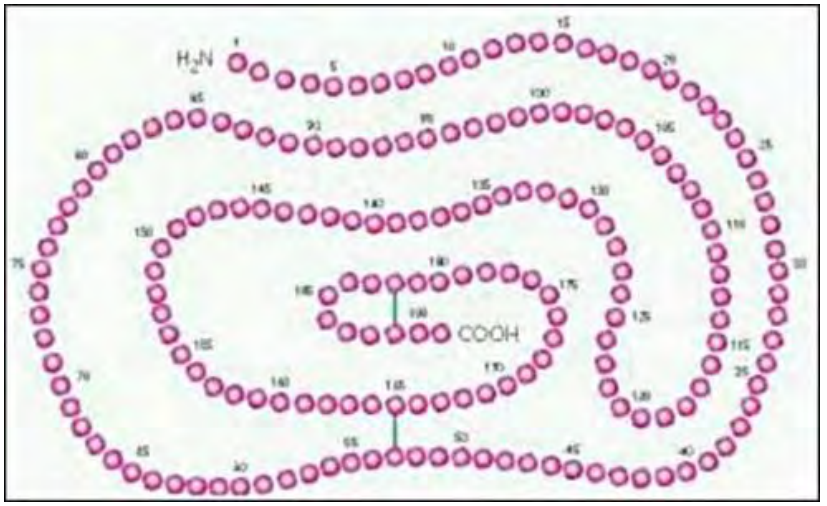
1986 – now Expansion of indications to Turner syndrome, Renal insufficiency, muscle wasting in AIDS etc

1986 – now Growing off-label black market use by athletes and as “anti-aging” therapy

1990 Cheaper versions like Tev-Tropin is approved by FDA

2006 Biosimilar of somatropin approved in EU by Sandoz

rGH – Replace Lacking Protein

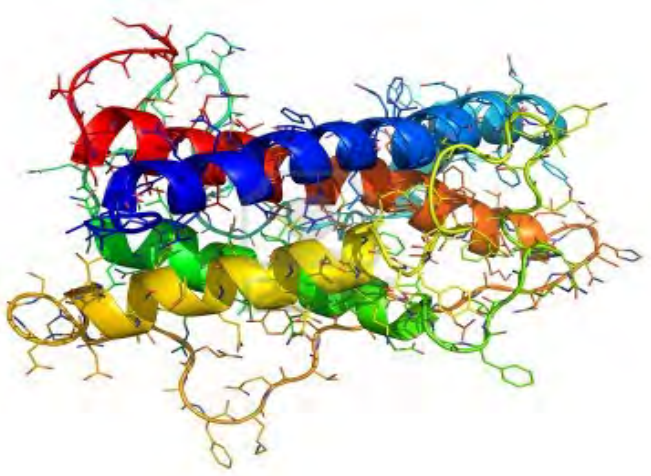


191 amino acids single, two S-S bonds,

Human Growth Hormone (HGH)

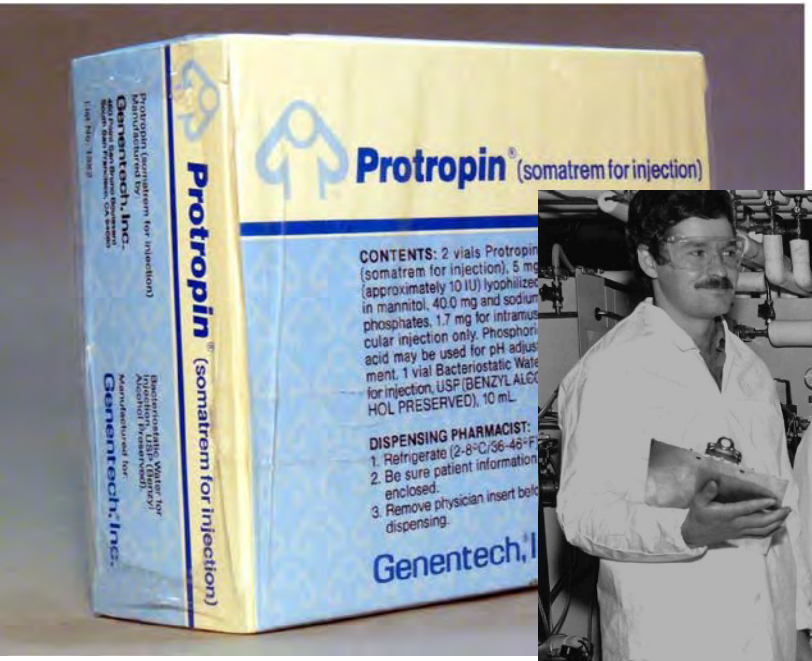
- Humatrope® (Lilly), Genotropin® (Pfizer), Norditropin® (Novo Nordisk), Nutropin®

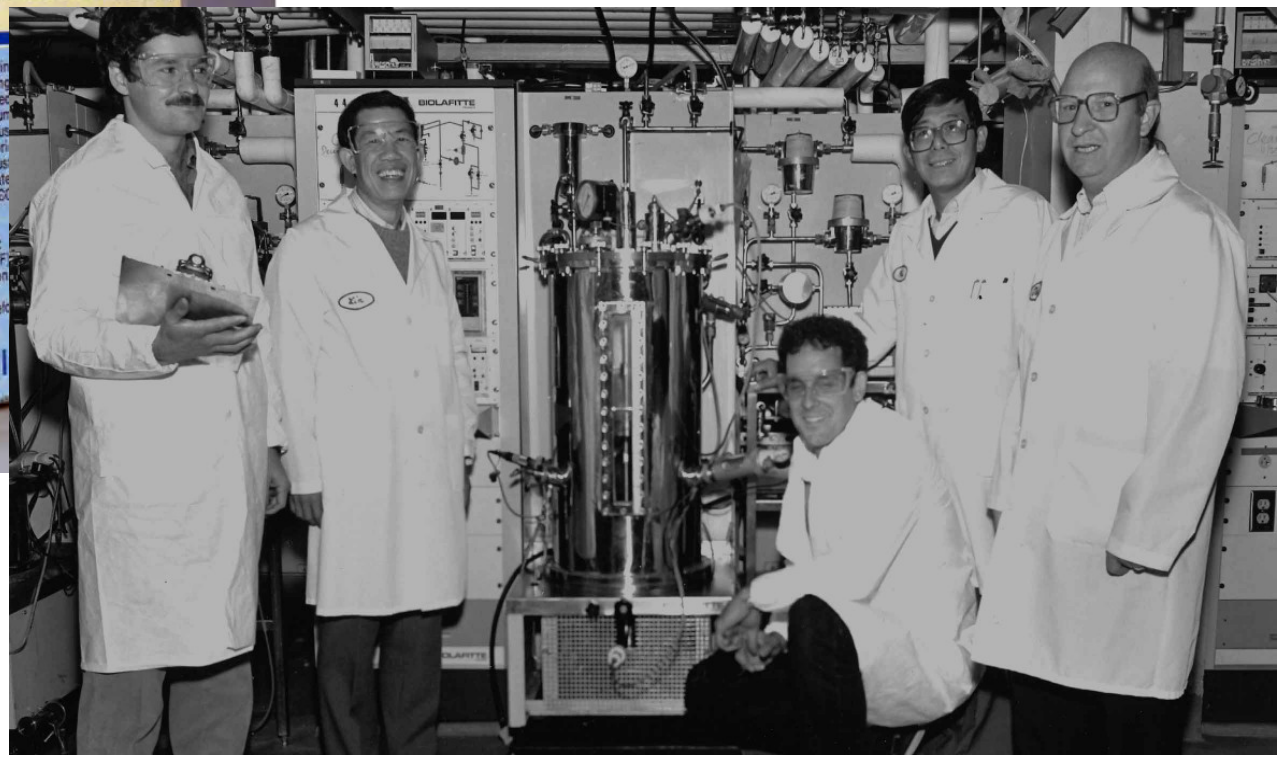
- First bio-similars: Teva-tropin (Teva), Hypertropin (NeoGenica, China), Jintropin (GeneScience, China)



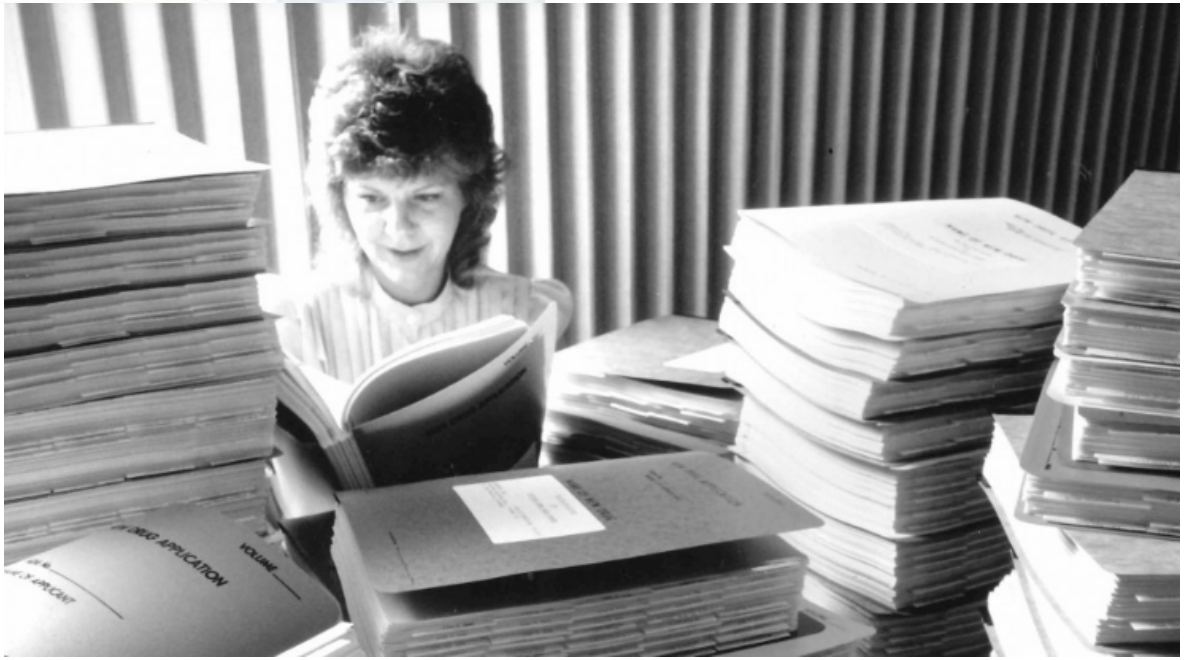
FDA Approves Genentech's Drug to Treat Children's Growth Disorder
South San Francisco, Calif. -- October 18, 1985 –

The FDA today approved Genentech's first human pharmaceutical, a biosynthetic growth hormone for treating growth retarded children.

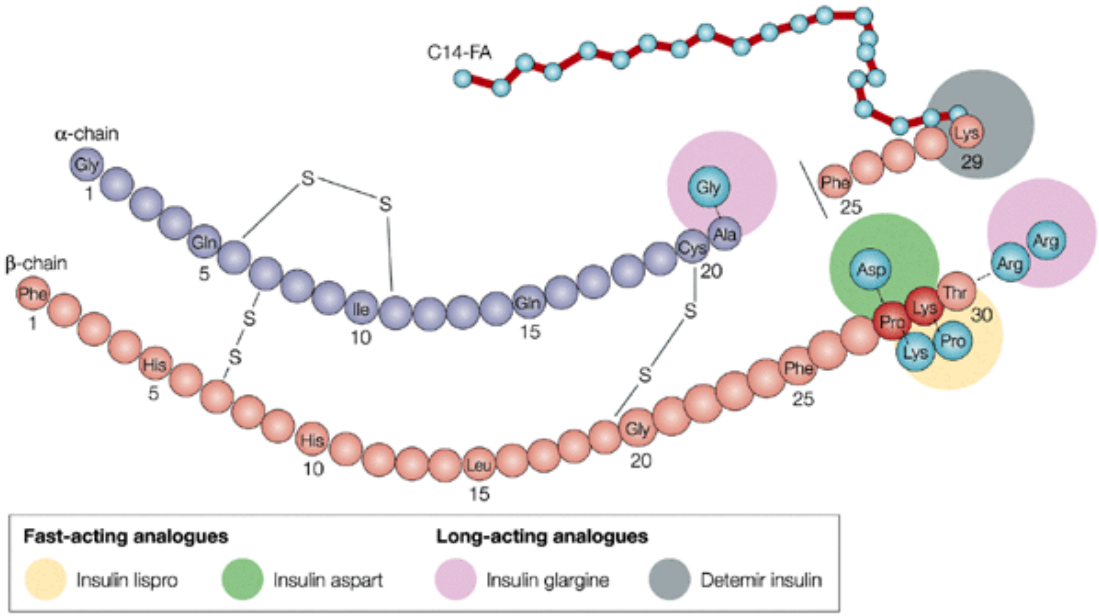





DRUG MANUFACTURING LICENSE


VOLUME

Insulin – Replace Lacking Protein



Nature Reviews | Drug Discovery

Insulin:

Novolog®, Novolin®, Novolin® 70/30 (Novo Nordisk)

Humalog®, Humulin®, Humulin® 50/50, Humalog® 75/25 (Lilly)

Lantus (Aventis)

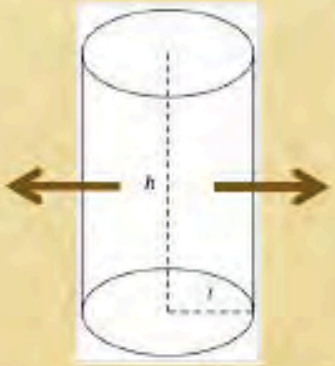
Some motivations to control the release of drugs

- ✧ Poor patient compliance
- ✧ Short plasma half-lives
- ✧ Insufficient control of plasma drug levels
- ✧ Drug targeting or local administration needed because of hard to reach tissues and/or systemic drug-induced toxicity
- ✧ Convenience and comfort to patients (e.g., fewer needles)
- ✧ Drug administration may be improved in under-privileged areas
- ✧ Poor bioavailability by noninvasive routes

(*adapted* from Langer and Peppas, *Biomaterials*, 1981)

5-year controlled release of a progestin for birth control

Norplant – Levonorgestrel releasing silicone rubber reservoir via solid-state diffusion



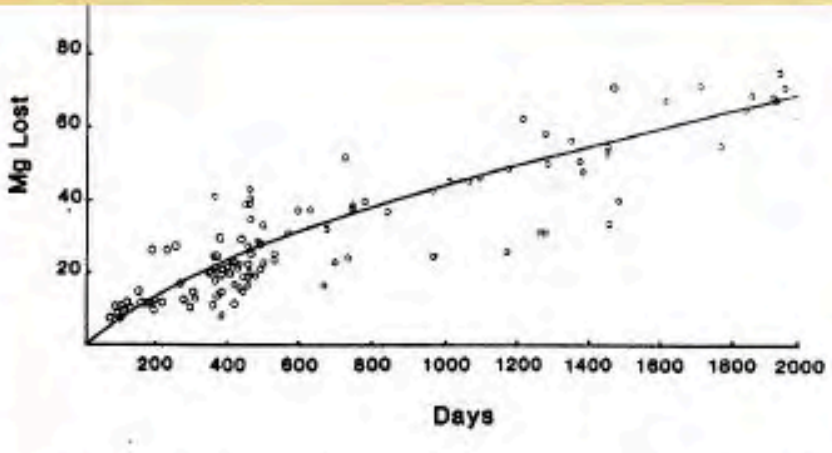


FIGURE 5. Levonorgestrel loss from sets of six Silastic capsules (2.4-mm O.D., 1.57-mm I.D., 30-mm filled length) in human subjects. (From Robertson, D. N., unpublished data. With permission).

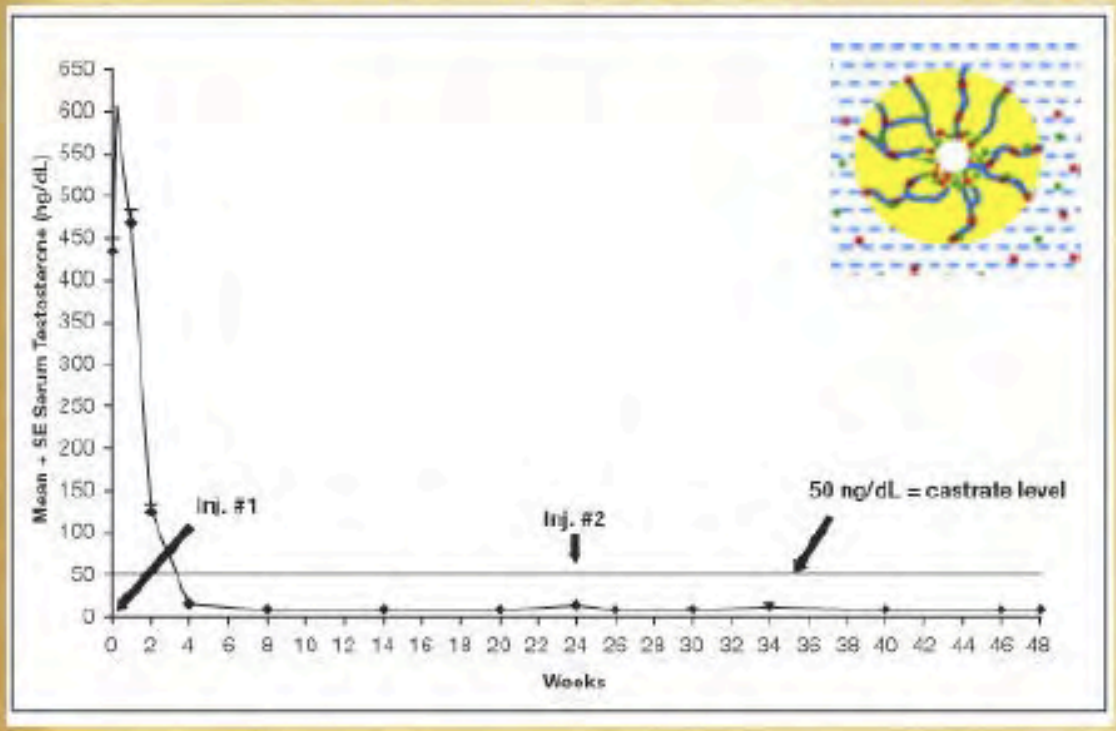
Table 3
LEVONORGESTREL LEVELS IN SUBJECTS USING SIX SILASTIC CAPSULES (NORPLANT®) FOR PERIODS UP TO 5 YEARS

Month of treatment	No.	ng levonorgestrel per ml plasma ± SEM
1—12	27	0.35 ± 0.03
13—24	23	0.31 ± 0.02
25—36	30	0.34 ± 0.02
37—48	8	0.32 ± 0.03
49—60	42	0.29 ± 0.02

From Croxatto, H. B., Diaz, S., Miranda, P., Elamsson, K., and Johansson, E. D. B., *Contraception*, 22, 583, 1980. With permission.

1, 3, 4 & 6-month controlled release of leuprolide from biodegradable polymers

Lupron Depot – Injectable poly(lactic/glycolic acid) biodegradable polymer microspheres <u>continuously lose mass by bioerosion</u>



Jain et al., *J. Control. Rel.*, **95**, 2360–2366 (2010)
http://www.drugs.com/pro/lupron-depot.html

What is the dose Dose and Dosing Rate ?

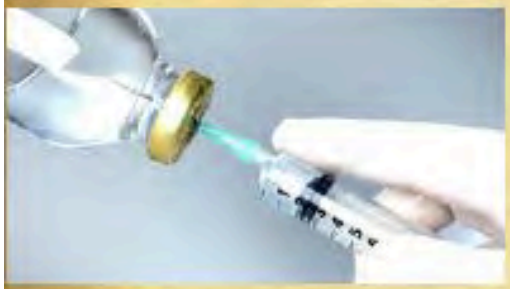
Reasonable — Unreasonable

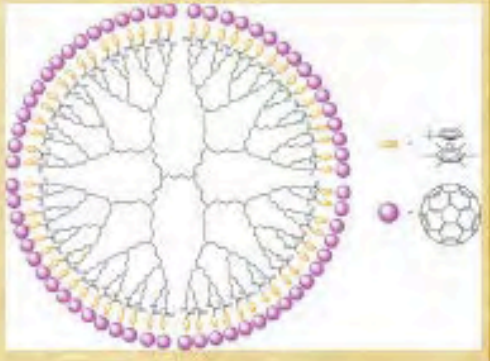
- Short-term or chronic therapy
- Route of administration, dosage form
- Bioavailability (K)
- Therapeutic drug concentration (potency)
- Drug clearance (rate of drug elimination)

$$\frac{dM}{dt} = \frac{C_{ther} \cdot CL}{K}$$

$$M_t = \frac{dM}{dt} \cdot \Delta t$$

Can we manufacture it?





High confidence

Low confidence

- Cost of raw materials
- Cost of manufacturing (scalability, testing/batch release)
- Residual solvents, aseptic processing/terminal sterilization, pyrogens
- Shelf-life

Patients don't always like what we make

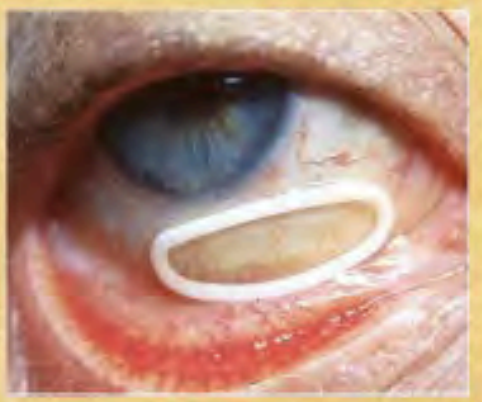
Market Scan
Pfizer Writes Off Exubera
Carl Gutierrez, 10.18.07, 11:45 AM ET

Cutting its losses, Pfizer has removed the disappointing diabetes drug Exubera from its roster. Now Wall Street hopes other cost cuts will bring the world's largest pharmaceutical company back on track.

- **Exubera** – Pfizer/Nektar dry inhaler of rapid-acting insulin.

Ocusert pilo-20/-40
Not well tolerated by patients
Difficulties with retention
never reached wide spread use

- **Ocusert** – Alza's pilocarpine controlled release insert.



Need for minimally invasive peptide/protein delivery and controlled release

- Hundreds of biotech drugs in clinical trials:
  (Mostly peptides and proteins)
- Poor bioavailability by noninvasive routes
  and short plasma half-lives
  → Need frequent injections

- Poor patient convenience, compliance, and comfort with injections
  (e.g., undesirable daily injections of protein drugs)
- Short duration of action during site-specific delivery
  (e.g., growth factors for tissue regeneration)

Major issue with peptide/protein delivery

Injection frequency of some important therapeutic peptides and proteins

Peptide or Protein	Injection Frequency
Leuprolide	Once a day
Octreotide	2-4 times a day
Exenatide	Twice a day
Interferon-γ	3 times a week
Growth hormone	Once a day
Erythropoietin	3 times a week

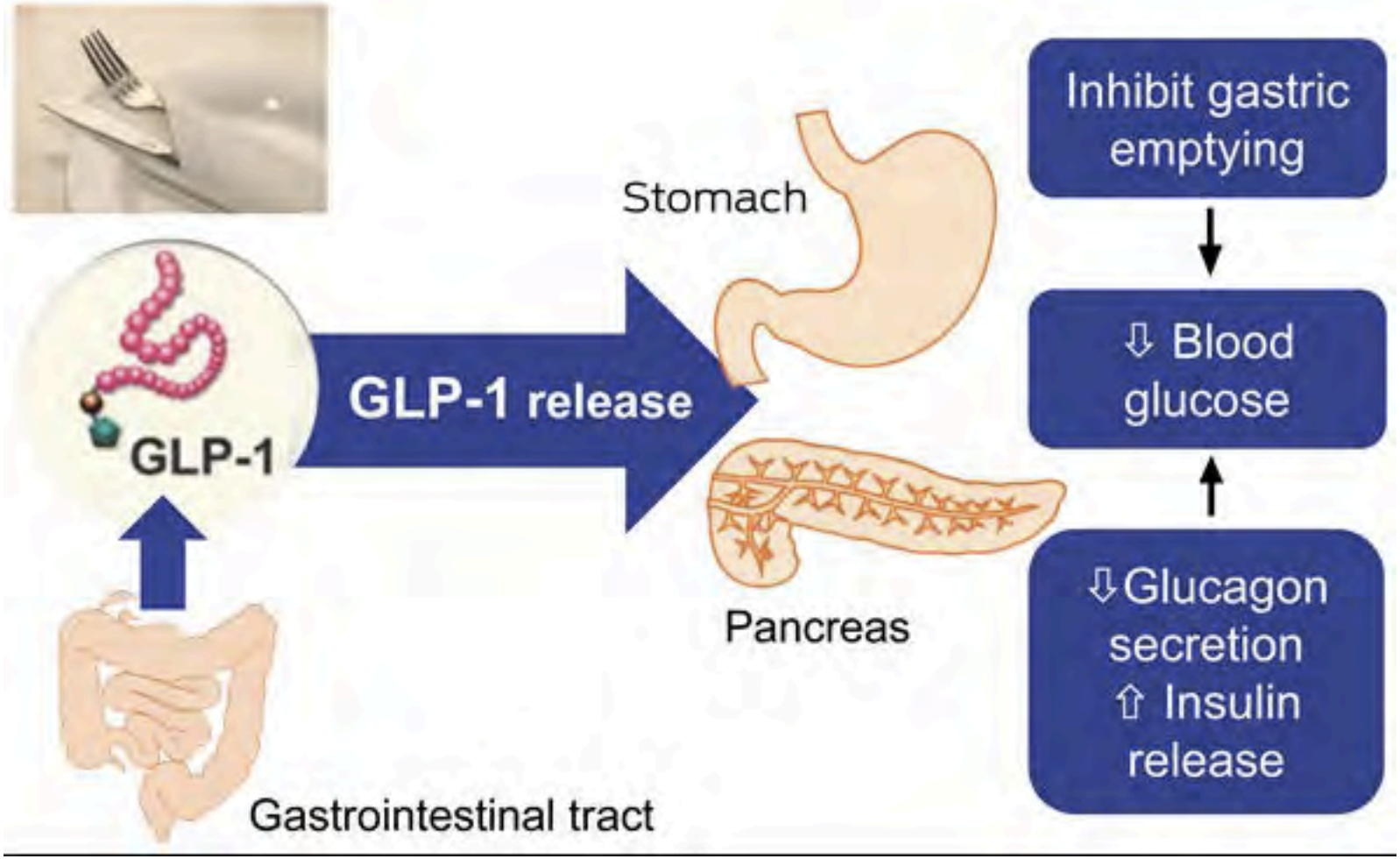
Examples and application of peptides/proteins for clinical use

Therapeutic peptide or protein	Application
Tissue necrosis factor	Carcinoma
Proleukin	Carcinoma
y-Interferon	Carcinoma
Epidermal growth factor	Wound healing
Transforming growth factors	Wound healing
Fibroblast growth factor	Wound healing
Insulin-like growth factors	Wound healing
Hirudin	Fibrinolytic
Tissue plasminogen activator	Fibrinolytic
Streptokinase	Fibrinolytic
Erythropoietin	Erythropoieais stimulation
Factor VIII	Haemophilia
Factor IX	Christmas disease
Triproamylin	Glucose regulation
Insulin	Glucose regulation
Somatostatm	Glucose regulation
Proinsulin	Glucose regulation
α-Interferon	Viral diseases/hairy cell leukemia
β-Interferon	Multiple sclerosis
Glucocerebrosidase	Gaucher'disease
Cerezyme	Type I Gaucher's disease
Pulmozyme	Cystic fibrosis
Calcitoninh	Bone disease
Oxytocin	Labour induction
Growth hormone	Short stature
α1 Antitrypsin (aat)	aat deficiency
Superoxide dismutase	Respiratory disorders

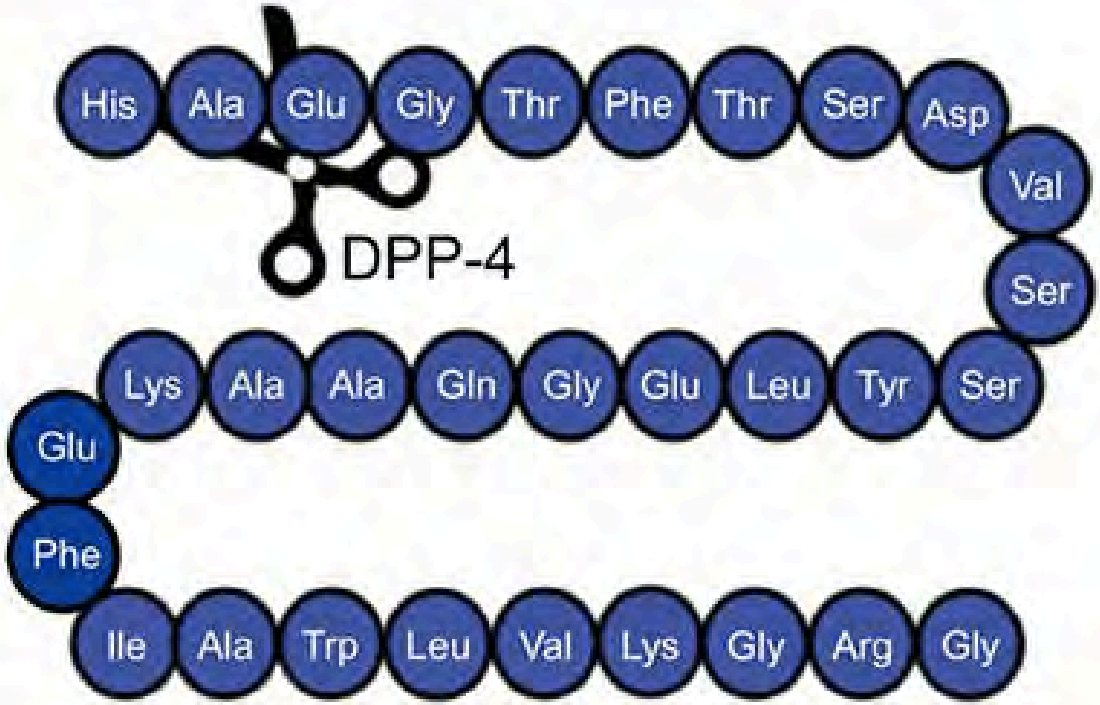
Mackay M, Phillips J, Hastewell J. Peptide drug delivery: colonic and rectal absorption. Adv Drug Del Rev 1997; 28: 253-273.

Peptide and Protein Delivery
Extension of Half-Life

GLP-1 Peptide Case Study


Stomach
Inhibit gastric emptying
⇩ Blood glucose
GLP-1 release
GLP-1
Pancreas
⇩Glucagon secretion
⇧ Insulin release
Gastrointestinal tract

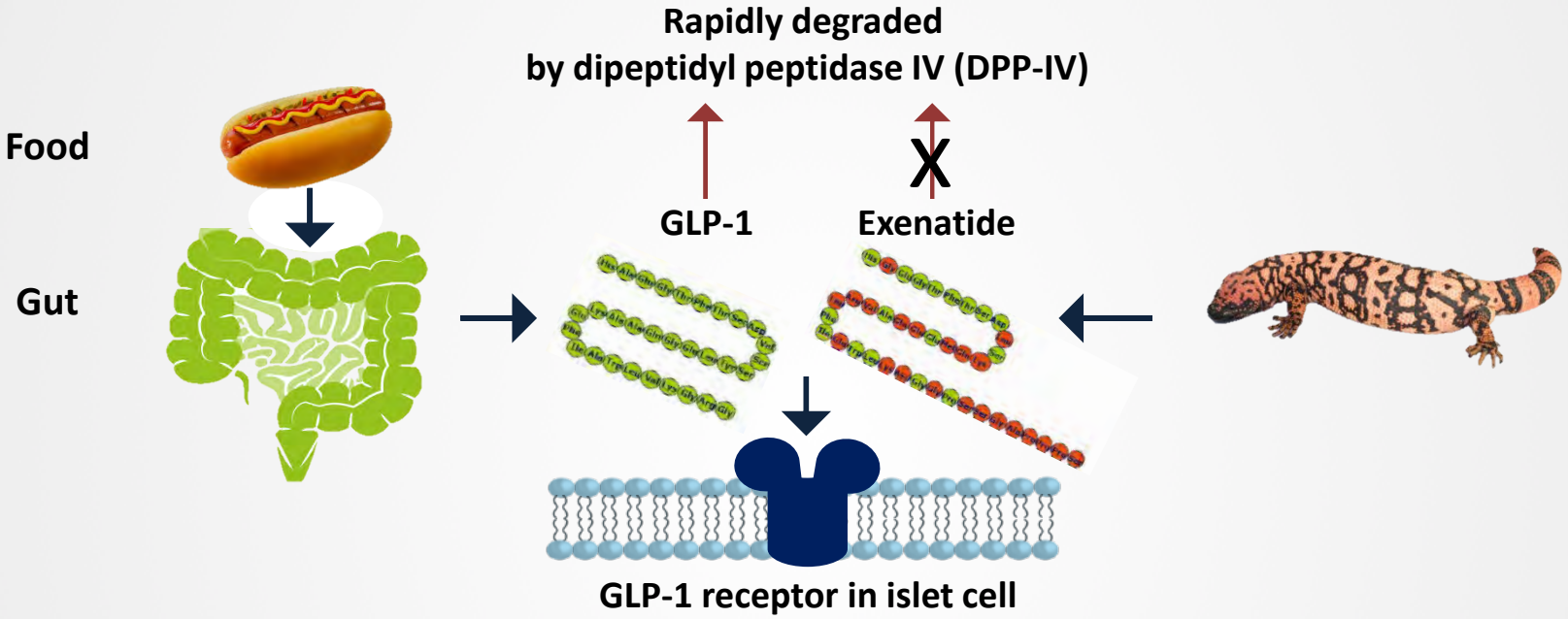
GLP-1 Is Rapidly Inactivated by the Ubiquitous Enzyme DPP-4



Half-time=15 min

Vilsbøll T, et al. *J Clin Endocrinol Metab*. 2003;88:220-224.[29]

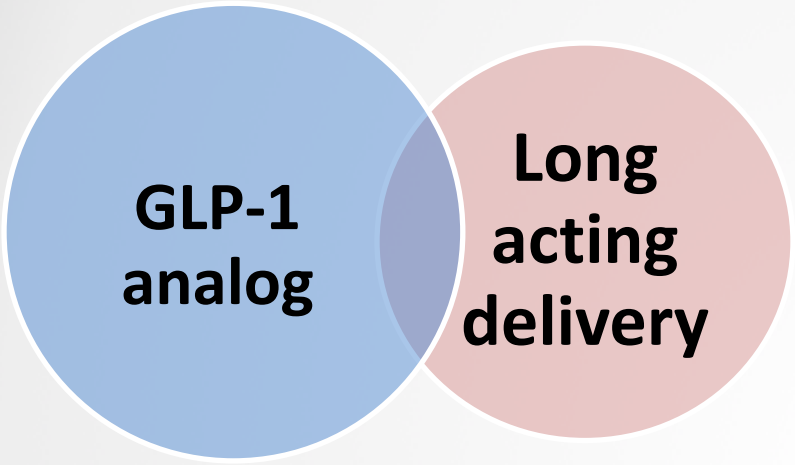
What is Exenatide? (Byetta®)

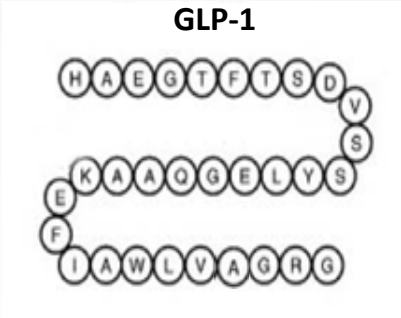


Glucose-stimulated insulin secretion↑↑
B-cell proliferation ↑
Delays gastric emptying
Satiety → reduces food intake

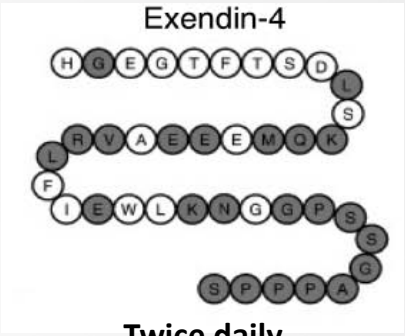
→ In April 2005, Exenatide (Byetta) was approved by the FDA as an adjunctive therapy for type 2 diabetes

Studies so far – GLP-1 analog





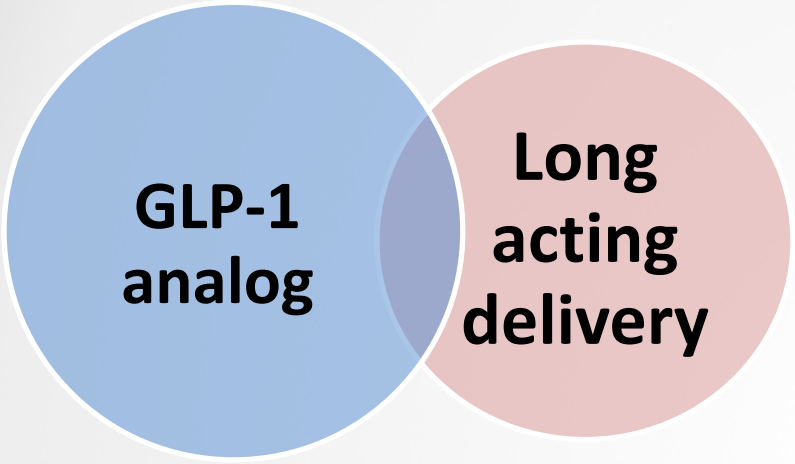
T1/2 = 1.5-5 min



Twice daily

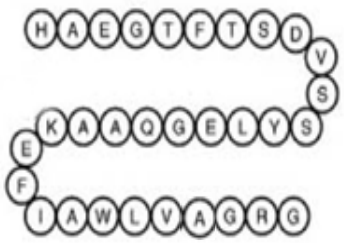
T1/2 = 2.4h

Studies so far – GLP-1 analog



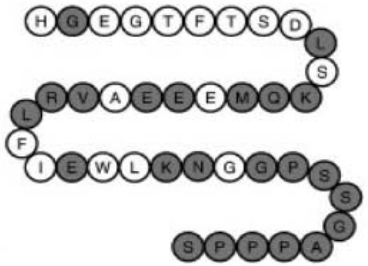
T1/2= 5 days

GLP-1



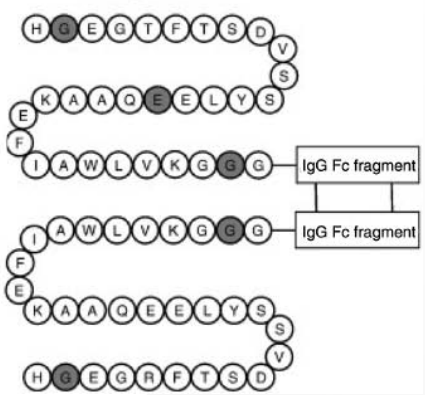
T1/2 = 1.5-5 min

Exendin-4

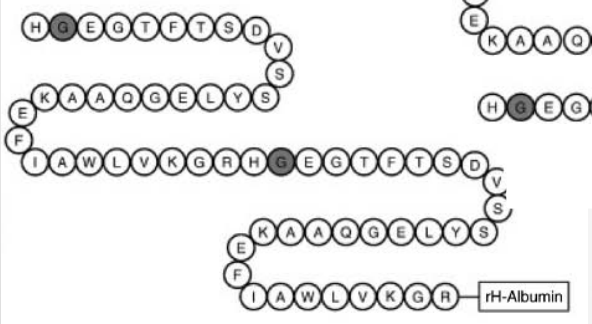


Twice daily
T1/2 = 2.4h

Dulaglutide



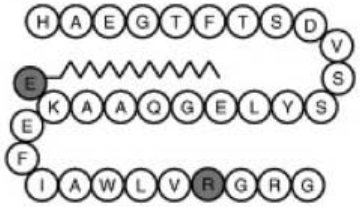
Albiglutide



once weekly

T1/2 = 4-7 days

Liraglutide

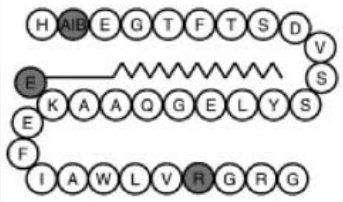


Once daily
T1/2 = 10h

Lixisenatide

Once daily
T1/2 = 2-4h

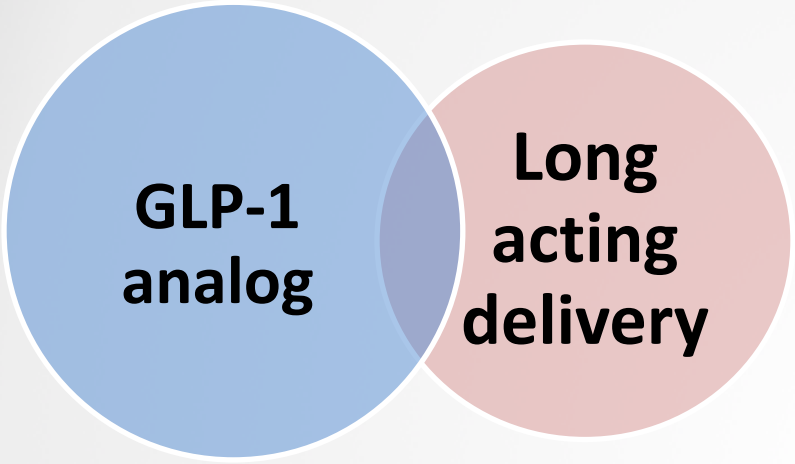
Semaglutide



T1/2 = 7 days

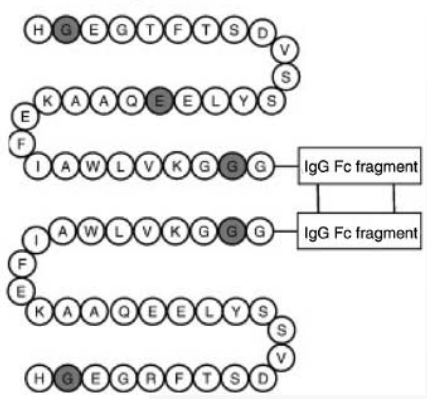
Yanwei et al., Reviews in the neurosciences (2016)

Studies so far – GLP-1 analog

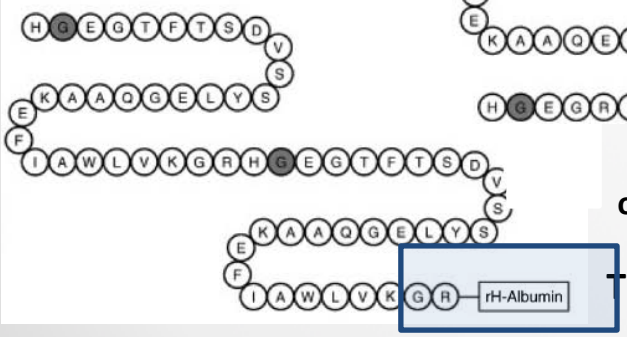


T1/2= 5 days

Dulaglutide



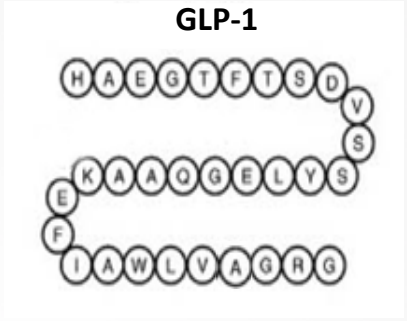
Albiglutide



once weekly

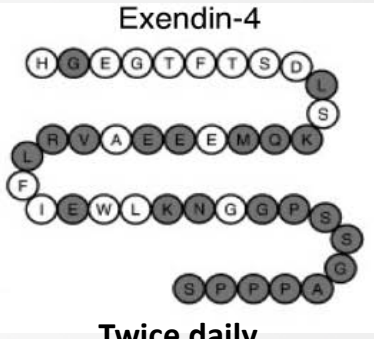
T1/2 = 4-7 days

GLP-1



T1/2 = 1.5-5 min

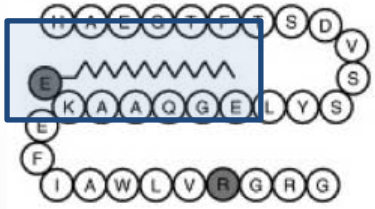
Exendin-4



Twice daily

T1/2 = 2.4h

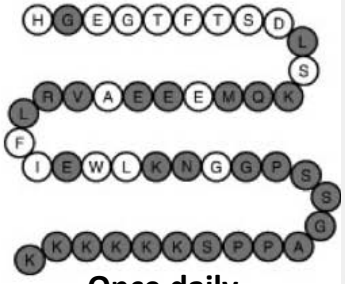
Liraglutide



Once daily

T1/2 = 10h

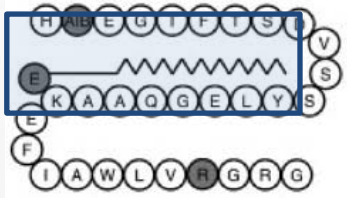
Lixisenatide



Once daily

T1/2 = 2-4h

Semaglutide



T1/2 = 7 days

Yanwei et al., Reviews in the neurosciences (2016)

Fatty Acid Attachment to Bind Albumin

Liraglutide

7 9

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Glu Lys Ala Ala Gln Gly Glu Leu Tyr Ser Glu Phe Ile Ala Trp Leu Val Arg Gly Arg Gly

37

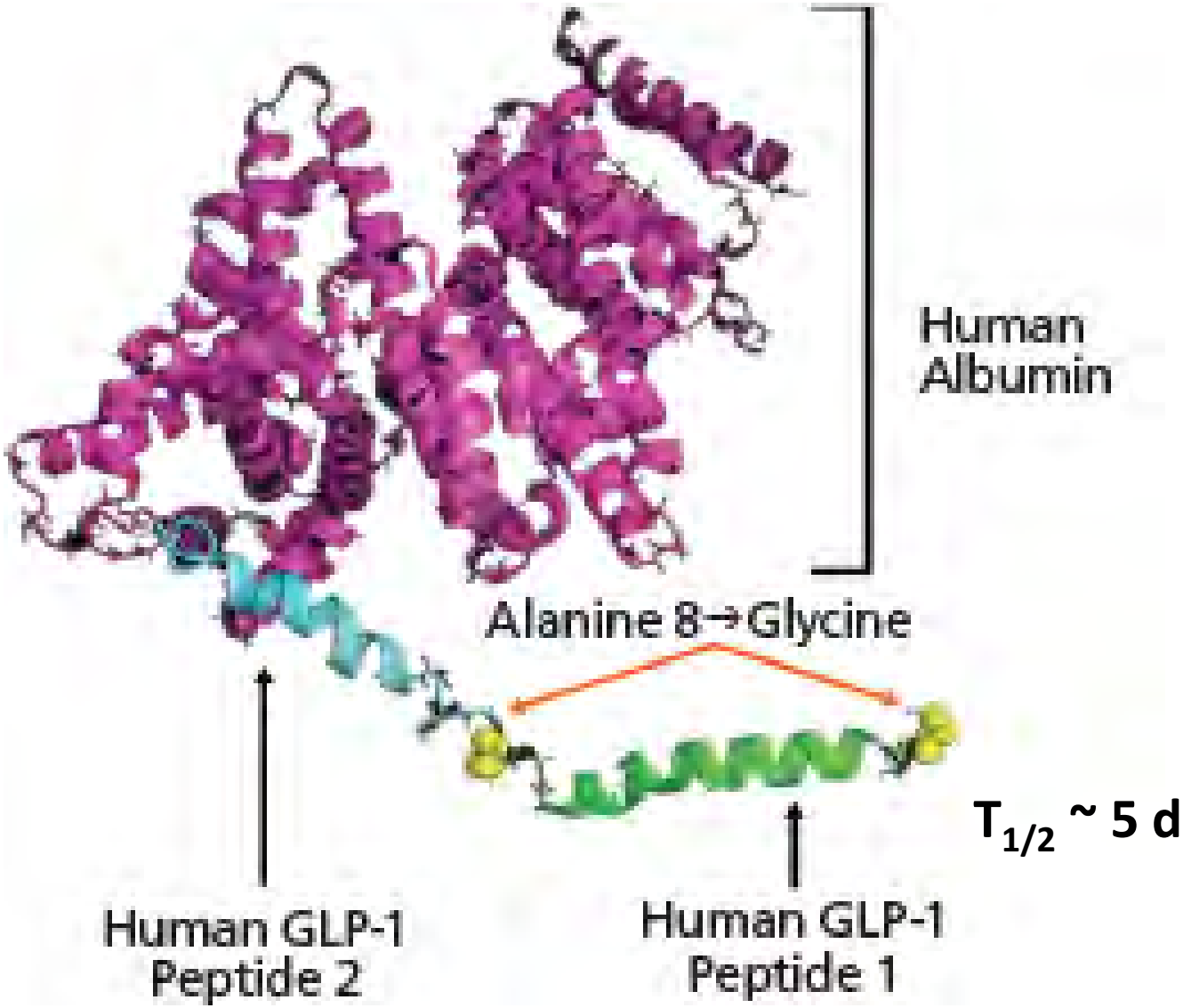
C-16 fatty acid (palmitoyl)

97% homology to human GLP-1

Improved PK: albumin binding; self-association

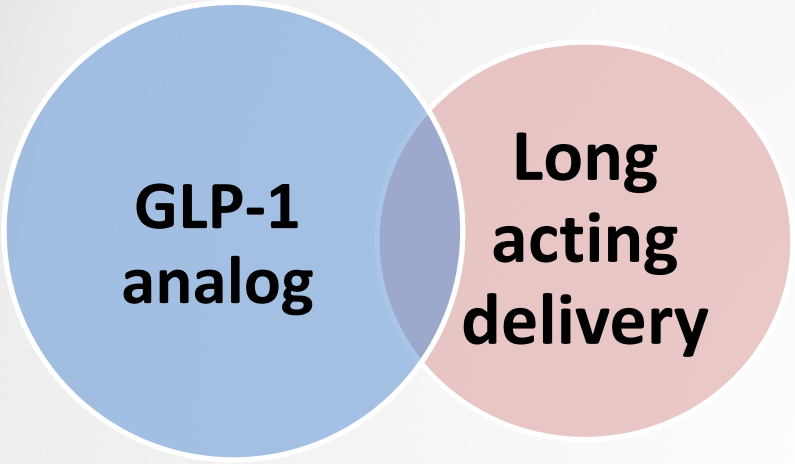
- Slow absorption from subcutis
- Stable against DPP-4
- Long plasma half-life (T_{v_2} = 13 h; T_{max} 10-13h)

Albumin-Fusion Extension



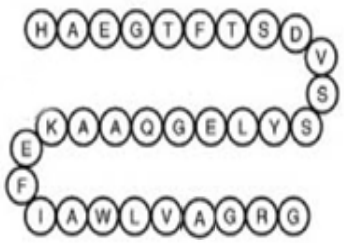
$T_{1/2}$ ~ 5 days

Studies so far – GLP-1 analog



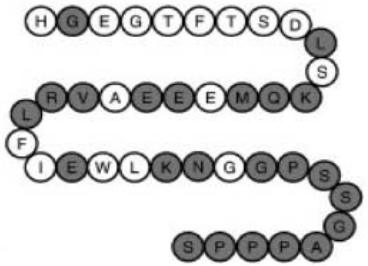
T1/2= 5 days

GLP-1



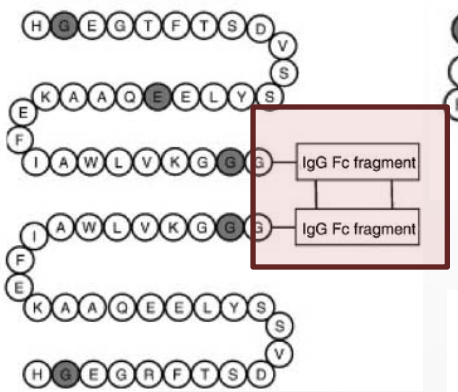
T1/2 = 1.5-5 min

Exendin-4



Twice daily
T1/2 = 2.4h

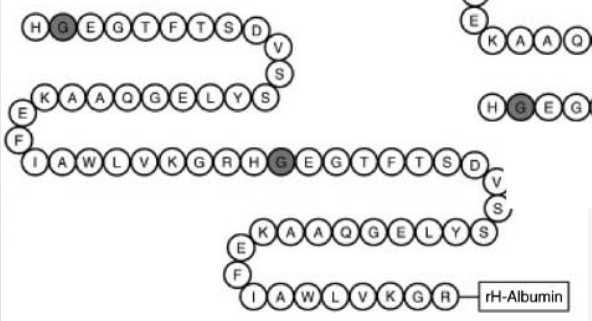
Dulaglutide



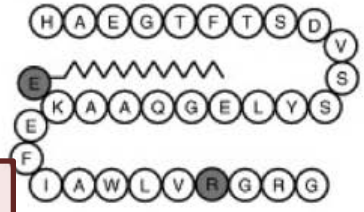
once weekly

T1/2 = 4-7 days

Albiglutide



Liraglutide

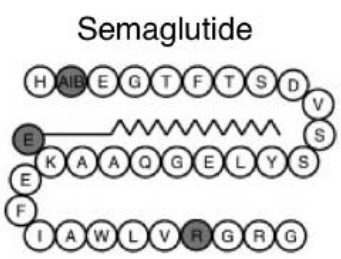


Once daily
T1/2 = 10h

Lixisenatide

Once daily
T1/2 = 2-4h

Semaglutide

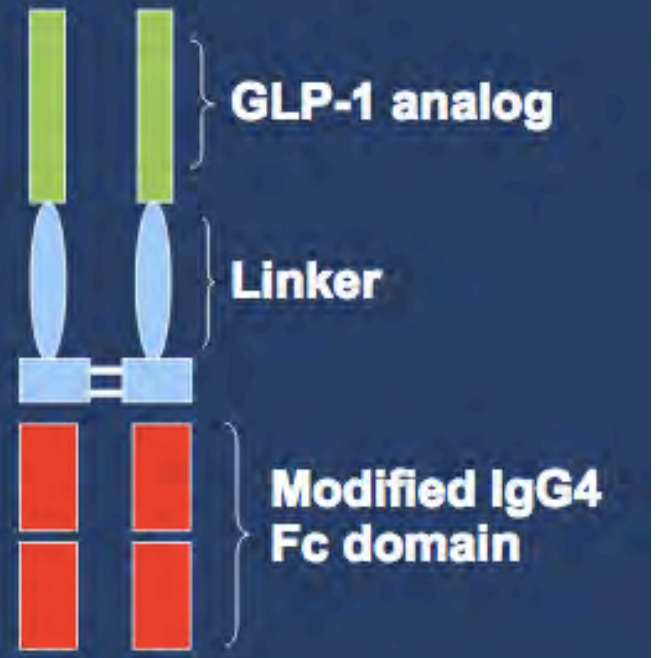


T1/2 = 7 days

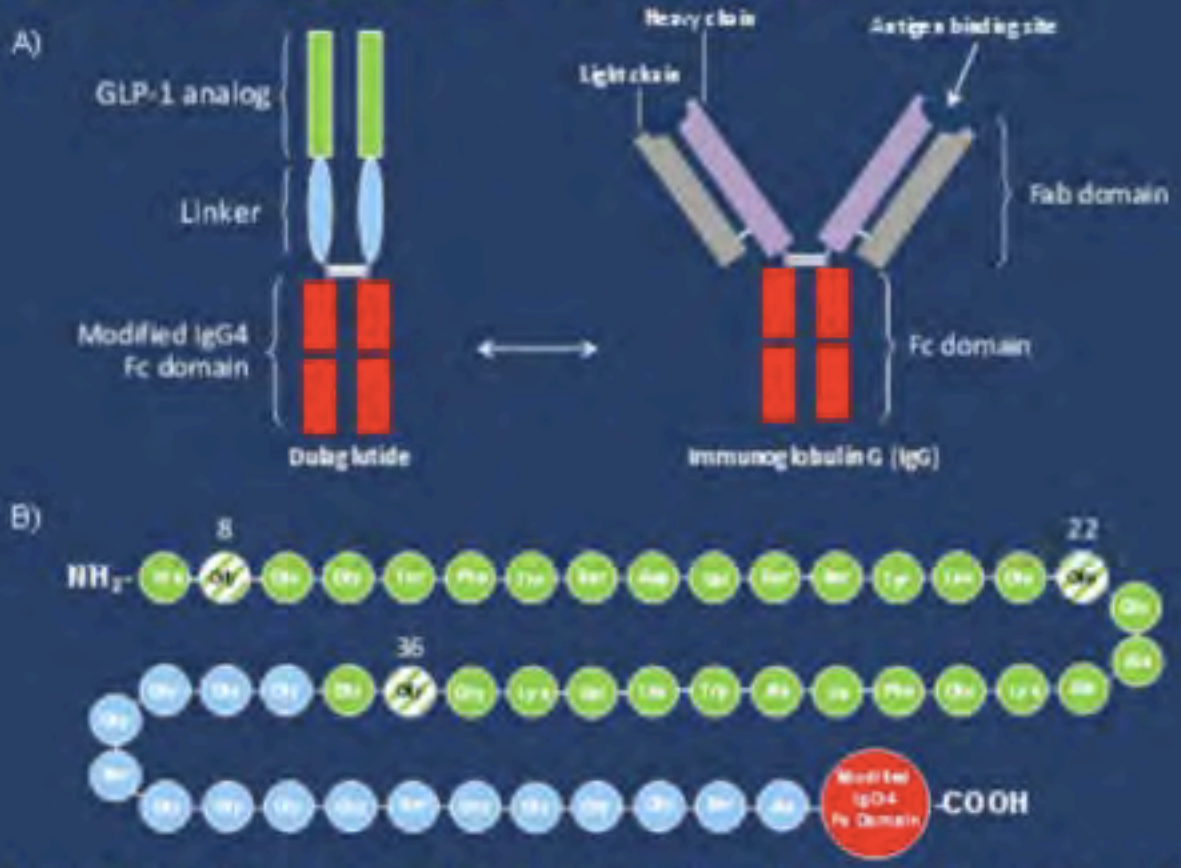
Yanwei et al., Reviews in the neurosciences (2016)

Fc-Fusion Extension

- A recombinant GLP-1 Fc fusion protein linking a human GLP-1 peptide analog and a variant of a human IgG4 Fc fragment[1,2]
  - Extended plasma half-life (~5 days)
  - Minimal renal clearance
  - Once-weekly dosing
  - Solution injection: no reconstitution needed
  - Low immunogenic potential



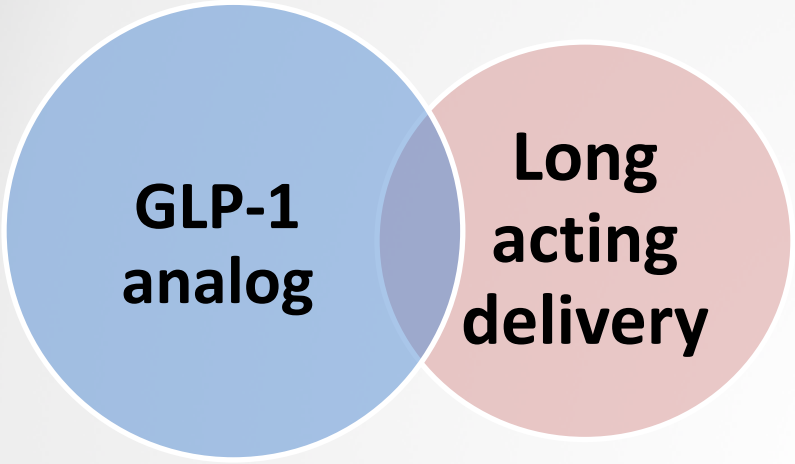
Structure of Dulaglutide and IgG4



- A) The Fc (fragment crystalizable) region in dulaglutide is altered at two amino acid residues to reduce binding to cellular Fc receptors and prevent dimerization of the dulaglutide molecule with endogenous IgG4 (half-antibodies). A disulfide linker acts to dimerize dulaglutide molecules.[1] Fab = Fragment antigen binding
- B) The amino acid composition of the GLP-1 (7-37) and linker region of dulaglutide. Striped positions indicate modified amino acids and their position from wild type human GLP-1.[1] Color code in B is representative of region color in A.

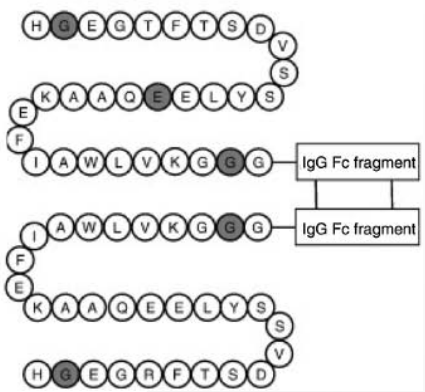
1. Glaesner W et al. Diabetes Metab Res Rev 2010;26:287-96

Studies so far – GLP-1 analog

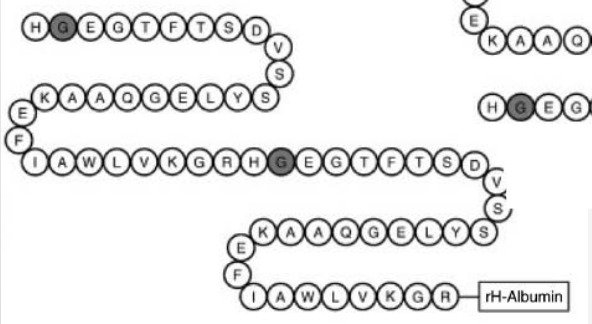


T1/2= 5 days

Dulaglutide



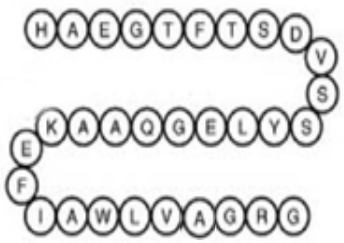
Albiglutide



once weekly

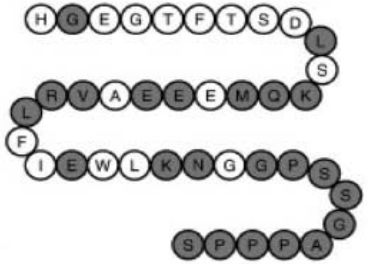
T1/2 = 4-7 days

GLP-1



T1/2 = 1.5-5 min

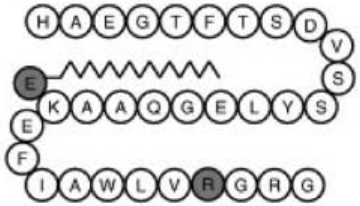
Exendin-4



Twice daily

T1/2 = 2.4h

Liraglutide



Once daily

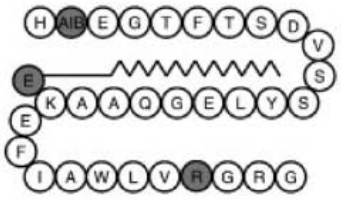
T1/2 = 10h

Lixisenatide

Once daily

T1/2 = 2-4h

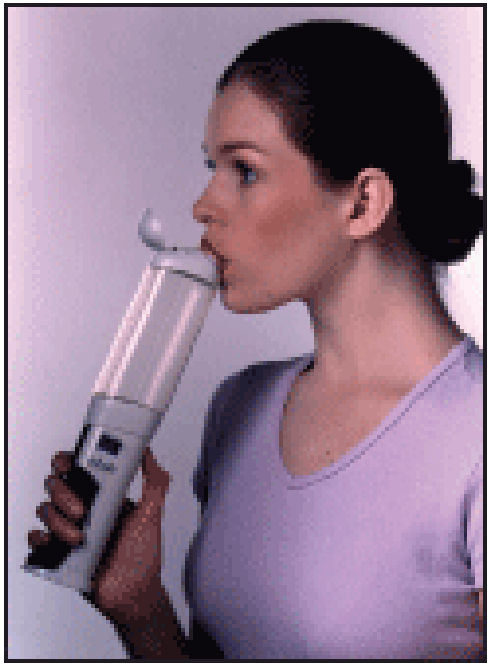
Semaglutide



T1/2 = 7 days

Yanwei et al., Reviews in the neurosciences (2016)

Medscape® www.medscape.com



Exubera

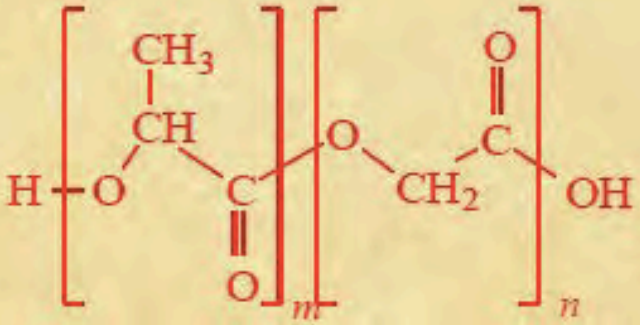
With permission from Pfizer

Source: Br J Diabetes Vasc Dis © 2006 Sherboume Gib

afrezza®
(insulin human) Inhalation Powder

Poly(lactic-co-glycolic acid) (PLGA) for delivery of peptides and proteins

- Structure



e.g., PLGA 50/50, m/n = 1
PLGA 75/25, m/n = 3

Mw ~ 10 kDa - 100 kDa

- Advantages
  - wide range of properties
  - ease of processing
  - predictable *in vivo* degradation kinetics
  - FDA approval for use in humans
  - No daily injections
  - Control release rate
  - Lower systemic toxicity
  - Reduce booster doses (vaccines)

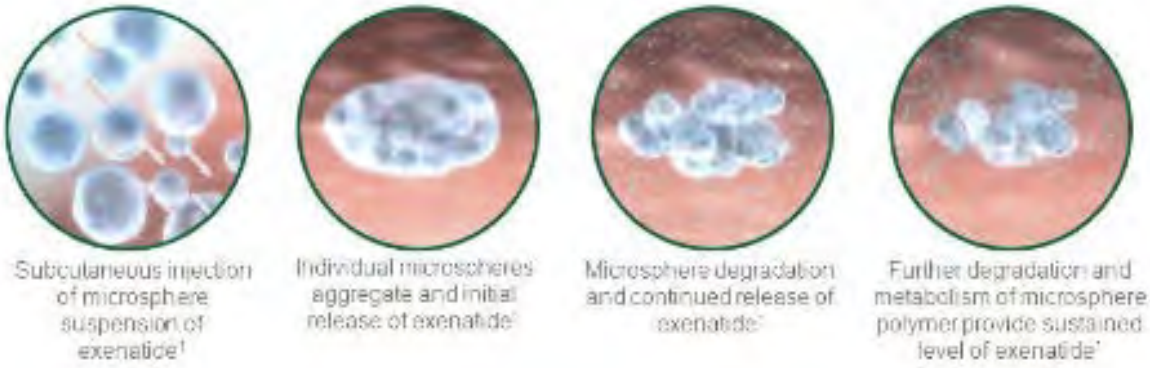
- Major configurations of injectable devices

microspheres (1 - 100 μm)

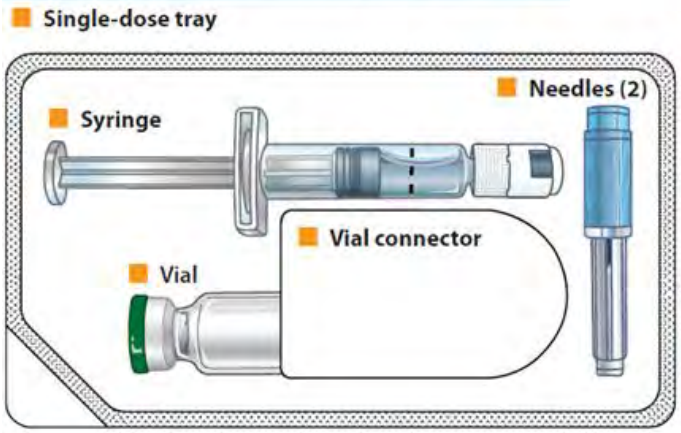
millicylinders (∅ = 0.8-1.5 mm)

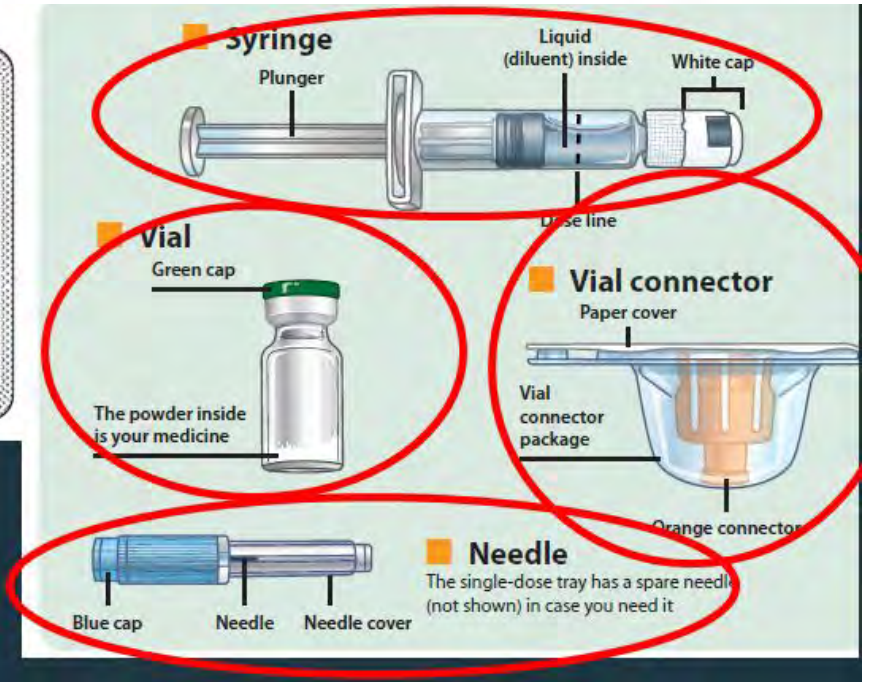
in-situ forming implants

Polymer Microsphere Depot



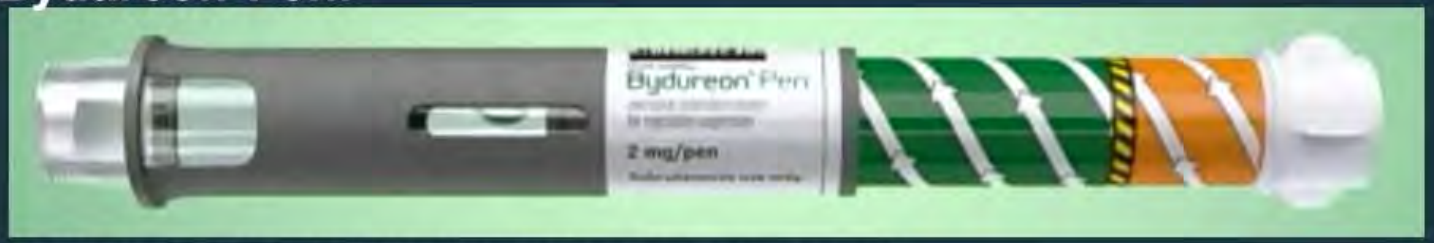
Bydureon Single Dose





1. Powder (Bydureon)
2. Liquid microspheres
3. Orange connecting device
4. Needle

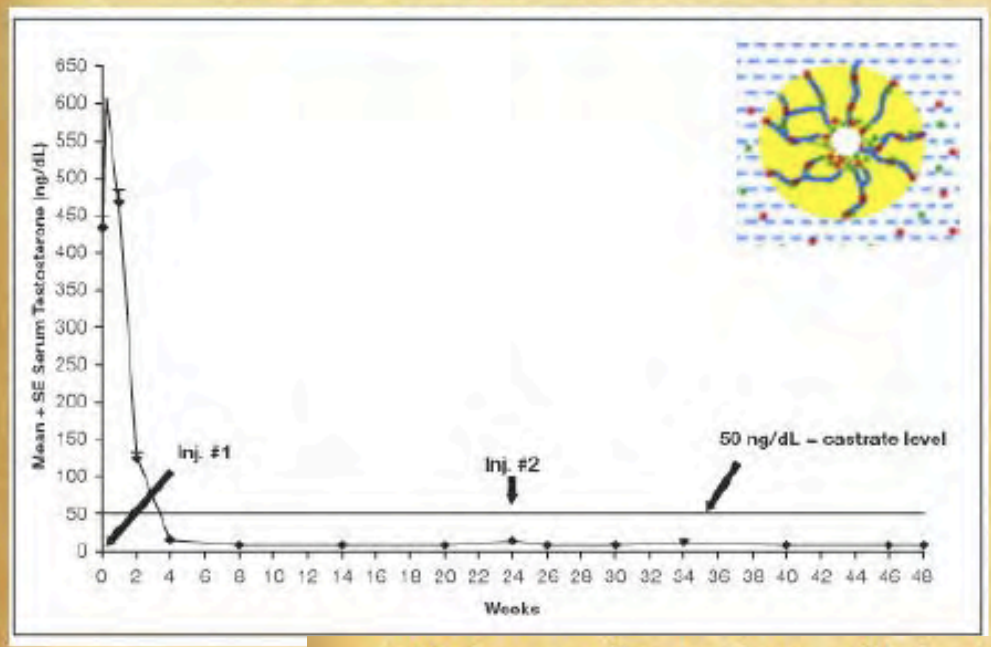
Bydureon Pen:



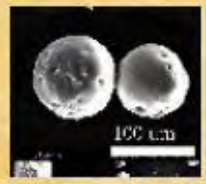
http://www.azpicentral.com/bydureon/ifu_bydureon.pdf

1, 3, 4 & 6-month controlled release of leuprolide from biodegradable polymers

Lupron Depot – Injectable poly(lactic/glycolic acid) biodegradable polymer microspheres continuously lose mass by bioerosion



- Major configurations of injectable devices



microspheres (1 - 100 μm)

millicylinders (∅ = 0.8-1.5 mm)

in-situ forming implants

No polymer depots for proteins

Tuesday, Jun 1, 2004
Genentech and Alkermes Announce Decision to Discontinue Commercialization of Nutropin Depot

South San Francisco, Calif. and Cambridge, Mass. -- June 1, 2004 -- Genentech, Inc. (NYSE: DNA) and Alkermes, Inc. (Nasdaq: ALKS) today announced their decision to discontinue commercialization of Nutropin Depot® (somatropin [rDNA origin] for injectable suspension). The decision is based on the significant resources required by both companies to continue manufacturing and commercializing the product.

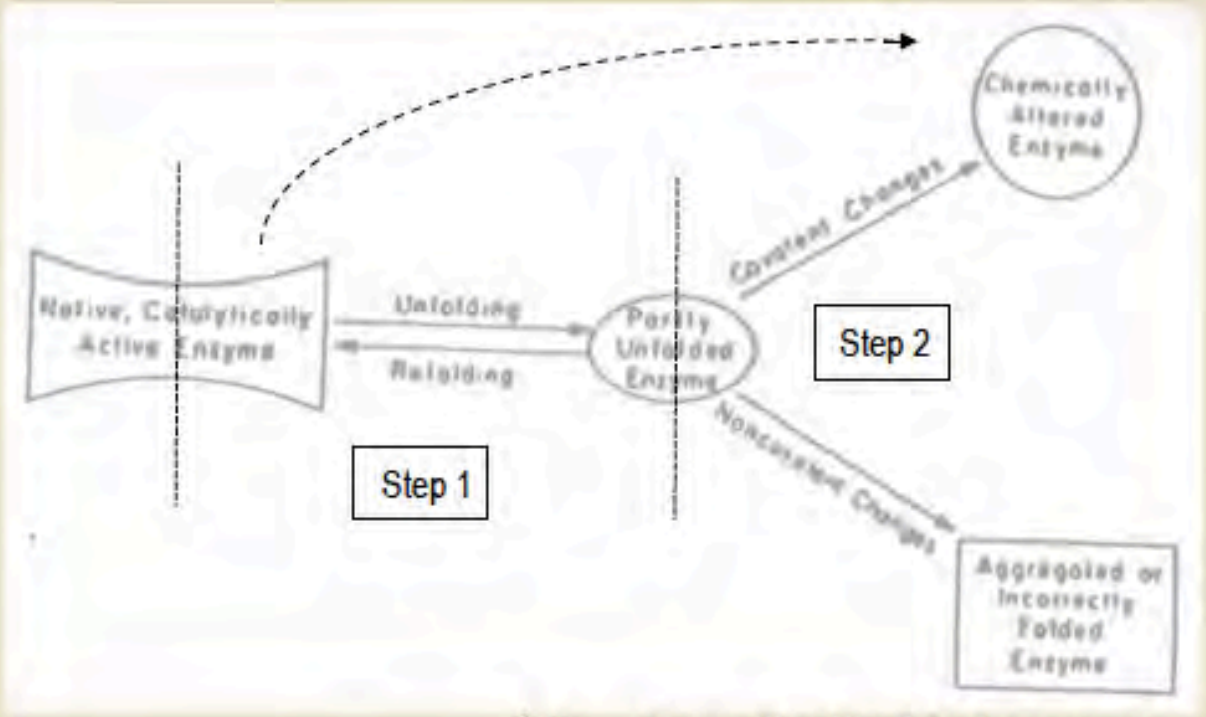
- **Nutropin Depot**– Once-monthly injection of human growth hormone was only polymer controlled-release product ever to be FDA approved.

Major issues limiting injectable PLGA depots for peptide/protein delivery

- Peptide/protein instability
- Elevated manufacturing costs (e.g., aseptic processing/scaling up)
- Difficulties associated with organic solvent use
- Insufficient control of release kinetics (e.g., initial burst release)
- Needle size too large

Irreversible inactivation of proteins

General scheme of 2-step instability (can be more complex)



Step 1: Native → part. unfolded

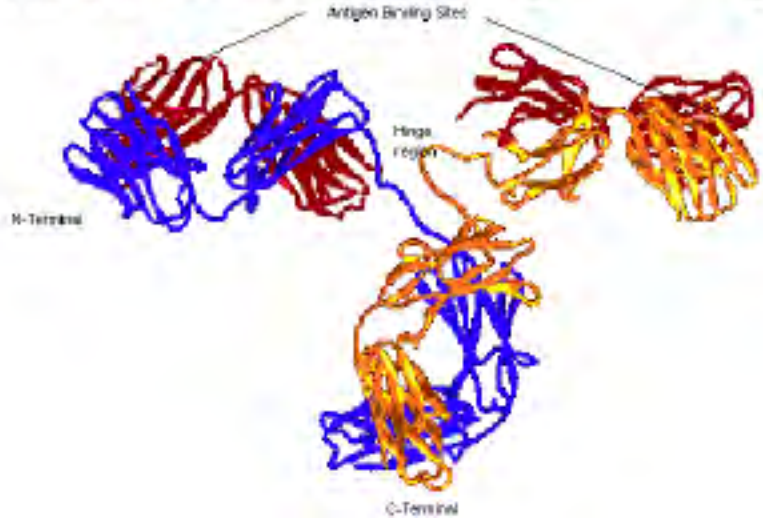
1) Generally still relatively compact
2) Retains significant secondary structure
3) Loss of most tertiary structure
4) Hydrophobic regions more exposed
5) Generally more susceptible to chem. rxns

(Volkin & Klibanov, in *Protein Function: A Practical Approach*, Creighton (ed.), Oxford University Press, Oxford, 1989)

Main Chemical and Physical Degradation Pathways of Proteins

Physical Instability

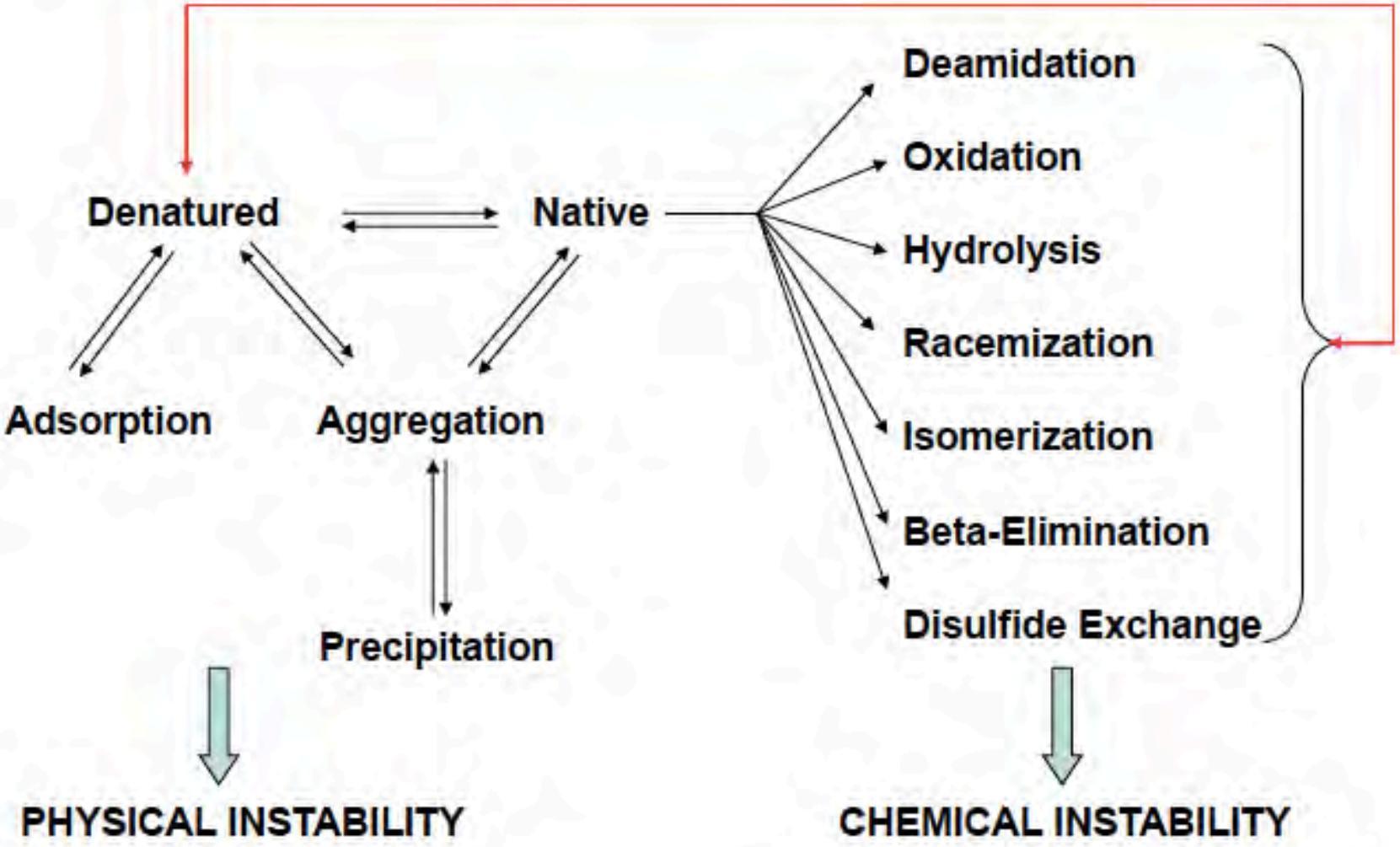
- Conformational Changes (denaturation/misfolding)
  - –Adsorption
  - –Aggregation
  - –Precipitation



Chemical Instability

- Fragmentation /hydrolysis
- Deamidation
- Oxidation
- Isomerization
- Racemization
- Transpeptidation
- Disulfide scission/reduction
- Disulfide exchange
- Deglycosylation

Protein Degradation Pathways



Li et al., Pharm. News, **2**, 12 (1995)

Deamidation of Proteins

- Non-Enzymatic reaction which occurs at Asn (Asparagine) and Gln (Glutamine)

- Hydrolysis reaction requiring water

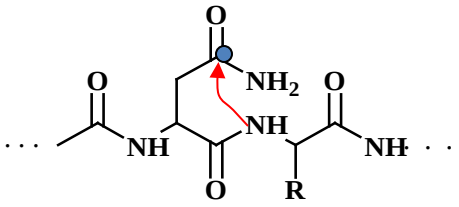
- Pathways depend on pH and temperature
  - Minimum rate in pH range ~ 3 – 4
  - Buffer catalytic effect in pH range ~ 7 – 12

- Rate is 5-10x faster for Asn than for Gln

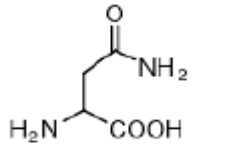
- Rate is affected by the N+1 residue: Asn-Gly (fastest),

Deamidation of Asparagine

Asn
z = 0



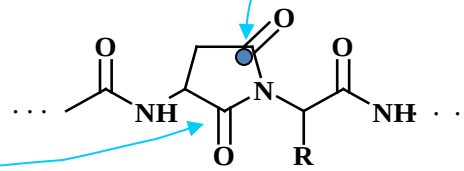
pH range (~ 5 – 12)



Asparagine (Asn, N)
MW: 114.11

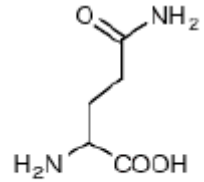
NH3
- 17 Da

β-carbonyl



α-carbonyl

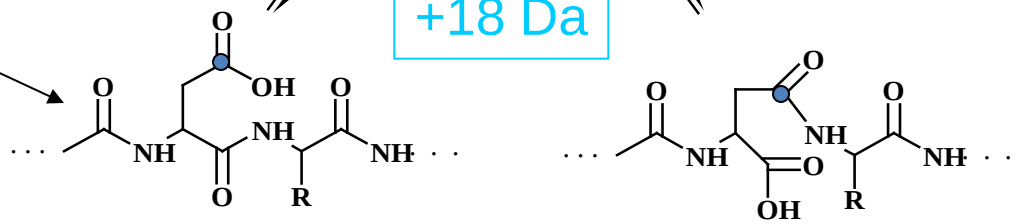
Succinimide
(cyclic imide)



Glutamine (Gln, Q)
MW: 128.14

Isomerization of Asp

H_2O
+18 Da



Asp, +1 Da, z = -1
by hydolysis at β-carbonyl

iso-Asp, +1 Da, z = -1
by hydrolysis at α-carbonyl

Adapted from JCarroll

Impact of Deamidation on Activity

- **mAb:** Asn30 to Asp on LC: Potency ~70%. Asp102 to isoAsp on HC: Potency < 30% *(Harris et al, JChromB, **752**, 233, 2001)*
- Presence of < 5% deamidated **Amylin** 20-29 leads to aggregation and amyloid formation (*Nilsson, ProtSci, **11**, 342 , 2002*)
- Enhanced bioactivity of mammalian **somatotropin** through selective deamidation (Asn to isoAsp in region 96 -101) (*WO 1987/01708*)
- **Protein activity may or may not be impacted by deamidation dependent upon:**
  - **Position and impact on structure/conformation**
  - **Extent of deamidation**

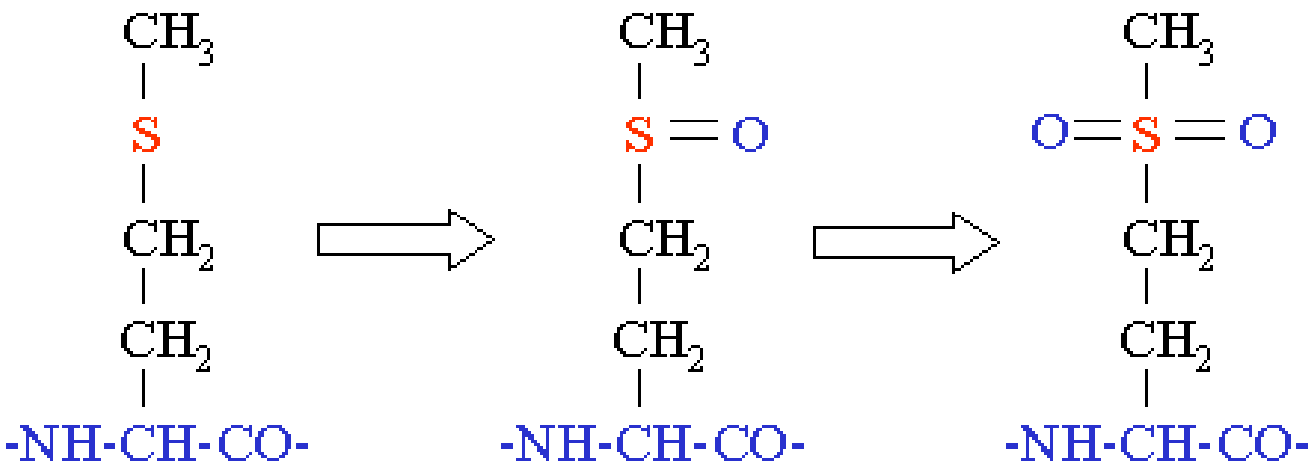
Hydrolysis of Peptide Bonds

- Catalyzed by acid or base
- -x-Asp-y sequence is considered labile
- Hydrolysis in dilute acid at least 100X faster than other peptide bonds
- Mechanisms similar to deamidation $\Rightarrow$ succinimide formation, followed by racemization and isomerization (possible at physiological pH)
- Asp-Pro (Pro-Asp?) susceptible (8-20X faster compared to other Asp-x or x-Asp) under acidic conditions
- -x-Ser-, -x-Thr-: Cleavage at N-terminal side faster than other peptide bonds

Protein Oxidation

- Covalent modification of a protein
- Induced by reactive oxygen intermediates or other oxidants
- Can be caused by
  - Chemical reagents (H_2O_2, $\cdot O_2$, metal ions, excipients)
  - UV light
- Commonly modifies the residues
  - Met, Cys, Trp, His, Tyr
- May cause reduced potency of protein, depending on site
- May cause conformational change in protein, leading to aggregation

Methionine Oxidation



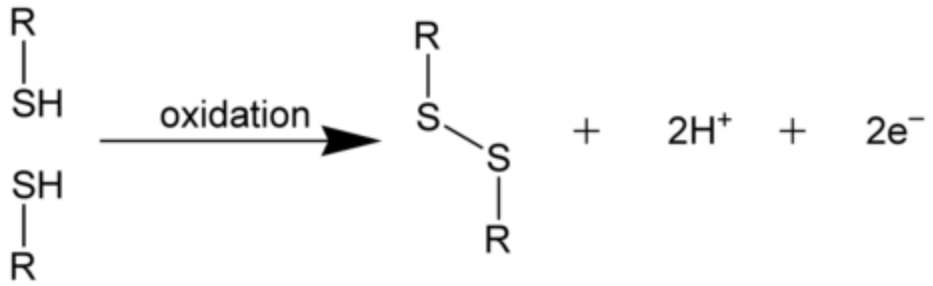
Methionine

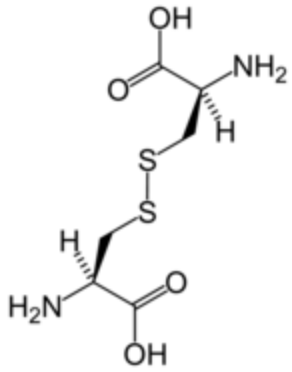
Methionine Sulfoxide

Methionine Sulfone

Disulfides: Cleavage and Scrambling

cysteines

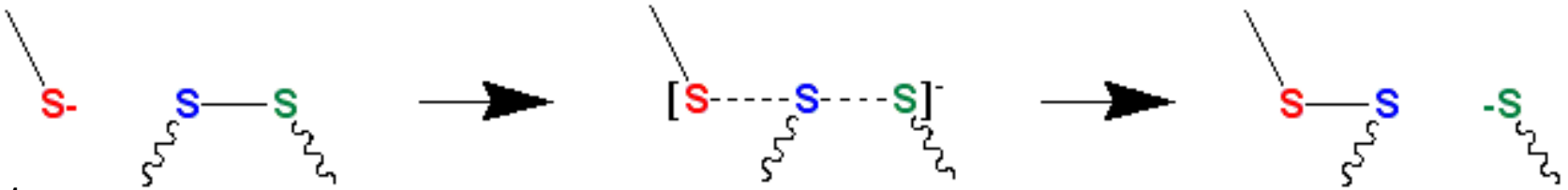




cystine

Cleavage: Reducing conditions e.g. in cytosol

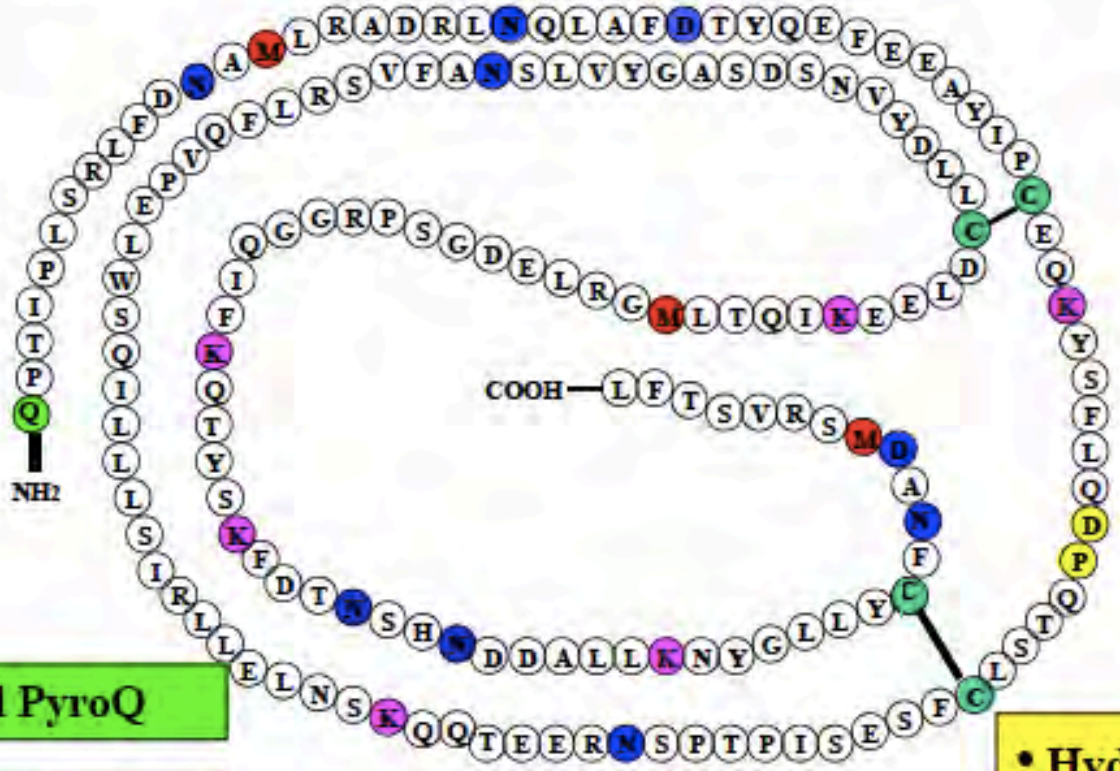
thiolate



Thiol-disulfide exchange
(neutral or alkaline environments; pH > ~8;
protonated thiols are not reactive, pKa ~ 8.3)

Disulfide reshuffling faster than formation/reduction of disulfides

Protein Degradation Hot-Spots



- N-terminal PyroQ
- Glycation at Lys
- Thioether Formation
- Scrambling
- Deamidation of Asn
- Isomerization of Asp
- Hydrolysis of Asp-Pro
- Oxidation of Met
- Aggregation

JCarroll

Protein Conformational States

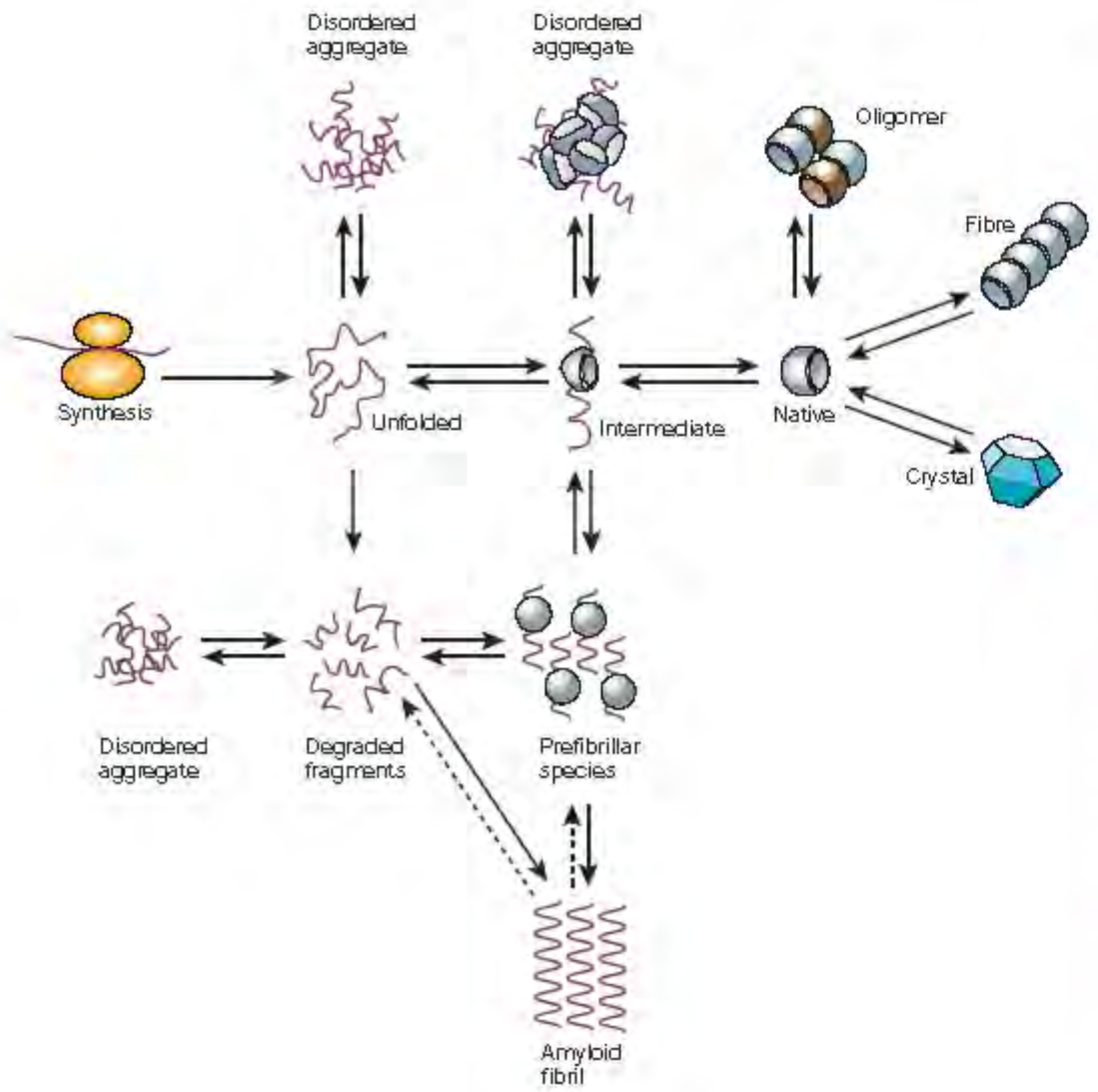
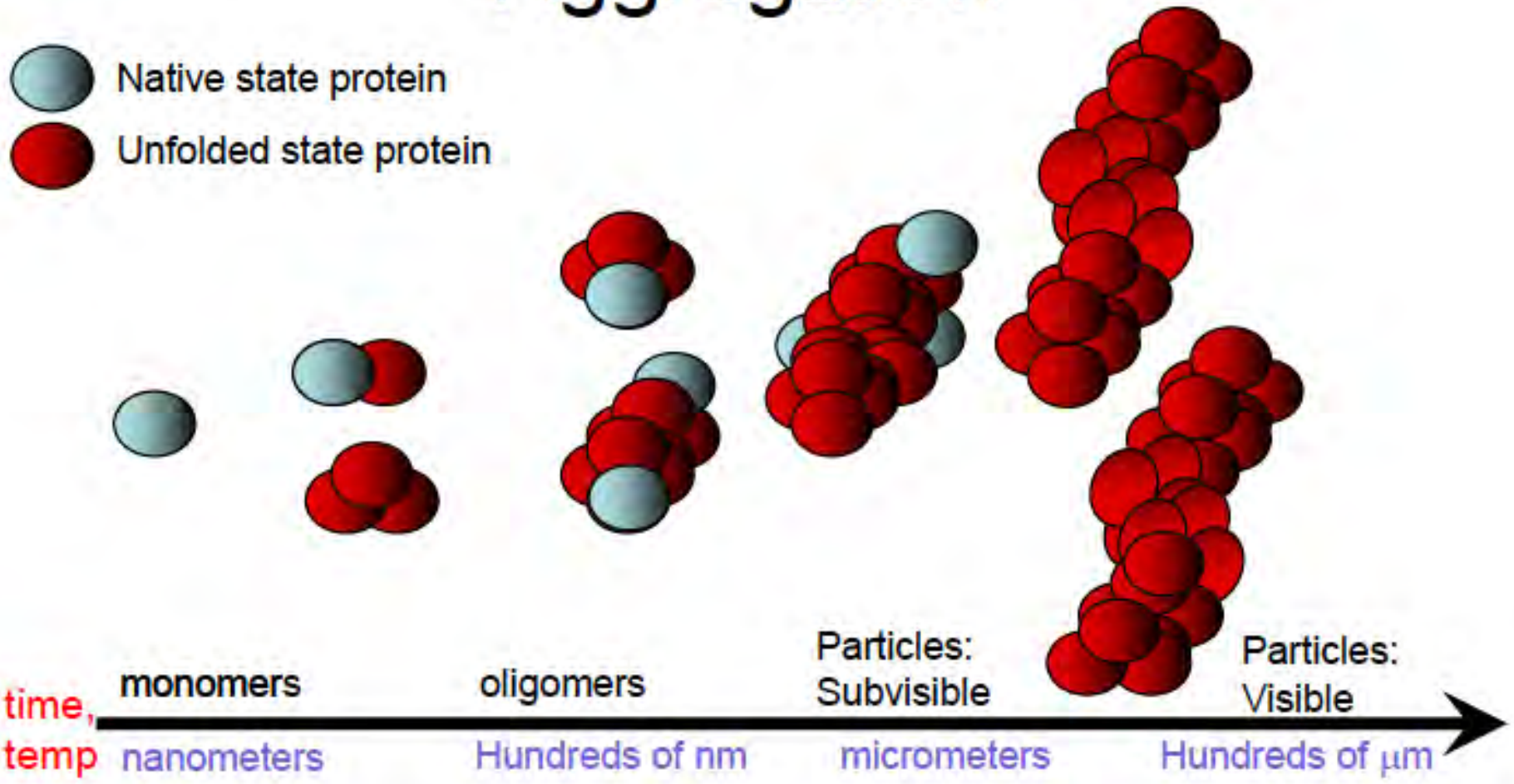


Figure 4 A unified view of some of the types of structure that can be formed by polypeptide chains. An unstructured chain, for example newly synthesized on a ribosome, can fold to a monomeric native structure, often through one or more partly folded intermediates. It can, however, experience other fates such as degradation or aggregation. An amyloid fibril is just one form of aggregate, but it is unique in having a highly organized 'misfolded' structure, as shown in Fig. 3. Other assemblies, including functional oligomers, macromolecular complexes and natural protein fibres, contain natively folded molecules, as do the protein crystals produced *in vitro* for X-ray diffraction studies of their structures. The populations and interconversions of the various states are determined by their relative thermodynamic and kinetic stabilities under any given conditions. In living systems, however, transitions between the different states are highly regulated by the environment and by the presence of molecular chaperones, proteolytic enzymes and other factors. Failure of such regulatory mechanisms is likely to be a major factor in the onset and development of misfolding diseases. Adapted from ref. 54.

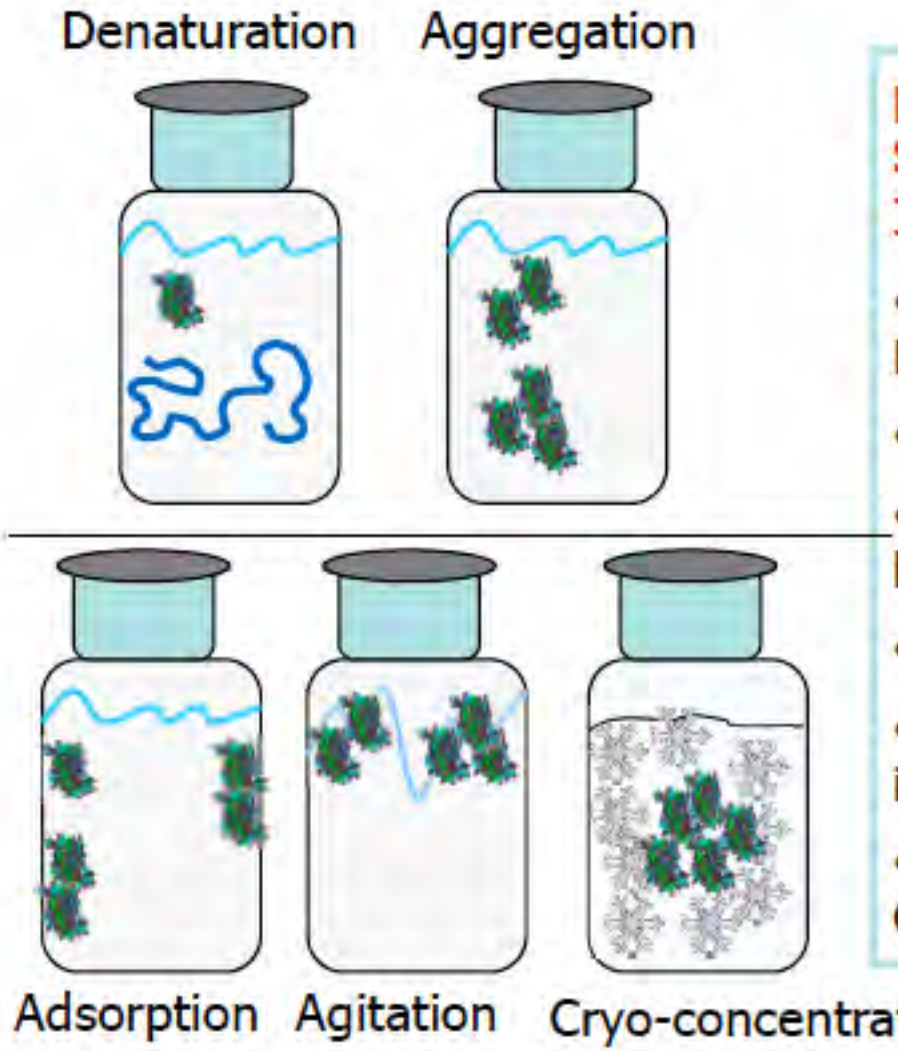
Aggregation



Aggregation → Native-state aggregation

Aggregation → Non-native aggregation

Risk Factors for Protein Aggregation



pH stress, Temperature stress, Solvent stress, Shear stress, Interfacial stress

- Fermentation/Expression, Purification, Filtration (shear, interfaces)
- Fill/Finish (shear, interfaces)
- Freeze/Thaw (cryo-concentration; ice-liquid interfaces)
- Agitation (air-liquid interfaces)
- Storage and Shipping (temperature, interfaces)
- Manufacturing equipment, Containers / Closures (leached compounds and metals)

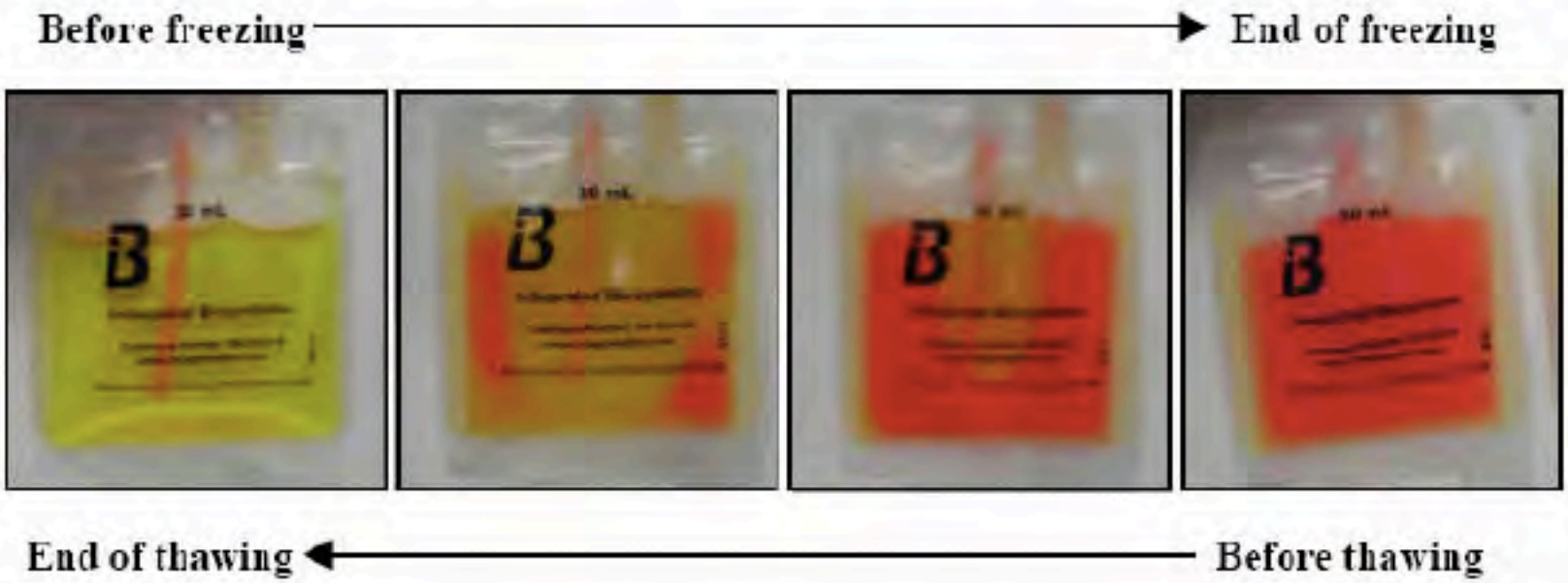
Stresses During Freezing

- Freeze Concentration
  - Cryo-concentration of solutes
    - ↑collision rates → ↑ reaction rate
  - Buffer crystallization / pKa shift → shift in pH
  - Ionic strength increase (0.15 M NaCl reaches 3 M at –10C)
  - Increase in protein concentration
  - Dessication
    - Dilute aqueous solution → ≈ 20% H_2O
  - Enhanced concentration of oxygen in unfrozen solution (solubility of O_2 in ice is less than in aqueous solutions)

- Frozen State
  - ✓ Unfolding due to interaction between ice surface and protein molecule
  - ✓ Decrease in Hydrophobic bonds strength
  - ✓ Subunit dissociation

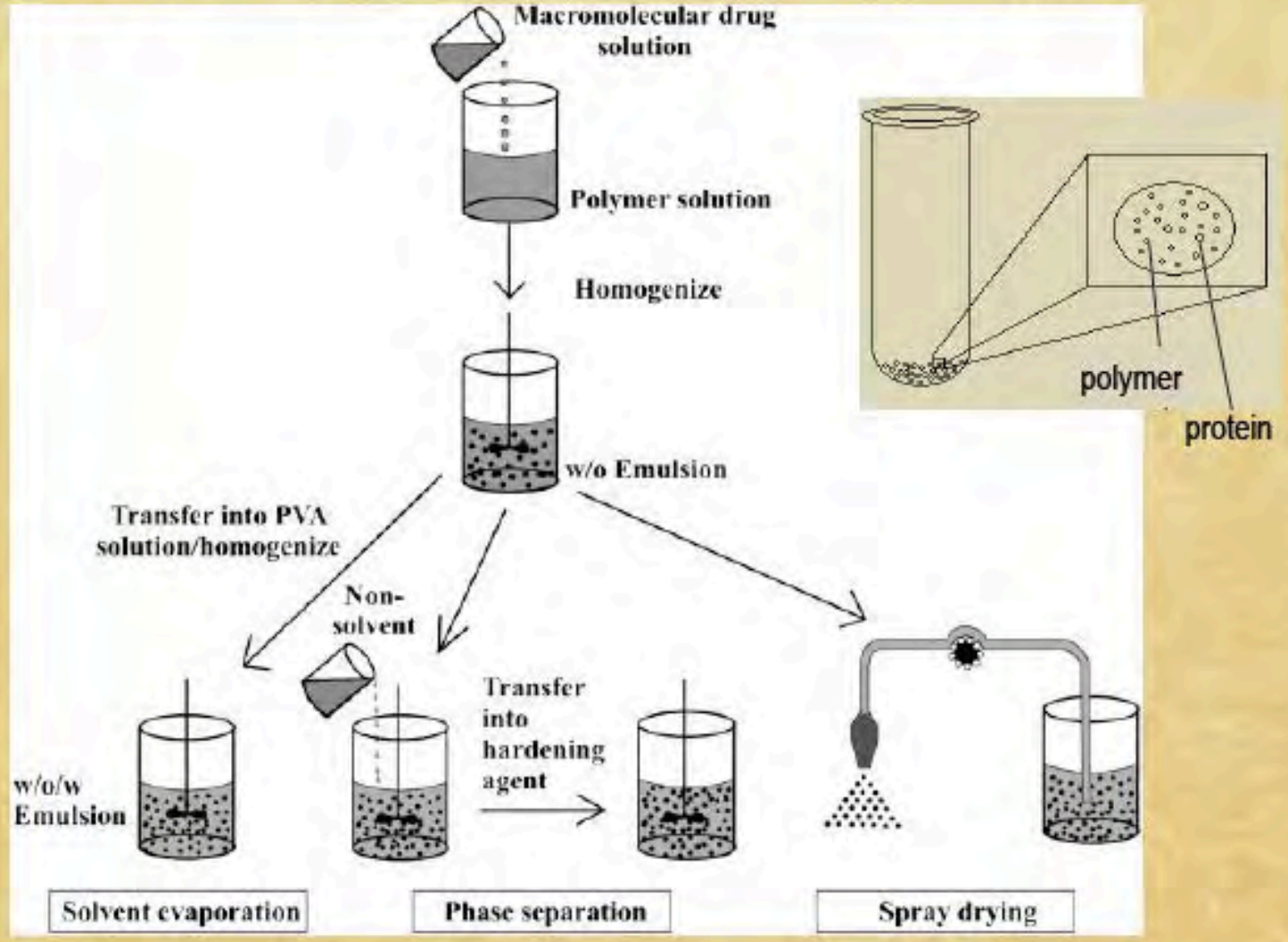
pH Changes on Freezing

Effect of freezing upon pH of buffered aqueous solutions



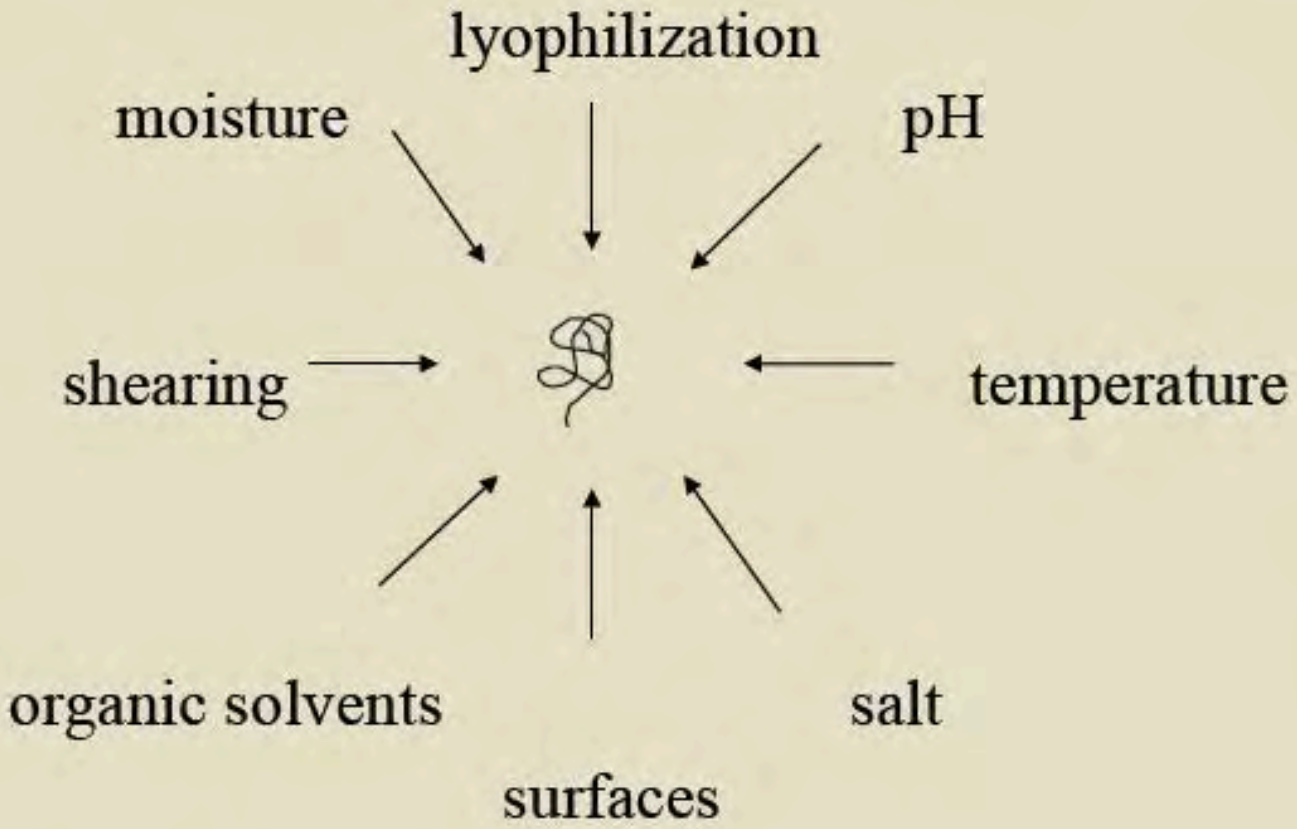
NaPhosphate buffer (green pH 7, red pH 4)
- pH shift caused by precipitation of the di-basic salt

Common methods to microencapsulate proteins in PLGA microspheres?



(Mundargi et al., *J. Cont. Rel.*, **125**, 193-209 (2008))

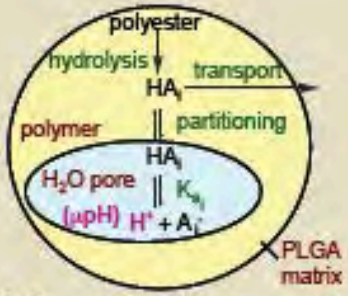
What causes instability of proteins during encapsulation?



(*adapted from* Kissel & Koneberg, in *Microparticulate Systems for the Delivery of Proteins and Vaccines*, Cohen, S. and Bernstein, H. (eds.), Marcel Dekker, New York, 1996, pp. 51-87)

What causes instability during release?

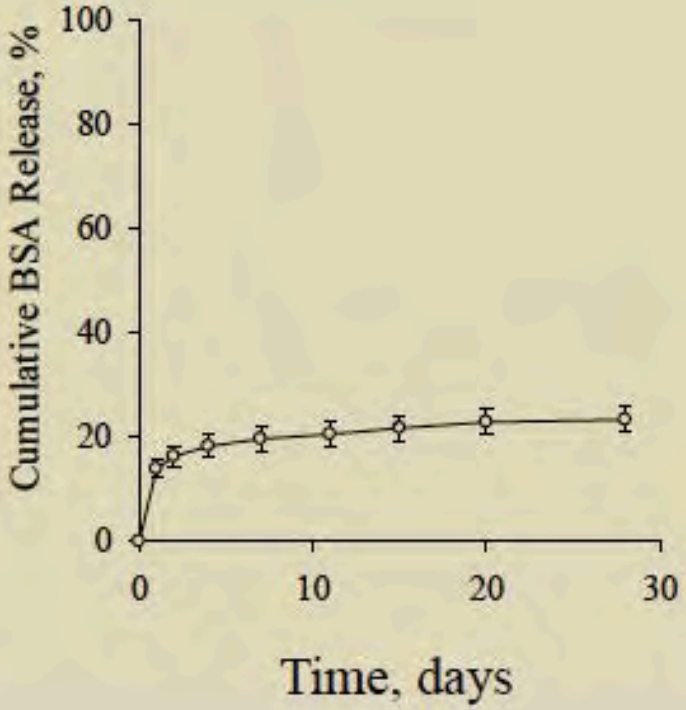
Potential Source of Protein Inactivation	Example
Initial rehydration-Reduced water content (Increased protein conformational mobility)	water (↑ mobility) → aggregation
Adsorption to polymer surface (Increased hydrophobic environment)	Adsorption → Unfolding → irreversible adsorption or denatured desorbed protein
Ionization of carboxylic end groups (Acidic microclimate pH)	irreversible acid denaturation (e.g., unfolding, aggregation, hydrolysis)



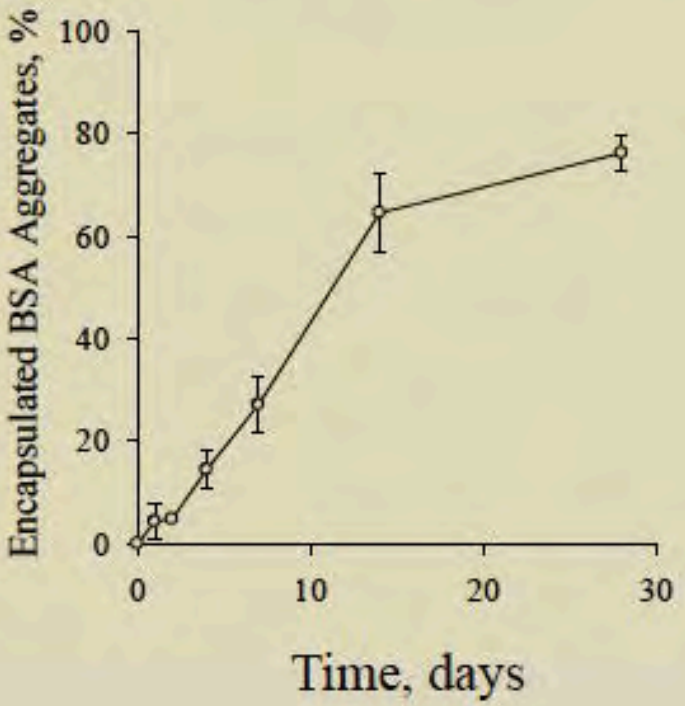
(*adapted from* Schwendeman et al. in *Microparticulate Systems for the Delivery of Proteins and Vaccines*, Cohen, S. and Bernstein, H. (eds.), Marcel Dekker, New York, 1996, pp. 1-49)

Kinetics of instability of BSA in 15% BSA/PLGA 50/50 millicylinders

- Release Profile

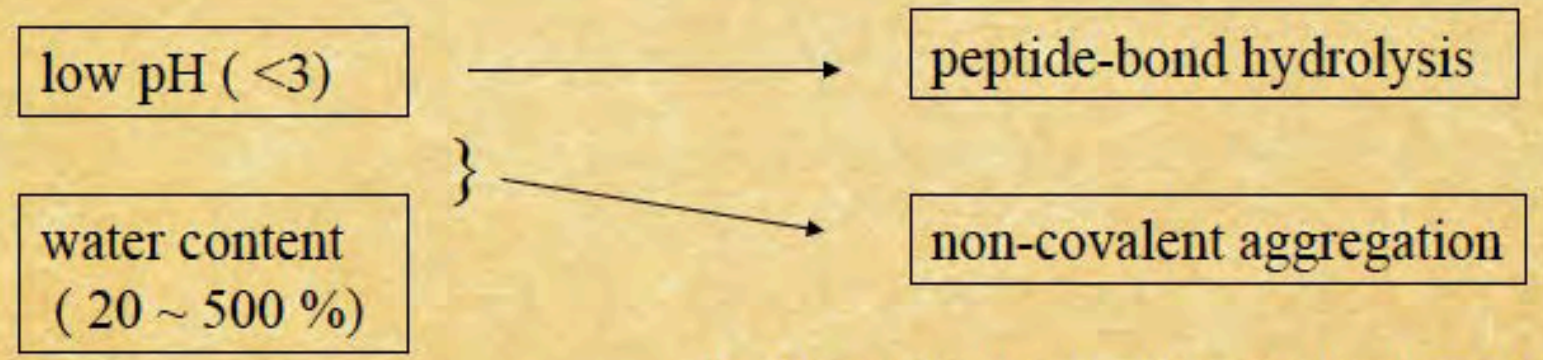


- Aggregation Profile



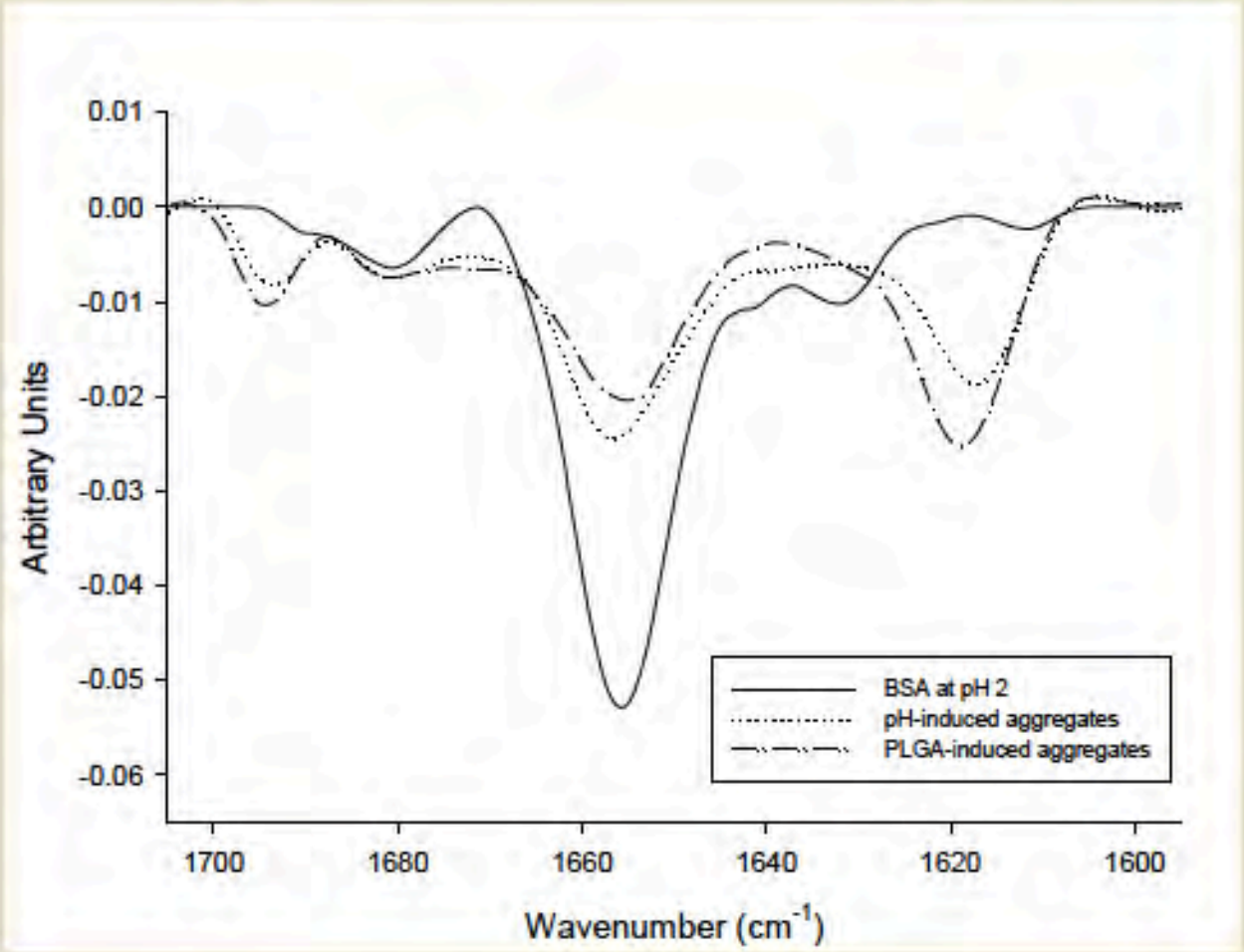
Summary of BSA instability

	Encapsulated	Simulated (86% rh, pH 2)
Solubility of aggregates in denaturing solvent	~ 98 %	~ 94%
Degradation products	~ 55, 45, and 20 kDa	~ 55, 45, and 20 kDa
Time to 50% aggregation	~ 12 days	~ 8 days



(Zhu, Mallery & Schwendeman, *Nat. Biotechnol.*, **18**, 52-57 (2000))

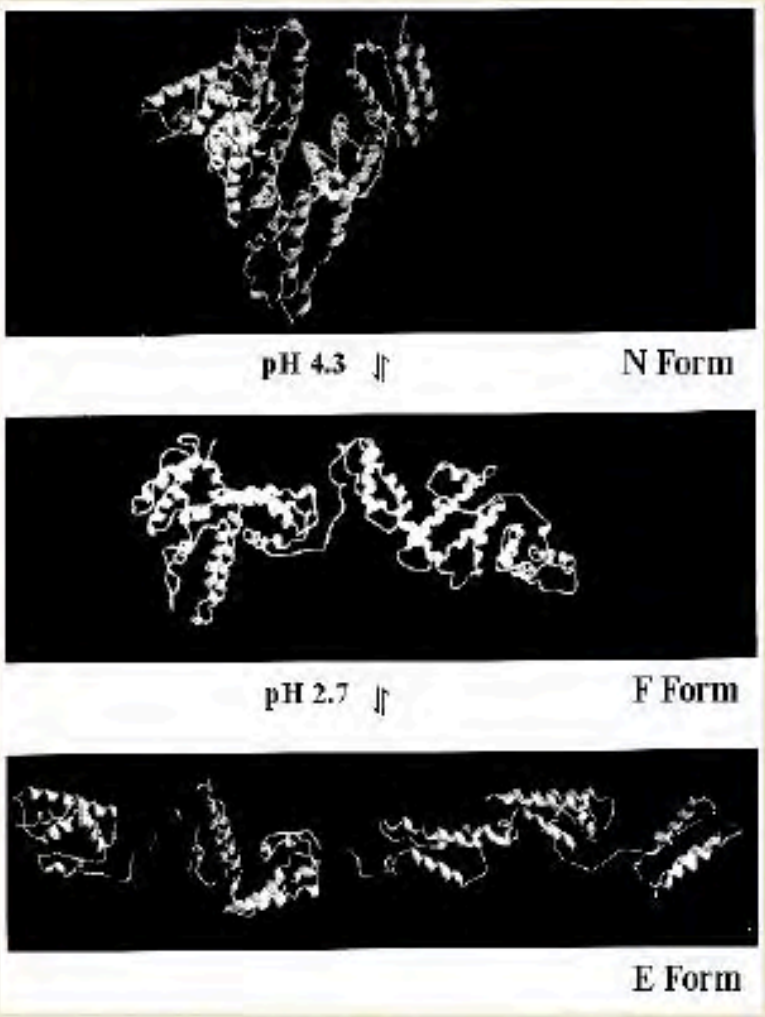
FTIR comparison of simulated and encapsulated BSA aggregates



(Estey et al., *J. Pharm. Sci.* 2006)

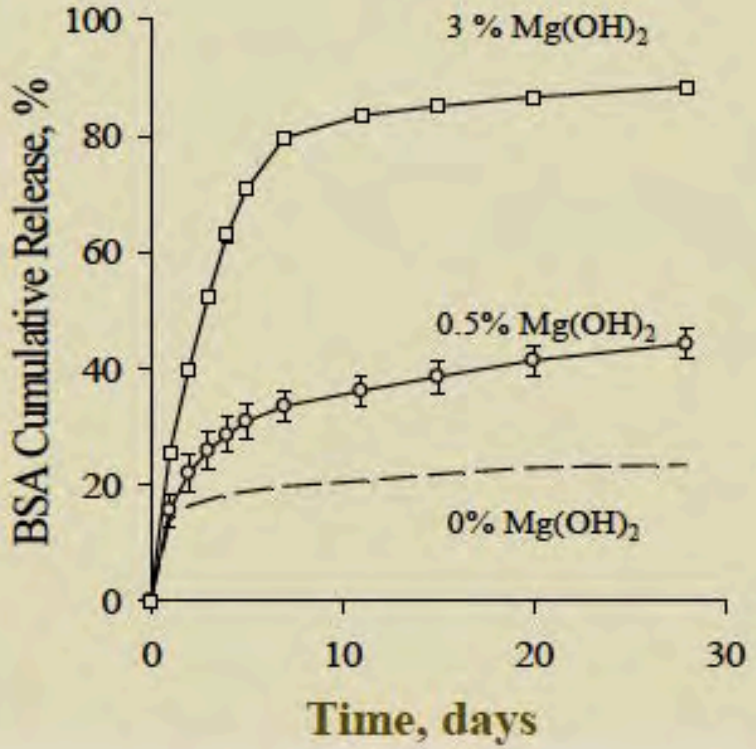
Hypothesis for instability of encapsulated BSA

- acid-induced cleavage at acid-sensitive peptide bonds (e.g., Asp-X) causes peptide-bond hydrolysis
- acid-induced unfolding (E form) initiates non-covalent aggregation

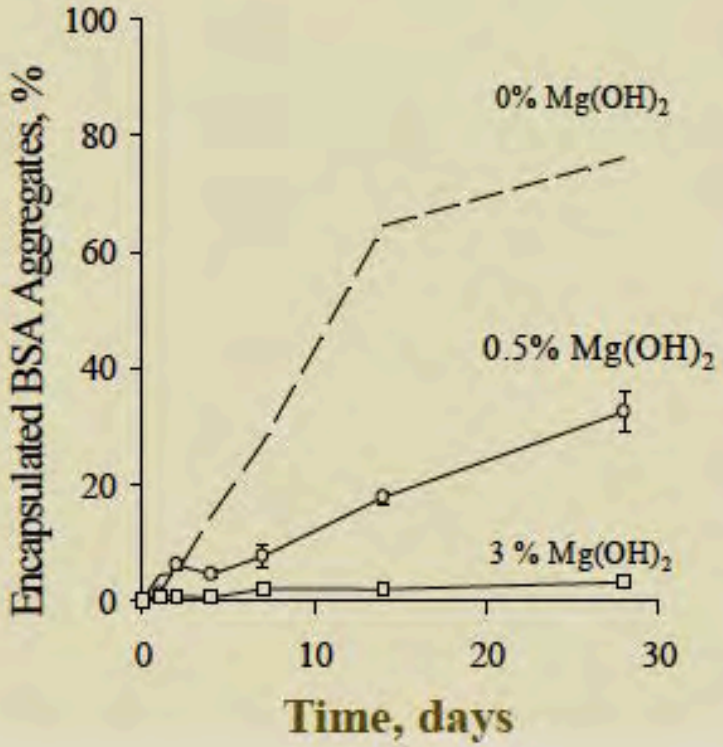


$Mg(OH)_2$ stabilizes encapsulated BSA

- Release profile

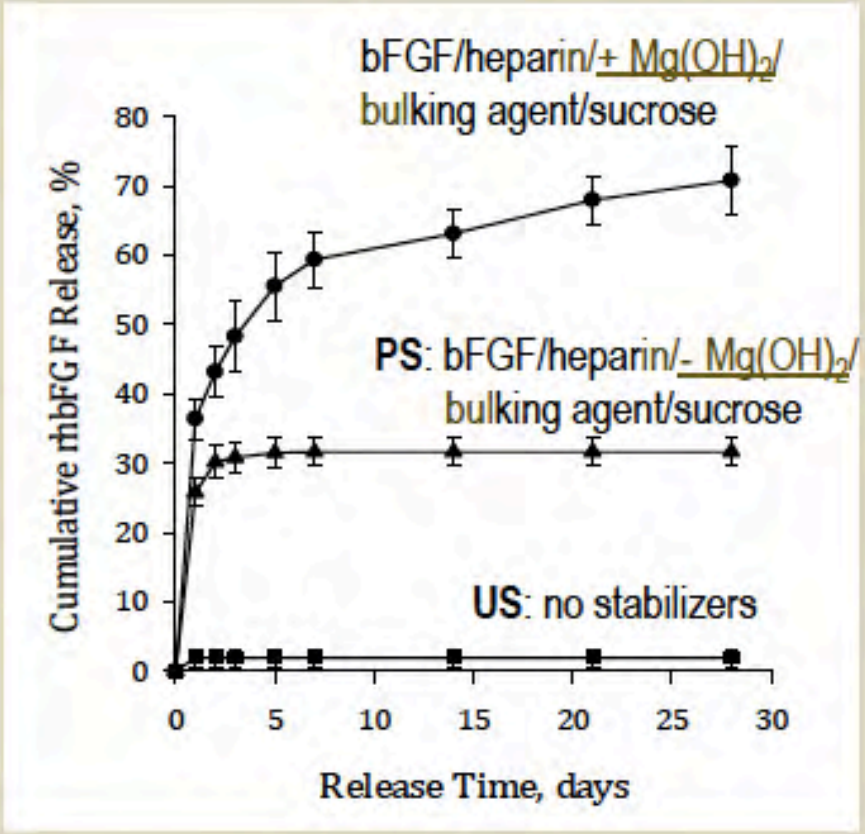


- Aggregation profile



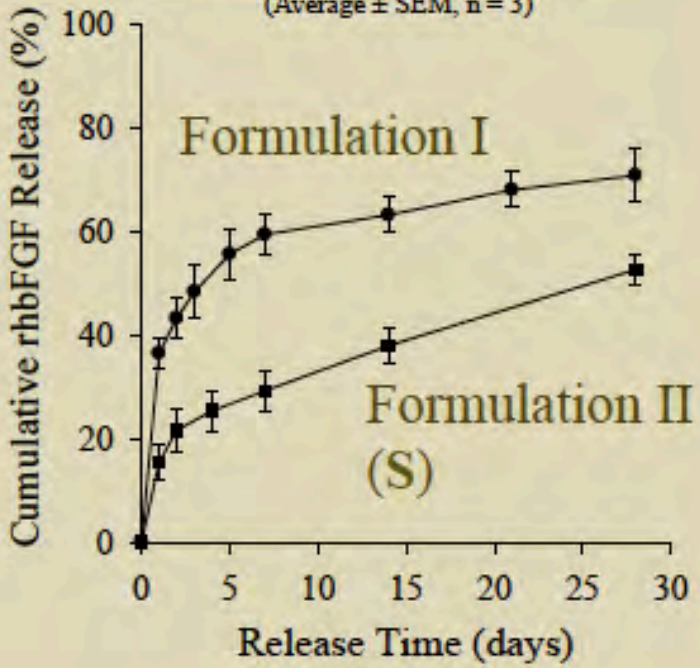
(Zhu, Mallery & Schwendeman, *Nat. Biotechnol.*, **18**, 52-57 (2000))

Stability and controlled release of immunoreactive bFGF in vitro



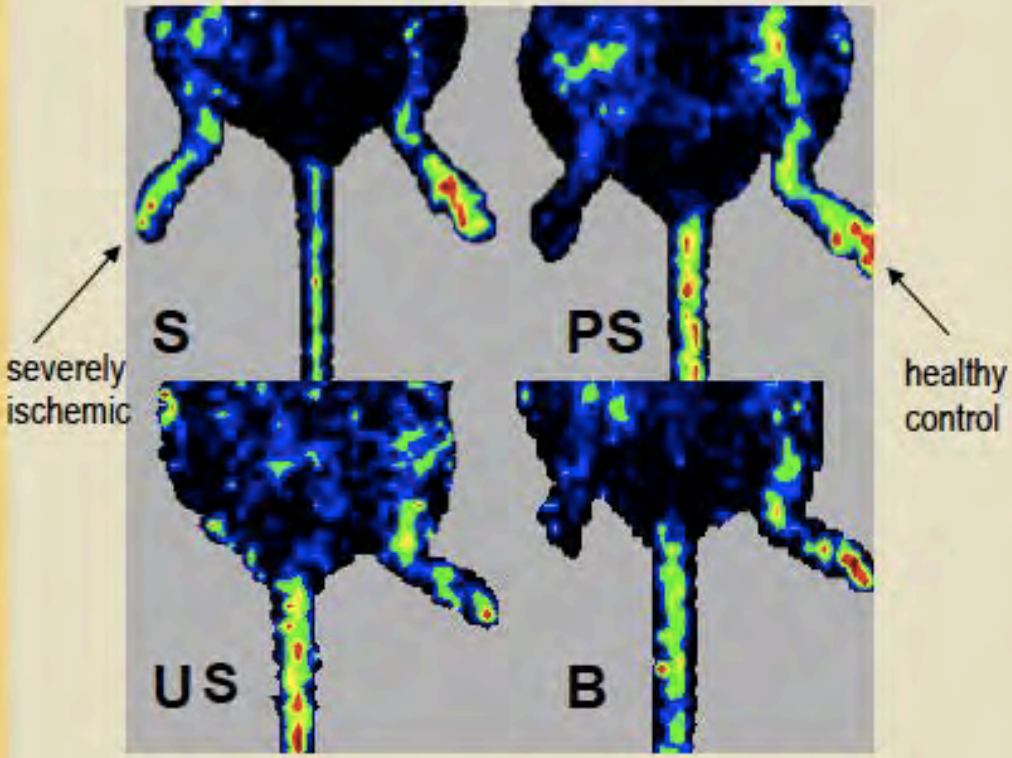
	Released after 4 weeks	Remained
I:	71 ± 5%	21 ± 2%
II:	49 ± 2%	45 ± 3%

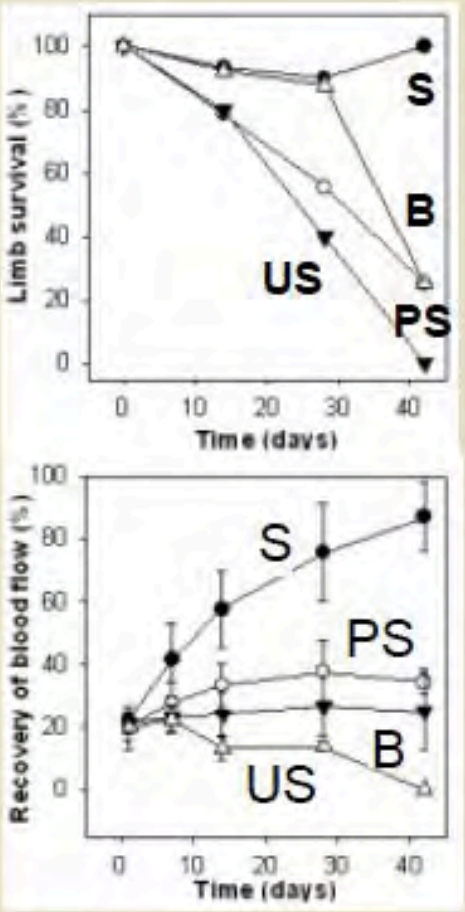
(Average ± SEM, n = 3)



Stabilized PLGA/bFGF rescue murine ischemic hindlimbs

Laser Doppler Perfusion Imaging after 6 weeks



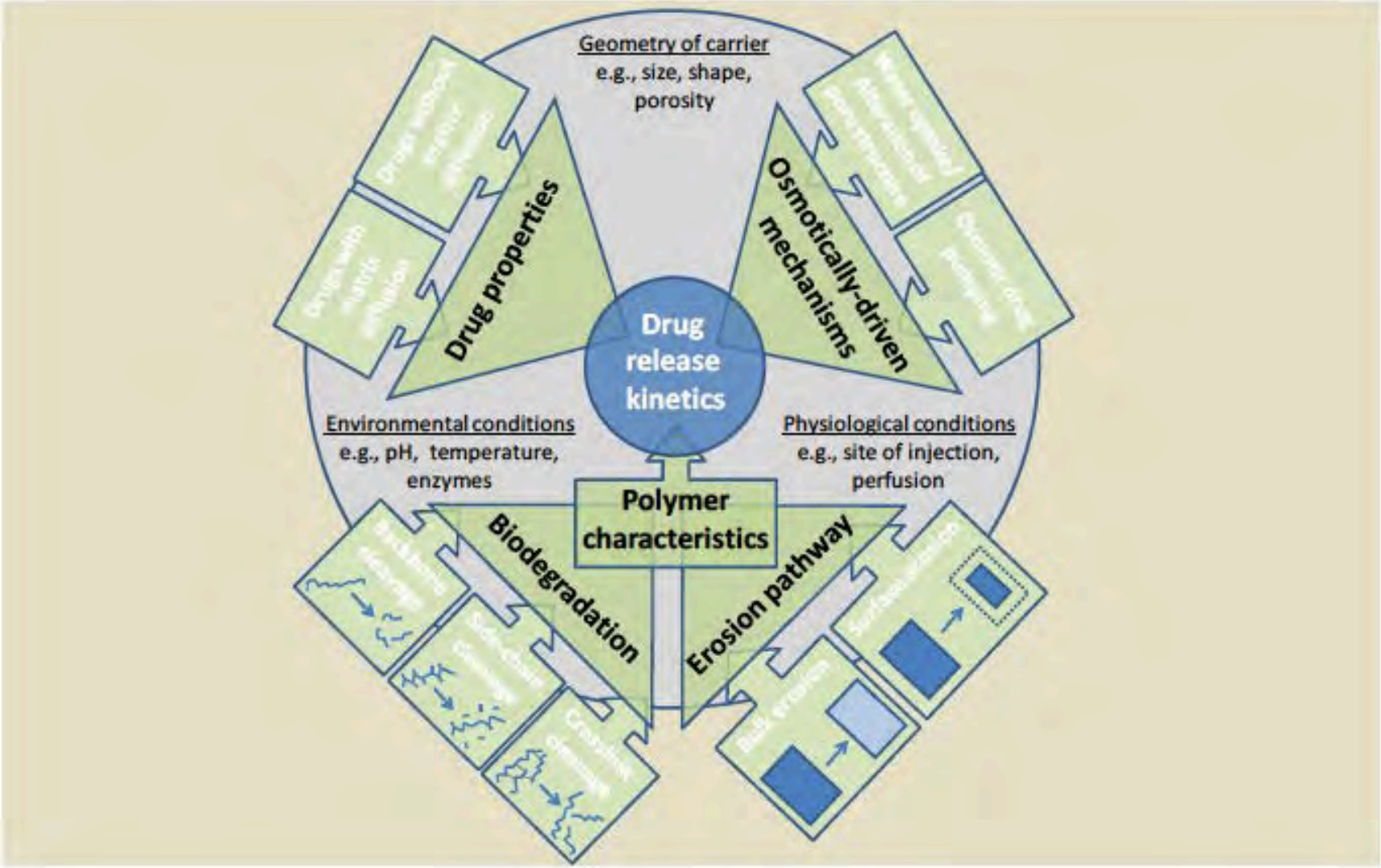


- PLGA/bFGF implanted at time and site of injury

Symbols mean ± SEM (n = 5 – 15)

(Zhong et al., *J. Controlled Release*, ***122***, 331-337 (2007))

Factors controlling release of drugs from PLGA

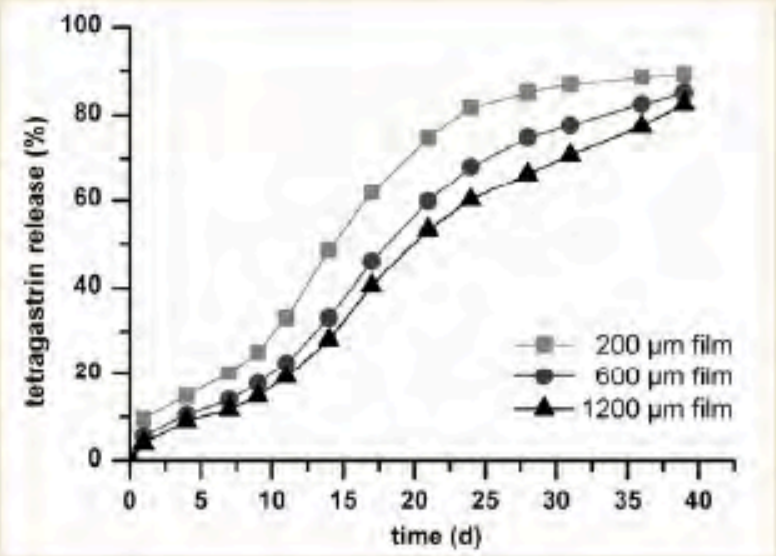


5 Examples of attaining continuous release from PLGA
Example 1 – Use of low MW fraction

Rationale: low MW fraction (as a blend or 100%) helps to eliminate induction time of mass loss for sustained erosion-controlled release

Low-MW fraction is expected to:
- increase water content
- increase polymer permeability
- provide increased soluble PLGA fraction to be released immediately

(- elevated drug loading also helps)



PLGA 50/50, Mw = 15.2 kD

(Hutchinson, EP 058481, 1982)

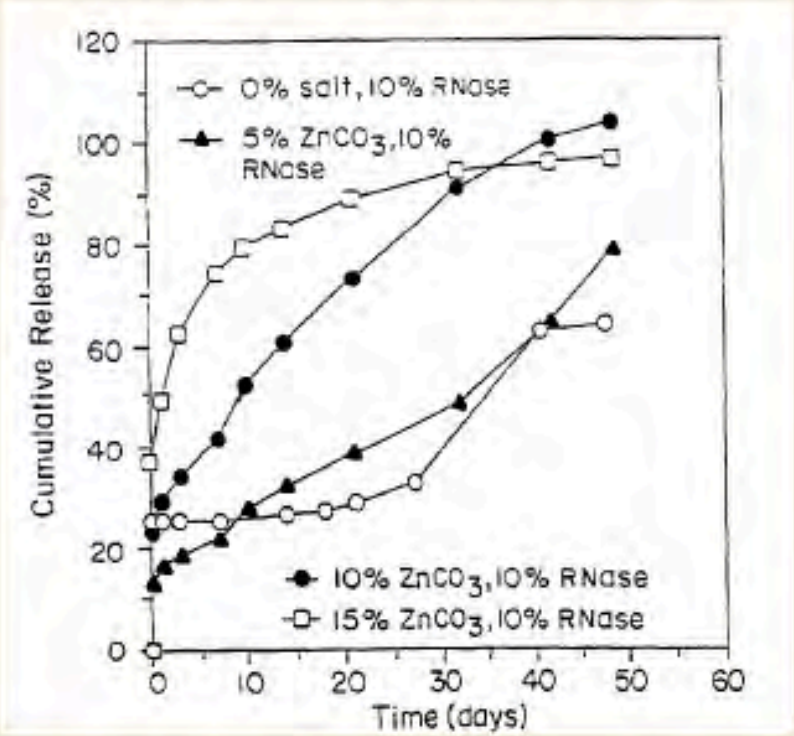
Example 2 – Incorporate poorly soluble base to create pores

Rationale: Increase porosity as base reacts with acid produced by polymer

Antacid type bases are expected to:

- React w/ acid produced to create osmotic salts to cause swelling
- extracts water-soluble acids to to make polymer more brittle and reduce degradation rate

(- also cause large changes to polymer morphology)

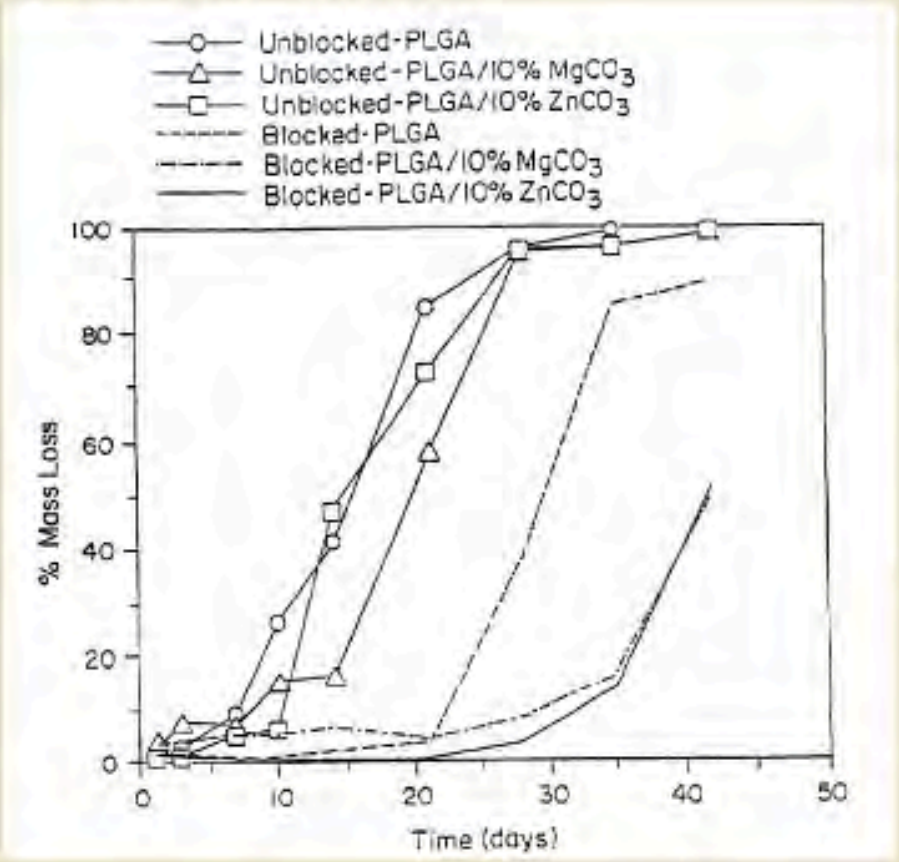


PLGA 50/50, Mw = 42 kD

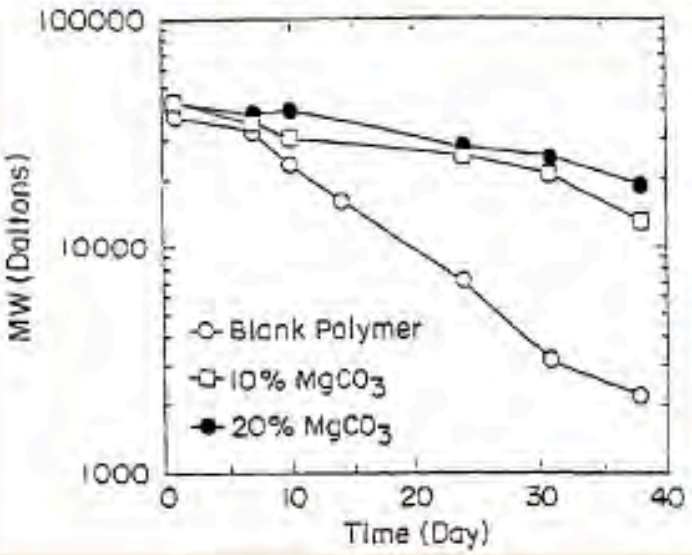
(Bernstein et al., US 6,749,866, 2004)

Example 2 – Incorporate poorly soluble base to create pores

Continuous mass loss



Reduced hydrolysis



PLGA 50/50, Mw = 42 kD

(Bernstein et al., US 6,749,866, 2004)

Example 3 – Blend water-soluble polymer with PLGA (e.g., PEG or poloxamer)

Rationale: blending above percolation threshold (of PEG additive) should allow release of PEG providing pore path for drug diffusion

>20% PEG required

PLA, i.v. = 1.07 dL/g

(Jiang & Schwendeman, Pharm. Res., 2001)

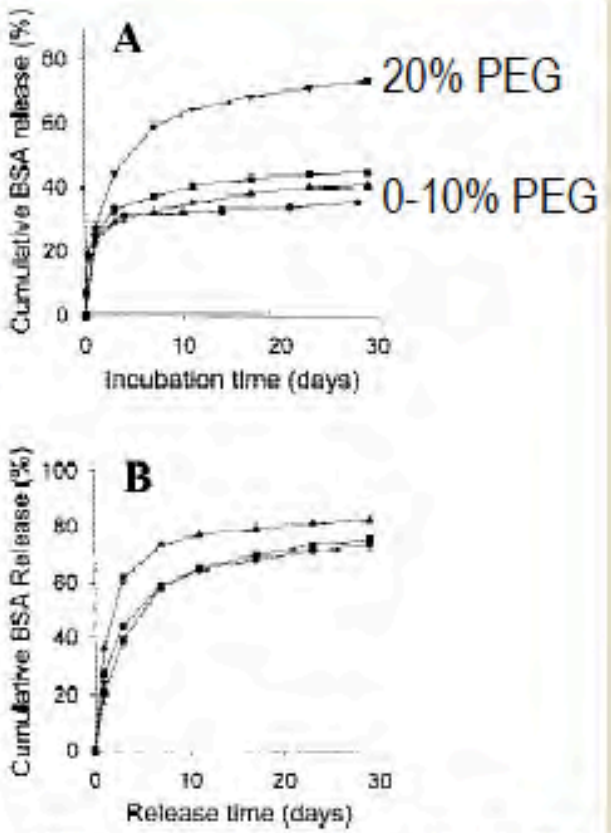
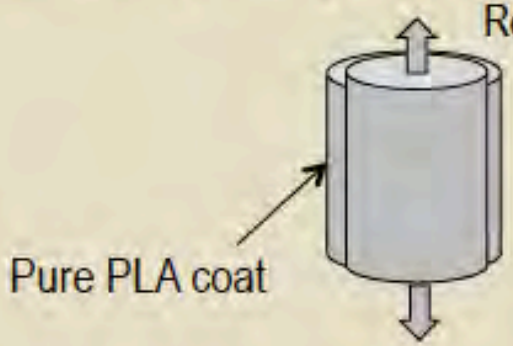


Fig. 3. The effect of PEG content and molecular weight in the PLA/PEG blend on the release kinetics of BSA. (A) PEG 10,000 content was 0% (●), 5% (■), 10% (▲), and 20% (▼); (B) PEG molecular weight and content were 20% PEG 10,000 (■) and 20% PEG 35,000 (●), 30% PEG 35,000 (▲). (average ± SD, n = 3)

Example 4 – Cases 1 – 4 for non-degradable polymers

Rationale:

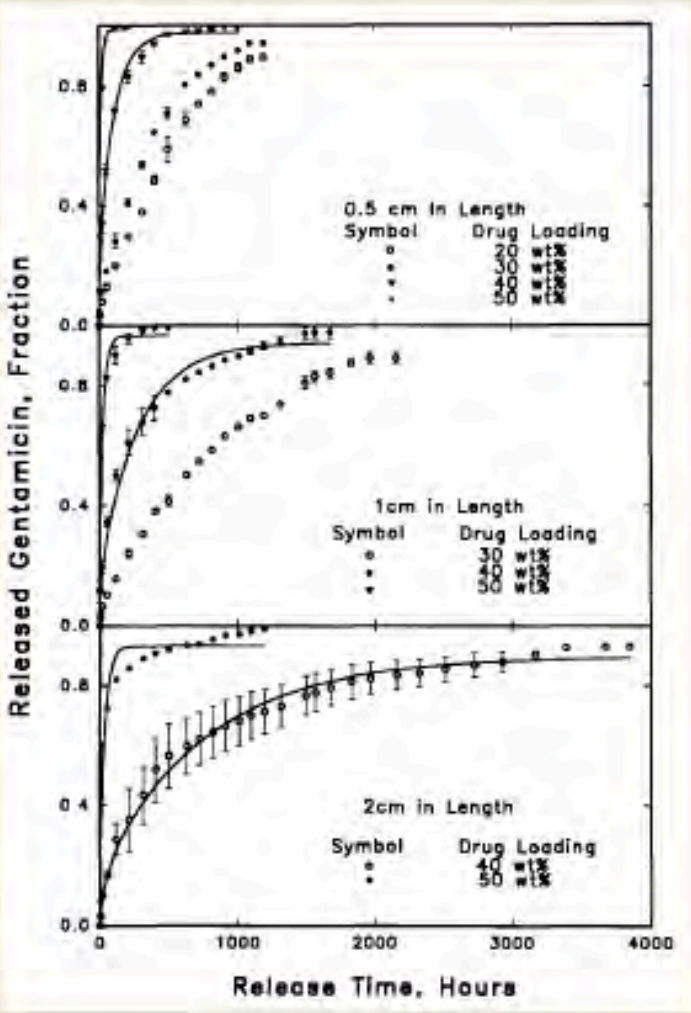
- If diffusion in polymer is much more rapid than polymer degradation, get cases 1 or 2
- If use very high loading >> percolation threshold, then get cases 3 or 4



Case 2
> 40% load

PLA, Mv = 32.6 kD

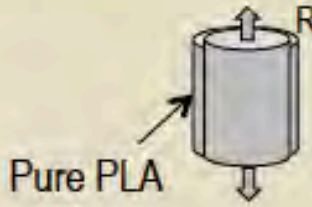
(Zhang *et al.*, *J. Cont. Rel.*, 1994)



Example 5 – Use direct osmotic agents (or drug itself) at or below percolation threshold

Rationale:

- If diffusion slower than osmotic pumping (e.g., by increasing cylindrical length) then strong dependence on external π



30% load

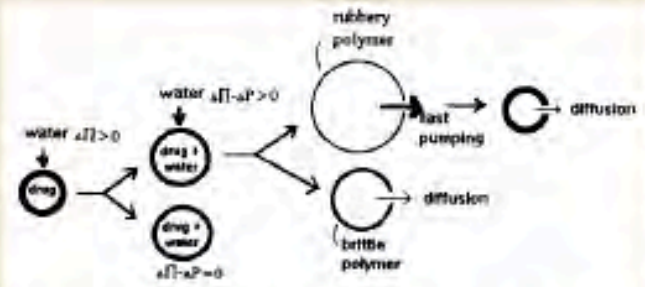


Fig 9 Illustration of osmotic pressure release mechanism.

PLA, Mv = 32.6 kD

(Zhang *et al.*, *J. Cont. Rel.*, 1994)