Hydrogels for the Controlled Release of Pharmaceutical Proteins

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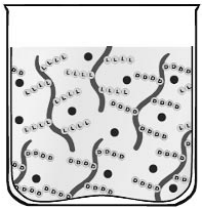

The authors describe the problems and opportunities of hydrogel technology and focus on the PolyActive polymer and chemically or physically cross-linked dextran-based hydrogels. The hydrogel delivery systems are shown to be ideal candidates for improving the performance of a large number of pharmaceutical proteins lacking proper pharmacokinetic and dynamic properties.

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Many new pharmaceutical proteins need controlled-release systems for constant (lasting weeks or months) or pulsed release of the intact protein. The "Candidate proteins" sidebar lists several proteins that are potential candidates for controlled-release strategies. Current routes of administration that are alternatives to injection show little promise, with the exception of the pulmonary route. Although this article focuses on injection delivery systems, we use controlled-release delivery systems for these proteins to

- produce the desired pharmacokinetic profile (e.g., to prevent high initial plasma concentrations [it is often said "the flatter, the better"])
- reduce the injection frequency and improve patient-friendliness
- reduce the involvement of trained medical personnel.

During the past few years a number of strategies have been developed to control the release of proteins on the basis of time (1). First, proteins have been formulated as crystals or in an amorphous form (e.g., insulin) to ensure release during a period of one or two days. Second, pumps with a catheter and a fixed needle can administer drugs for local or systemic purposes. The advantages of these pumps are that the pump rate is controlled, and the technology is reliable. Upon subcutaneous injection, liposomal dispersions release their content during periods as long as three weeks. But even the miniaturized devices can be bothersome for patients during long-term use. In addition, the systems are costly. Third, liposomal dispersions release their content during periods as long as three weeks following subcutaneous injection.

Proteins are intrinsically fragile molecules. Their therapeutic action is highly dependent on proper folding of the amino acid string and rather weak physical interactions. S-S-bonds stabilize their complex three-dimensional structure. Proteins are sensitive to exposure to high and low temperatures, the presence of hydrophobic surfaces (many organic solvents), high shear, high and low pH, and the removal of water (2). These conditions should be avoided during preparation, storage, and adminis-

Candidate proteins*

EPO	**Erythropoietin**	G-CSF	Granulocyte-colony stimulating factor
CD40L	CD-40 ligand		
TNFR:Fc	Tumor necrosis factor receptor	TGF-β1	Transforming growth factor- β1
IL-2	Interleukin-2	a and bFGF	Acidic fibroblast growth factor and basic fibroblast growth factor
IL-1R	Interleukin-1 receptor		
IL-4R	Interleukin-4 receptor	LIF	Leukemia inhibiting factor
IL-17R	Interleukin-17 receptor	NGF	Nerve growth factor
INF	Interferons	VEGF	Vascular endothelial growth factor
GM-CSF	Granulocyte macrophage-colony stimulating factor	BMP-2	Bone morphogenetic protein-2
		IGF-1	Insulin growth factor-1

* W.R. Gombotz and S.F. Wee, "Protein Release from Alginate Matrices," *Adv. Drug Delivery Rev.* **31,** 267–285 (1998). M. Yamamoto, Y. Ikada, and Y. Tabata, "Controlled Release of Growth Factors Based on Biodegradation of Gelatin Hydrogel," *J. Biomater. Sci. Polymer Edn.* **12,** 77–88 (2001).

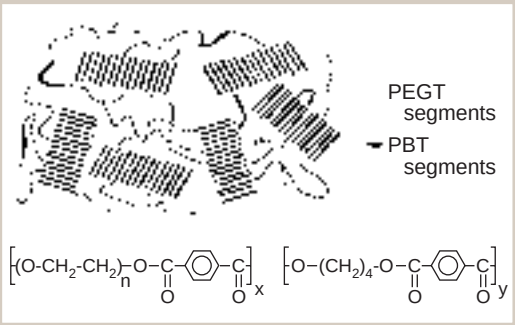

Figure 1: Schematic representation of the morphology of PEGT–PBT copolymers. The PBT segments form hydrophobic domains in the hydrophilic PEG matrix, thereby creating a physically cross-linked network (10).

tration of protein-containing microspheres. A complicating factor is that even when the full spectrum of modern analytical equipment is used, the assessment of a complete conformational structure of proteins is still a challenge (3,4).

A successful controlled-release device depends on technological factors such as protein-loading efficiency, protein integrity, and desired release characteristics. Recently, poly(lactide-co-glycolide) (PLG) polymer-based microspheres with hydrophobic characteristics were launched as protein-delivery systems (5). Upon subcutaneous injection, the microspheres released proteins at relevant rates during a period of two to three weeks (6). Proper selection of the polymers and production protocol allows some control over release characteristics, but it is difficult to avoid a burst release. In the production process of this first generation of polymeric microspheres, organic solvents often are used. In addition, the internal pH in these microspheres tends to drop during release. Ultimately, both the use of organic solvents and the acidic pH may affect the integrity of the protein.

A rather new development is the emergence of hydrogel-based technologies for the controlled release of proteins. Hydrogels are three-dimensional hydrophilic, polymeric networks capable of imbibing large amounts of water (7). Their high aqueous content offers a protein-friendly environment, and many of them show excellent biocompatibility (see "Hydrogel material" sidebar). pH drops do not occur during protein release, and the use of organic solvents often can be avoided during the preparation of formulations. Natural polymers can be used to form hydrogels, possibly after modification, and synthetic polymers also can be used (see "Hydrogel material" sidebar). The release of proteins can be controlled by bulk degradation of the hydrogel rather than by surface erosion in other systems. Some of these polymers are responsive to environmental changes, e. g., they have a low viscosity at room temperature (during administration) and form a gel after administration to the patient. This characteristic has yielded some promising results, as in the case of intratumoral delivery of the small molecule taxol. However, the in situ gelling means that there is limited control over the geometry of the gel upon injection (8).

Hydrogels are stabilized by cross-links between the polymeric units that form the backbone of the gel. These bridges can be chemical (covalent) or physical in nature (i.e., by ionic or hydrophobic interactions). Chitosan and alginate are examples of polymers that can be cross-linked by ionic interactions. The cationic chitosan polymer's complex formation is achieved by adding a negatively charged polymer, e.g., carboxymethylcellulose. The negatively charged alginate can be cross-linked by divalent cations. Amphiphilic polymers can form physical cross-links through hydrophobic interactions. One can attach hydrophobic units to hydrophilic polymers by grafting poly(glycolic acid) or poly(lactic acid) (PLA) to hydrophilic polymers such as poly(vinyl alcohol).

Synthetic hydrogel

PolyActive (IsoTis, The Netherlands) is an amphiphilic multiblock copolymer (9–11). Hydrophilic poly(ethylene glycol)-terephthalate (PEGT) segments are coupled to more hydrophobic poly(butylene terephthalate) (PBT) segments (see Figure 1). A family of copolymers can be synthesized with varying combinations of the PEGT/PBT, ranging from a PEGT content of 30–90 wt % and a molecular weight of the poly(ethylene glycol) (PEG) segments between 300 and 4000 g/mole. In the PolyActive copolymer, hydrophobic PBT domains dispersed in a hydrophilic PEG matrix stabilize the hydrogel. The mechanical, physicochemical, and biological properties of the polymers vary with the choice of the building

Hydrogel material

Based on natural materials

Collagen	Gelatin
Starch	Alginates
Chitosans	Dextrans

Based on synthetic polymers

N-vinylpyrrolidone
Poly(vinyl alcohol)
Polyphosphazenes
Poly(ethylene oxide)-b-poly(propylene oxide) Copolymers
PL(G)A/PEO/PL(G)A copolymers
PVA-g-PLGA graft-polymers
PEGT-PBT copolymers (PolyActive)
MA-oligolactide-PEO-oligolactide-MA

Responsive polymers

Methacrylates (pH-dependent swelling)
Poly(N-isopropylacrylamide) (LCST)
PEO-PPO-PEO (Pluronics)
PEO-PPO-PAA graft-copolymer (LCST)
PLGA-PEO-PLGA (LCST)

Abbreviations

BSE	Bovine spongiform encephalopathy
Dex	Dextran
DS	Degree of substitution
HEMA	Hydroxyethyl methacrylate
hGH	Human growth hormone
IgG	Immunoglobulin
(rh)IL-2	(recombinant human) Interleukine2
MA	Methacrylate
MW	Molecular weight
PBS	Phosphate-buffered saline
PBT	Poly(butylene terephthalate)
PEG	Poly(ethylene glycol)
PEGT	Poly(ethylene glycol)-terephthalate
PLA	Poly(lactic acid)
PLG	Poly(lactide-co-glycolide)

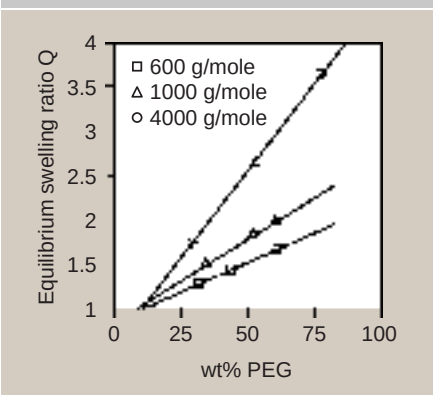

Figure 2: Equilibrium swelling ratio of the PolyActive copolymer as a function of PEG wt% of the copolymers. *Q* is defined as swollen volume divided by dry volume (n = 3; ± s.d.).

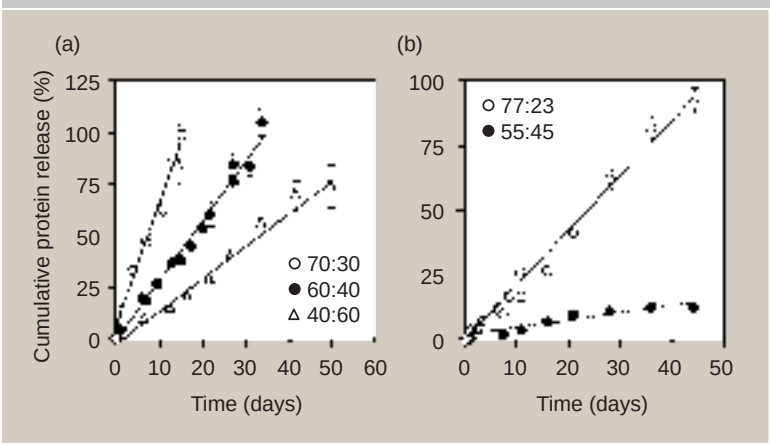

Figure 3: Cumulative release of lysozyme from PolyActive copolymer. (a) Various PEG:PBT ratios are shown for the molecular weight of the PEG segment at 1000 g/mole; (b) various PEG:PBT ratios are shown for the molecular weight of the PEG segment at 600 g/mole; (*n* = 3; ±s.d.).

blocks. For example, the swelling of the PolyActive copolymer in water increases with the increasing PEG content and molecular weight (MW) of the PEG segment length (see Figure 2) (9). Control of the swelling allows fine-tuning of the release kinetics for a wide range of drugs, including proteins and peptides. Upon administration, the PolyActive copolymer begins to degrade as a result of hydrolysis of the ester bonds and oxidation of the PEG ether bonds.

PolyActive copolymer has been used in thousands of patients as a degradable cement restrictor, bone replacement material, artificial skin, and an anti-adhesive barrier. An extensive biological safety file about the product is available (European/FDA approval for two small medical devices). PolyActive-based microspheres currently are being developed as protein-delivery systems that release the protein for periods of weeks to one year.

When PolyActive microspheres are prepared, the double-emulsion technique (W/O/W) is used. First, an emulsion of protein-containing water droplets in an organic polymer solution is prepared. Subsequently, that W/O emulsion is emulsified in a second, external water phase. The organic solvent then is removed by evaporation, and the protein-loaded microspheres can be collected. In general, high-protein entrapment efficiencies (≤100%) can be achieved.

Figure 3 shows how varying the PolyActive composition can generate various release rates. Interestingly, the continuous release of a model protein (lysozyme) can be obtained (12). This release behavior can be attributed to a combination of protein diffusion and polymer degradation. Upon release, the enzymatic activity of released lysozyme was preserved. Simply changing the microsphere preparation protocol is another way to create various release patterns (see Figure 4) (13). The curve showing the delayed release may be of interest when designing vaccine formulations with intrinsic booster effects.

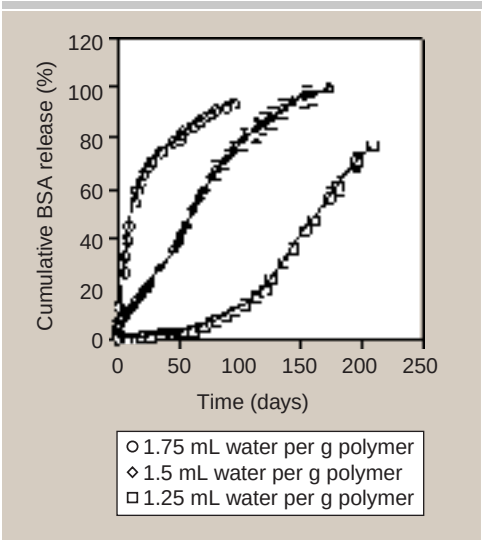

Figure 4: Effect of emulsion composition on the release of BSA from PolyActive microspheres in PBS at 37 °C.

Modified dextran hydrogels: chemically cross-linked

Dextran solutions have been used as plasma expanders for many decades. Under those conditions, large quantities (larger than gram doses) of dextran are administered intravenously. Dextran-based hydrogels also have been investigated for the controlled release of proteins. But to form a gel, the dextran chains must undergo some form of cross-linking, either chemically or physically (14). This article first will discuss chemical cross-linking strategies in combination with a process of microsphere formation that does not involve the use of organic solvents. The article then will discuss new developments regarding the physical cross-linking of dextrans.

The all-aqueous process for the preparation of modified dextran microspheres is based on the finding that phase separation occurs in a system consisting of an aqueous solution of conjugated dextran (dex-HEMA/dex-Lactate-HEMA) and a solution of (PEG) in water (15). This phase separation is used to create an emulsion of aqueous dextran conjugate–enriched droplets in a continuous, aqueous, PEG-enriched phase. Subsequent polymerization of groups containing a double bond attached to the dextran chain (dex-HEMA or dex-Lactate-HEMA) results in cross-linking of the dextran chains in the droplets. Thus, microspheres are formed (see Figure 5). No organic solvents such as chloroalkanes are used. An additional advantage of this process is the enrichment of the dextran phase with the pharmaceutical protein so that protein encapsulation effi-

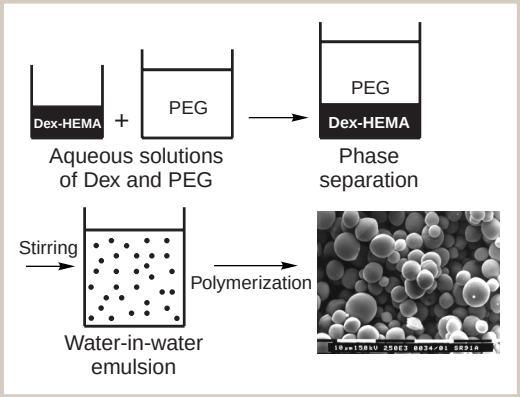

Figure 5: Schematic representation of the microsphere preparation process (10).

Table I: In vivo efficacy of degradable, (rh)IL-2–loaded dextran microspheres in an animal tumor model (SL2 lymphosarcoma-bearing mice) (18).

Treatment	Cures (%)†
Diluent	0:5 (0%)
Empty microspheres	0:45 (0%)
Free-(rh)IL-2*	19:28 (68%)
(rh)IL-2–loaded microspheres**	14:18 (78%)

* Total dose (50% 1×10^6 IU, 50% 2×10^6 IU) given in 5 days with one injection (of 1/5 of the total dose) per day.
**Dex-HEMA microspheres, DS 11, water content 60%, release of IL-2 over 10 days. Total dose (50% 1×10^6 IU, 50% 2×10^6 IU) given in one injection.
† Number of mice tumor-free per number of mice treated.

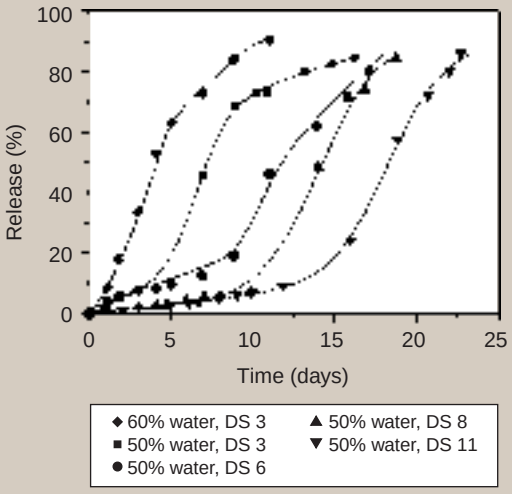

Figure 6: Cumulative release of IgG from degrading dex-HEMA microspheres in vitro at pH 7, 37 °C. The values are the mean of two independent measurements that deviated <5% of the encapsulated amount of IgG (17).

ciencies of >90% can be reached. By choosing the proper reaction conditions, oxidative damage of the encapsulated protein could be minimized, e.g., for IL-2 and hGH (16). Typically the microsphere particle diameter ranges between 2 and 50 µm.

The release of the entrapped protein from the microsphere is initially controlled by its rate of diffusion in the gel. Diffusional release will occur only when the pore size in the microspheres is larger than the hydrodynamic radius of the protein. The pore size changes initially by swelling of the gel and later by gel degradation. The release kinetics of entrapped proteins from dextran microspheres (at 37 °C, pH 7) depend on the initial water content and on the cross-link density (degree of substitution) of the gel. Therefore, it is necessary to carefully control the initial water content and the cross-link density of the hydrated particles. Moreover, the nature of the cross-links influences degradation kinetics and therefore the release profiles. For instance, methacrylate (in dex-MA) forms very stable links, whereas hydroxyethyl methacrylate (in dex-HEMA) bridges degrade faster. Dex-HEMA-lactate bridges degrade even more rapidly.

Another factor that affects the release of encapsulated proteins is the pore size of the microspheres. The hydrogel network allows passage of low-MW molecules. Thus, during the release process no pH drop will occur while the acidic degradation products are rapidly released.

Figure 6 illustrates the release of a model protein (IgG, MW = 150 kD) from degradable dex-HEMA microspheres with different network densities. Under certain conditions, delayed-release profiles were observed for these biodegradable dex-HEMA microspheres. The release of the protein is fully controlled by the degradation of the microspheres, and the delay time increases with increasing network density (17).

In vivo, the therapeutic performance of one injection of IL-2 in dex-HEMA microspheres was compared with five intratumoral injections of free IL-2 in a mouse model (see Table I) (18). Total IL-2 doses were the same for both treatments. The microspheres showed at least similar survival rates as in the daily injected free IL-2. In another experimental setup, similar growth rates were obtained in a dwarf mouse model injected once with microspheres of human growth hormone when compared with daily injections of human growth hormone.

Larger structures such as liposomes, viruses, and cells also can be entrapped in dextran microspheres. Figure 7 shows the release of liposomes from dextran microspheres (19). Typically, a pulsed or delayed release during prolonged periods is observed. As with proteins, the delay time depends on the initial water content, the cross-link density, and the type of polymeric precursor (dex-HEMA versus dex-HEMA-lactate).

In vivo biocompatibility studies of dextran hydrogels were performed. Current results demonstrate that the hydrogels fully disintegrate upon injection as the cross-links hydrolyze. No adverse reactions are observed in rats (20). In a direct comparative study, degrading dextran microspheres showed a similar or decreased tissue reaction when compared with PLG microspheres.

Modified dextran hydrogels: physically cross-linked

The modified dextran hydrogels previously described were designed to be cross-linked by chemical bridges. Physical cross-linking strategies also are possible. De

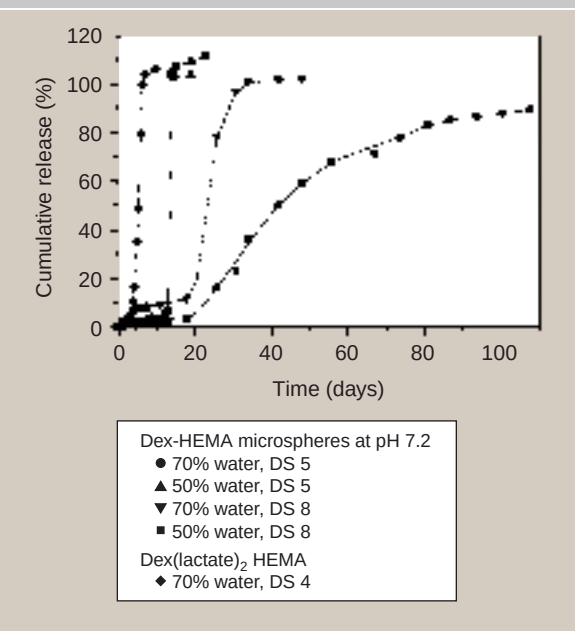

Figure 7: The release of liposomes from dex-HEMA microspheres at pH 7.2 by various formulations and the release of liposomes from dex(lactate)$_2$HEMA. Values are the mean of at least two measurements (19).

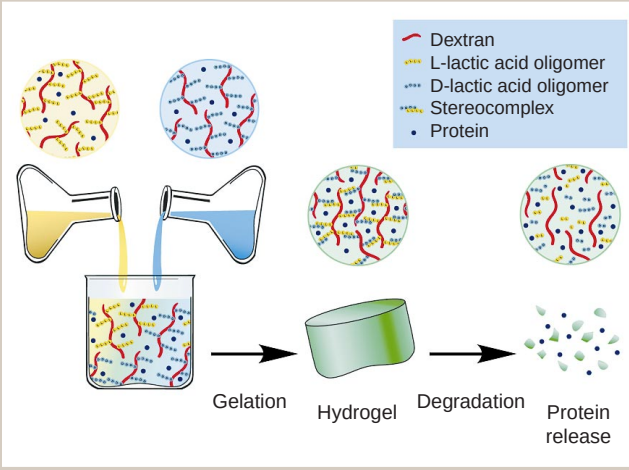

Figure 8: Schematic picture of a hydrogel formation based on cross-linking of dextran chains by oligomeric lactide stereoisomers. The hydrogel is simply obtained after mixing aqueous solutions of dex-L-lactate (L-lactic acid oligomer grafted to dextran) and dex-D-lactate (22).

Desired characteristics for hydrogels to be used for parenteral protein delivery

- Biodegradable with safe degradation products
- Biocompatible in standard tests:
  - Low cytotoxicity
  - No sensitization
  - No lasting histological, morphological, systemic toxic effects
  - No genotoxicity
  - No carcinogenicity
  - No pyrogenicity
  - No immunogenicity
  - BSE-free
- Protein integrity ensured
- High protein loading
- High encapsulation efficiency
- Stable in storage (2 years)
- Release-profile tunable
- Ease in upscaling
- Inexpensive and high-quality material readily available

Jong et al. (21,22) describes an approach in which modified dextran is stabilized as a hydrogel by PLA stereocomplexing in an all-aqueous environment. Lactic acid contains a chiral center. D- and L-lactic acid oligomers can be grafted to dextran. The formed dextran-L-lactic acid and dextran-D-lactic acid polymers can be dissolved in water.

Choosing the appropriate length of the side chains results in a system in which gelation occurs only when the two isomers are mixed. Figure 8 is a schematic illustration of this process. Proteins were mixed with the modified dextran solutions and fully encapsulated in the gels. When they were mixed with excess water, the gels first swelled and then started to degrade into lactic acid and dextran, two degradation products considered to be harmless. The gels fully released the proteins in their active form during one week. Release kinetics depended on the degree of substitution, oligomer chain length, and molecular weight of the protein (22). Again, the proteins are exposed neither to organic solvents during gel formation nor to pH drops during degradation.

Conclusions

Hydrogels are versatile delivery systems for protein delivery. Basically, one can choose from a long list of potential sources (see "Hydrogel material" sidebar). The sidebar "Desired characteristics for hydrogels to be used for parenteral protein delivery" presents the wish list for hydrogels that could be used in parenteral protein-delivery systems. This article focused on two types of hydrogel materials that meet many, if not all of the items on this list: one completely synthetic (PolyActive) and one of a hybrid type, both modified dextrans. These examples offer numerous opportunities for achieving prolonged- and delayed-release patterns, which makes them excellent candidates for improving the performance of a large number of pharmaceutical proteins that lack proper pharmacokinetic and pharmacodynamic properties.

References

1. D.J.A. Crommelin and R.D. Sindelar, "Formulation of Biotech Products, Including Biopharmaceutical Considerations," in *Pharmaceutical Biotechnology: An Introduction for Pharmacists and Pharmaceutical Scientists* (Harwood Academic Publishers, OPA Amsterdam BV, Amsterdam, The Netherlands, 1997).
2. M.C. Manning, K. Patel, and R.T. Borchardt, "Stability of Protein Pharmaceuticals," *Pharm. Res.* **6,** 903–918 (1989).
3. "Physical Methods to Characterize Pharmaceutical Proteins," in *Pharmaceutical Biotechnology,* J.N. Herron, W. Jiskoot, and D.J.A. Crommelin, Eds. (Plenum Press, New York, NY, 1995).
4. D.J.A. Crommelin, E. van Winden, and A. Mekking, "Formulation of Pharmaceutical Proteins," in *Pharmaceutics,* M.E. Aulton, Ed. (Churchill Livingstone, New York, NY, 2002), in press.
5. Genentech (2001), http://www.gene.com/gene/products/nutropin_depot/insert.html.
6. D.M. Cook et al., "Results of Pilot Study: Pharmacokinetics (Pk) and Pharmacodynamics (Pd) of Nutropin Depot (Somatropin [rDNA Origin] for Injectable Suspension) in Adult Growth Hormone–Deficient (AGHD) Men and Women," poster (P3-326) presented at the 83rd annual meeting of the Endocrine Society, Endo2001, Denver, Colorado, 20–23 June 2001.
7. N.A. Peppas et al., "Hydrogels in Pharma-

ceutical Formulations," *Eur. J. Pharm. Biopharm.* **50,** 27–46 (2000).
8. G.M. Zentner et al., "Biodegradable Block Copolymers for Delivery of Proteins and Water-Insoluble Drugs," *J. Controlled Rel.* **72,** 203–215 (2001).
9. J.M. Bezemer et al., "A Controlled-Release System for Proteins Based on Poly(ether ester) Block-Copolymers: Polymer Network Characterization," *J. Controlled Rel.* **62** (3), 393–405 (1999).
10. OctoPlus (2001), http://www.octoplus.nl/pages/technologies_prop-00.html.
11. PolyActive is a trademark of IsoTis, The Netherlands.
12. J.M. Bezemer, et al., "Zero-Order Release of Lysozyme from Poly(ethylene glycol)/Poly(butylene terephthalate) Matrices," *J. Controlled Rel.* **64** (1–3), 179–192 (2000).
13. J.M. Bezemer et al., "Control of Protein Delivery from Amphiphilic Poly(ether ester) Multiblock Copolymers by Varying Their Water Content Using Emulsification Techniques," *J Controlled Rel.* **66** (2–3), 307–320 (2000).
14. W.E. Hennink and C.F. van Nostrum, "Novel Cross-Linking Methods to Design Hydrogels," *Adv. Drug Delivery Rev.* (2001), in press.
15. R.J.H. Stenekes et al., "The Preparation of Dextran Microspheres in an All-Aqueous System: Effect of the Formulation Parameters on Particle Characteristics," *Pharm. Res.* **15,** 557–561 (1998).
16. J.A. Cadée et al., "Oxidation of Recombinant Human Interleukin-2 by Potassium Peroxodisulfate," *Pharm. Res.* **18** (10), in press (2001).
17. O. Franssen, et al., "Chemically Degrading Dextran Hydrogels: Controlled Release of a Model Protein from Cylinders and Microspheres," *J. Controlled Rel.* **60,** 211–221 (2000).
18. J.A. Cadée, "Biocompatible Dextran Hydrogels for the Controlled Release of Interleukin-2 in Cancer Immunotherapy," PhD Thesis, Utrecht University, Utrecht, The Netherlands (2001).
19. R.J.H. Stenekes et al., "Controlled Release of Liposomes from Biodegradable Dextran Microspheres: A Novel Delivery Concept," *Pharm. Res.* **17,** 690–695 (2000).
20. J.A. Cadée et al., "A Comparative Biocompatibility Study of Microspheres Based on Cross-Linked Dextran or Poly(lactic-co-glycolic) Acid after Subcutaneous Injection in Rats," *J. Biomed. Mater. Res.* **50,** 397–404 (2000).
21. S.J. de Jong et al., "Novel Self-Assembled Hydrogels by Stereocomplex Formation in Aqueous Solution of Enantiomeric Lactic Acid Oligomers Grafted to Dextran," *Macromolecules* **33,** 3680–3686 (2000).
22. S.J. de Jong et al., "Physically Cross-Linked Dextran Hydrogels by Stereocomplex Formation of Lactic Acid Oligomers: Degradation and Protein Release Behavior," *J. Controlled Rel.* **71,** 261–275 (2001). **PT**