ELSEVIER

Journal of Controlled Release 61 (1999) 267–279

journal of controlled release

www.elsevier.com/locate/jconrel

Mechanisms controlling diffusion and release of model proteins through and from partially esterified hyaluronic acid membranes

L.D. Simon[a,b,1], V.J. Stella[a], W.N. Charman[b], S.A. Charman[b,*]

[a]*Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS 66045, USA*

[b]*Department of Pharmaceutics, Victorian College of Pharmacy, Monash University, 381 Royal Parade, Parkville, Victoria 3052, Australia*

Received 9 November 1998; received in revised form 8 April 1999; accepted 28 May 1999

Abstract

The effects of polymer percent esterification and protein molecular weight on the diffusion of two model proteins, deoxyribonuclease (DNase) and ribonuclease A (RNase A), through and from partially esterified hyaluronic acid membranes were compared. The permeability of the polymer membranes was inversely related to the degree of polymer esterification and the molecular weight of the protein. Transport rates of proteins through the membranes decreased dramatically over narrow ranges of polymer esterification. As expected, the apparent diffusivity of the larger protein in the polymer matrix was more sensitive to changes in membrane hydration than that of the smaller protein. These observations demonstrated the dependence of the mobility of large molecular weight proteins on polymer hydration and chain relaxation. The relationship between protein diffusion through and release from the modified hyaluronate matrices was also investigated using RNase A as a model. The release profiles from fully esterified membranes showed lag behavior and varied with protein load and hyaluronate hydrolysis rates, while release from less esterified membranes was rapid and independent of polymer esterification or hydrolysis. Potential applications of modified hyaluronate matrices in the controlled delivery of proteins are discussed.

Keywords: Protein release; Protein permeability; Hyaluronic acid esters; Polymer membranes; Protein load

1. Introduction

Research in the area of controlled protein and peptide delivery is continuing to expand due to the increasing availability of proteins for potential therapeutic use. Of particular interest has been the development and characterization of implantable polymeric matrices for the extended release of proteins [1–3]. Such matrices are designed to overcome some of the limitations of the conventional intravenous route of administration by eliminating the need for frequent dosing and reducing undesirable side effects. Both non-biodegradable and biodegradable polymers have been studied for use in implantable devices. While non-biodegradable polymers such as poly(vinyl alcohols) [4], poly(ethylene-co-vinyl ace-

*Corresponding author. Tel.: +61-3-9903-9626; fax: +61-3-9903-9627.

E-mail address: susan.charman@vcp.monash.edu.au (S.A. Charman)

[1]Current address: Department of Pharmaceutics Research and Development, Bristol-Myers Squibb Company, New Brunswick, NJ 08903, USA.

0168-3659/99/$ – see front matter
PII: S0168-3659(99)00123-6

tate) [5], and acrylates [6] are capable of releasing protein over prolonged periods, they have the disadvantage of requiring surgical removal following depletion of their drug load. Therefore, most effort has addressed the development of controlled release devices composed of biodegradable polymers such as polylactide or poly(lactide-co-glycolide) systems [7–9], polyanhydrides [10], and poly(ortho esters) [11].

Much research in the area of controlled drug delivery has focused on the use of polymers to achieve constant or zero-order release of therapeutic agents over extended periods of time. However, there are many applications for which continuous drug release may not be appropriate. For example, the releasing hormones of gonadotropins and growth hormones are most effective when delivered in a pulsatile fashion [12,13]. Other proteins, such as insulin and thrombolytic agents, may show toxicity when administered continuously [6,14]. Controlled release systems designed for pulsatile rather than sustained, constant release are also potentially valuable in the area of vaccine delivery [15] where multiple dose therapy often tends to reduce patient compliance. The rate of protein release from polymer-based delivery systems is generally dependent upon the diffusion rate of the protein through the polymeric network. Examination of the factors influencing the diffusion of macromolecules in hydrated polymers is therefore useful in determining the mechanisms of release from polymeric matrices in order to select the polymer system most appropriate for the intended application.

Hyaluronic acid is a naturally occurring polysaccharide found throughout the body in various tissues including connective tissue, the synovial fluid of joints and the aqueous humor of the eye [16]. Partially esterified hyaluronic acid is currently being evaluated as a potential biodegradable and biocompatible matrix for controlled protein and peptide delivery [17–22]. Preliminary studies of protein diffusion through hyaluronate membranes have suggested that these materials may be very well suited for use as pulsatile-release matrices for macromolecules [22]. The membrane permeability of RNase A has been previously described and was shown to occur primarily through the free water domain of the polymeric matrix, consistent with the ‘free volume’ theory of diffusion developed by Yasuda et al. [23]. The diffusion characteristics of the hyaluronates have indicated that zero-order protein release profiles would be unlikely, but that these polymers could be useful for applications requiring combinations of immediate and delayed release.

In the present study, the dependence of hyaluronate membrane permeation on molecular size of the permeant was evaluated by comparing the membrane diffusion characteristics of RNase A (molecular weight 13.7 kDa), deoxyribonuclease (DNase, molecular weight 31 kDa), and thymidine (Sung et al. [24], reproduced with permission). To investigate release mechanisms, the rate of release of membrane-incorporated RNase A was examined under conditions of variable protein load and polymer esterification and was compared with the rate of protein release from compressed hyaluronate pellets. The relationship between protein release and polymer ester hydrolysis was also determined.

2. Experimental

2.1. Materials

The partial benzyl esters of hyaluronic acid used in these studies are depicted in Fig. 1. Fully esterified and 77% esterified hyaluronic acid (denoted as HA p100 and HA p77, respectively) were supplied by Fidia Advanced Biopolymers, S.p.A (Abano Terme,

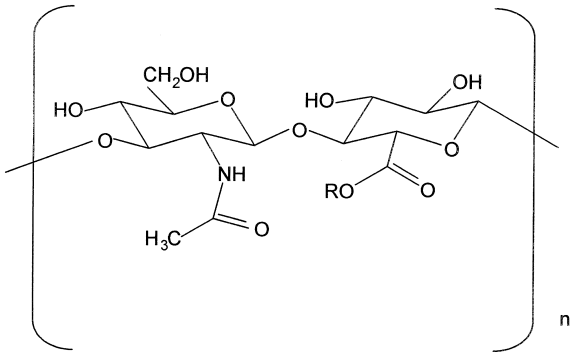

Fig. 1. Structure of the hyaluronic acid (HA) repeating unit, consisting of two sugar subunits: *N*-acetyl glucosamine and D-glucuronic acid. R represents either the benzyl ester or the sodium salt, depending on the degree of esterification (esterification range of 64 to 100%). The nomenclature HA p*X* is used to describe esterified HA where *X* represents the degree of esterification.

Italy). The HA used by Fidia in the preparation of the esterified derivatives was extracted from rooster comb and had an average approximate molecular weight of 160 kDa. Polymers of intermediate and lower percent esterification were prepared by hydrolysis of the HA p100 polymer as described previously [22]. Bovine pancreatic RNase A (Type III-A) and its substrate, cytidine 2′:3′-phosphate, were purchased from Sigma Chemical Company (St. Louis, MO, USA) and were used without further purification. Recombinant human DNase was generously donated by Genentech (San Francisco, CA, USA) and was also used without further purification. All other chemicals were reagent grade and were used as received.

2.2. Validation of DNase stability – exposure to physiological temperature/shear stress

The aqueous stability of DNase upon exposure to physiological temperature (37°C) with continuous shear stress (solution stirring) was evaluated over 120 h using size exclusion chromatography (SEC) and reducing and non-reducing SDS-PAGE. Details of the stability validation experiments have been presented elsewhere [22].

SEC analysis was performed at ambient temperature using a Superose 12 HR 10/30 size exclusion column (Pharmacia LKB Biotechnology, Uppsala, Sweden) with a flow-rate of 1.0 ml/min. Protein was detected by UV absorbance at a wavelength of 278 nm. The mobile phase consisted of 0.1 M phosphate buffer (pH 7.0) containing 0.02% sodium azide. Under these conditions, the retention volume of DNase was 13.2 ml, and the limit of detection was approximately 10 ± 3 μg/ml.

SDS-PAGE was conducted using a FisherBiotech Electrophoresis System (Model FB105; Fisher Scientific, Pittsburgh, PA, USA) and 12.5% SDS-PAGE gels cast from a solution of 30% acrylamide stock, buffer, and water in proportions listed in Table 1. Gels were polymerized overnight using *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) and a 10% solution of ammonium persulfate. The running buffer consisted of 0.0225 M tris buffer, 0.2 M glycine and 0.1% SDS. Following electrophoresis, the gels were stained overnight with Coomassie blue and then destained with a solution of 25% ethanol with 8% acetic acid. The molecular weight of DNase was estimated by use of a calibration curve of low molecular weight (14.4–97.4 kDa) protein standards.

2.3. DNase transport and membrane permeability

Partially esterified hyaluronate membranes of 76% esterification were prepared using a solvent extraction method, and membranes of 64 and 73% esterification were prepared by a membrane hydrolysis method. Dimethylsulfoxide (DMSO) was used as the solvent for these membranes, and a full description of solvent extraction and membrane hydrolysis matrix preparation methods were described previously [22]. The permeability of DNase through these membranes was measured in triplicate using Side-Bi-Side® glass diffusion cells. Prior to each experiment, three membrane disks (1.7 cm diameter) were cut, weighed, and the absence of holes confirmed by light microscopy (100–400× magnification). The dry thickness of each membrane disk was measured using an Ames micrometer ($n = 3$), and the disks were mounted on the diffusion cells, sealing with a thin layer of vacuum grease. A 3 ml solution of 1 mg/ml DNase in 0.01 M phosphate buffer (pH 7.4, $\mu = 0.15$, containing 0.02% sodium azide) was

Table 1
Components used in the preparation of SDS-PAGE gels

Stacking gel		Separating gel	
Component	Volume (μl)	Component	Volume (μl)
Stacking gel buffer	500	Stacking gel buffer	1250
30% acrylamide	350	30% acrylamide	2084
Water	1150	Water	835
10% APS	6	10% APS	18.4
TEMED	4	TEMED	2.5

added to the donor compartment of each diffusion cell, and 3 ml of buffer without protein was added to the receiver compartment. The temperature was maintained at 37°C throughout all diffusion experiments using a thermostated circulating water bath, and both donor and receiver solutions were continuously stirred at 600 rpm by a magnetic stirring console upon which each diffusion cell was mounted. The diffusional surface area of the cells was 0.693 cm^2.

Samples were periodically removed from the receiver solution and replaced with fresh buffer. In order to monitor both protein permeation and polymer benzyl ester hydrolysis rates, each sample was analyzed for both DNase and benzyl alcohol by SEC as described in the previous section. The retention volume of benzyl alcohol using these chromatographic conditions was 36.1 ml. The detector was programmed to change the wavelength from 278 to 205 nm between the elution of the DNase peak and the benzyl alcohol peak during each run. At the end of the experiment, the hydrated membrane thickness was measured.

Protein transport data were analyzed assuming that initial membrane hydration was rapid compared to protein diffusion (confirmed in preliminary studies [22]), that transport through the hydrated membranes occurred through simple (Fickian) diffusion, and that the protein was not degraded during permeation. The apparent permeability coefficient (P_{app}) was calculated using the initial slope of the cumulative mass of protein transported versus time curve and Eq. (1):

$$P_{app} = (D_{app})(K) = \frac{\partial M/\partial t}{(A)(C_d)} * h \quad (1)$$

where D_{app} is the apparent diffusivity of DNase in the hydrated membrane, K is the membrane/buffer partition coefficient, M is the cumulative mass of protein transferred to the receiver solution, A is the diffusional surface area of the membrane (0.693 cm^2), C_d is the protein concentration of the donor solution, and h is the hydrated membrane thickness. The membrane/buffer partition coefficient of DNase into the partially esterified hyaluronate membranes was measured at 37°C using a solution depletion method presented previously [22].

Membrane ester hydrolysis data were analyzed assuming that benzyl alcohol was released only from the hydrated polymer within the diffusional surface area of the membrane in the diffusion cell. The rate of change in membrane percent esterification was calculated based on the mass of exposed hydrated polymer and the rate of benzyl alcohol release.

2.4. Preparation of RNase A-loaded hyaluronate membranes

The solvent extraction and membrane hydrolysis methods used to prepare blank hyaluronate membranes for protein transport studies were not suitable for the preparation of protein-loaded membranes because of the potential for protein loss from the matrix during the solvent extraction process. Therefore, thin partially esterified hyaluronic acid monolithic membranes (blank and RNase A-loaded) were prepared using a solvent evaporation method in which solutions of polymer and protein (protein concentration variable depending on the desired final protein load) in 90% hexafluoroisopropanol (HFIP) in water were cast onto Teflon® petri dishes and slowly air dried. Resulting membranes contained two visually distinct regions, a transparent region in the membrane center and a translucent region around the perimeter. Since the transparent region of HA p100 membranes prepared by solvent evaporation showed similar permeability properties to HA p100 membranes prepared with the solvent extraction method, only the transparent regions of the solvent evaporation membranes were used in these studies. Further details of the membrane preparation method and the characteristics of the resulting membranes are described elsewhere [25]. Thicker membranes were prepared by casting larger volumes of the polymer/protein mixture onto the petri dish. The polymers and solvent compositions used to prepare membranes containing incorporated RNase A are listed in Table 2. Also listed are the percent protein load (determined using a ninhydrin total protein assay [26]) and dry membrane thickness, as measured by an Ames micrometer.

2.5. Preparation of RNase A-loaded compressed hyaluronate pellets

Protein-loaded HA p100 pellets, each composed of a physical mixture of polymer with either 5% or 30%

Table 2
Characterization of esterified hyaluronic acid membranes (HA p100) containing RNase A (mean±SD, $n=3$) prepared by the solvent evaporation method using 90% v/v HFIP and 10% v/v water

Theoretical % protein load (w/w)	Actual % protein load (w/w)	Unhydrated thickness (μm)
5	3.4±0.4	85±8
10	8.6±1.1	77±1
10	8.9±0.3	9±1
20	19.4±0.5	10±0
30	28.8±3.6	16±2
50	52.9±2.4	17±1
60	58.7±0.8	17±1

w/w RNase A, were prepared by direct compression at 8000 lbs for 1 min using a Model 2512 Carver tablet press (Fred S. Carver, Inc., Summit, NJ, USA). The pellets were 1.3 cm in diameter, approximately 0.05 cm thickness, and weighed 80 mg each. Following compression, all pellets were stored in a vacuum desiccator at room temperature over $CaSO_4$ for at least 72 h prior to use.

2.6. Release of RNase A from hyaluronate membranes

The release rates of RNase A from HA p100 and HA p77 membranes were measured in triplicate using Side-Bi-Side® glass diffusion cells as described elsewhere [25]. Samples were periodically removed from each compartment, replaced with fresh buffer, and analyzed for both RNase A and benzyl alcohol by SEC. Analysis was performed as described in Section 2.2 but using a flow-rate of 0.8 ml/min. Under these conditions, the retention volumes of RNase A and benzyl alcohol were 14.5 and 36.1 ml, respectively. The total mass of protein released from each membrane was taken to be the sum of the mass released into each of the two cell compartments. The percentage released was then calculated based on the mass of the membrane, the percent protein load (see Table 2), and the assumption that protein was released only from the hydrated membrane within the diffusional surface area of the cells.

The rate of change in membrane percent esterification during the release experiments was calculated as outlined elsewhere [22], and the integrity of RNase A following release from the membranes was confirmed by assay of enzymatic activity [27].

2.7. Release of RNase A from compressed hyaluronate pellets

The release of RNase A from compressed hyaluronate pellets was measured, in triplicate, by submerging the pellets in 25 ml of 0.01 M phosphate buffer (pH 7.4, $\mu = 0.15$, with 0.02% sodium azide) contained in a sealed bottle. The temperature was maintained at 37°C, and the solutions were agitated using a Model 25 Precision reciprocal shaking water bath (Precision Scientific, Chicago, IL, USA) with shaker speed of 100 rpm. Samples were periodically removed and analyzed for both RNase A and benzyl alcohol by SEC (see Section 2.6). The percentage of protein released from each pellet was calculated based on the mass of the pellet and the percent protein load. The protein load was obtained by SEC upon complete dissolution of the pellet. The rate of change in polymer percent esterification during protein release was calculated based on the weight, protein load, and initial polymer percent esterification of each pellet.

3. Results

3.1. Validation of DNase stability – exposure to physiological temperature/shear stress

No significant changes in the DNase peak area or peak shape were observed in the SEC chromatograms following 120 h of incubation in solution at 37°C under conditions of continuous solution stirring (data not shown). These results indicated that the apparent molecular weight of DNase remained unchanged during the study. Reducing and non-reducing SDS-PAGE confirmed that the protein did not undergo apparent peptide bond hydrolysis or covalent cross-linking. On the basis of these results, all conclusions from the permeability studies were based on the assumption that any undetected chemical alterations (e.g., deamidation, etc.) did not affect the permeability characteristics of DNase over the course of the experiments.

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.