International Journal of Pharmaceutics, 87 (1992) 21–29

IJP 02826

Hyaluronane derivative microspheres as NGF delivery devices: Preparation methods and in vitro release characterization

E. Ghezzo [a], L. Benedetti [a], M. Rochira [a], F. Biviano [b] and L. Callegaro [a]

[a] *Advanced Technology Division and* [b] *Chemistry Department, Fidia Research Laboratories, Via Ponte della Fabbrica 3/A, 35031 Abano Terme (Italy)*

(Received 26 June 1991)
(Modified version received 18 December 1991)
(Accepted 4 March 1992)

Key words: Hyauronane; Biopolymer; Microsphere; Nerve growth factor; Drug delivery

Summary

Nerve growth factor (NGF), a protein which plays an important role in the growth and maintenance of sympathetic and certain sensory neurons, was physically incorporated into four different types of hyaluronane derivative microspheres. In addition, four analogous formulations were prepared, introducing also GM1, a monosialoganglioside which seems to potentiate the neuritic outgrowth induced by NGF (Matta et al., *Devel. Brain Res.*, 27 (1986) 243–252). All the products obtained were analyzed to quantify the amount of incorporated protein and to characterise the in vitro release profiles. It has been demonstrated that the preparation techniques employed were useful for the development of microspheres able to protect, carry and release bioactive polypeptidic molecules in their pharmacologically active form. The protein release profiles were significantly influenced by the physico-chemical properties of the polymeric derivatives used and by the copresence of GM1 which increased the amount of NGF released.

Introduction

Hyaluronane (HA) is a naturally occurring mucopolysaccharide widely found in the fundamental substance of the connective tissue and in the synovial fluid of joints, where it acts as regulator of viscosity, tissue hydration, lubrication and repair (Comper and Laurent, 1978; Varma and Varma, 1983). Chemically, HA is an unbranched long-chain molecule of repeating monomeric units of D-glucuronic acid linked by a β1–3 glucosidic bond to *N*-acetyl-D-glucosamine (Fig. 1); a β1–4 glucosidic bond links the single units.

It has been possible to obtain a large number of hyaluronane derivatives (HYAFF) through an esterification reaction of the free carboxylic function with various therapeutically active or inactive alcohols (Della Valle and Romeo, 1987). They represent a new class of biomaterials, which are biocompatible, biodegradable, non-immunogenic and able to release HA in situ by enzymatic and/or chemical bond cleavage. Depending upon the degree of esterification and the esterifying alcohol, the resulting polymers have different physico-chemical properties and therefore can be selected to meet specific requirements.

Correspondence to: L. Benedetti, Advanced Technology Division, Fidia Research Laboratories, Via Ponte della Fabbrica 3/A, 35031 Abano Terme, Italy.

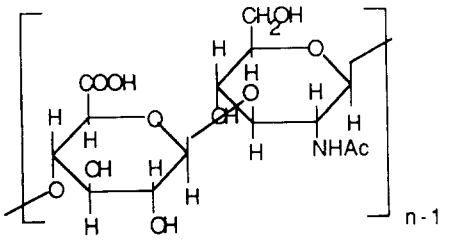

Fig. 1. Monomeric unit of hyaluronane.

Four of these derivatives were studied in the development of a delivery system for proteins and peptides (Table 1). The model protein in the present study was nerve growth factor (NGF), for which we will propose different formulations. NGF presents a dimeric structure composed of two identical peptidic chains linked by non-covalent interactions and performs its activity only in this dimeric form. NGF is known to be essential for the neuronal development and maintenance in the peripheral nervous system (Mobley et al., 1977; Levi-Montalcini, 1987) and acts as a neuronotrophic factor in the mammalian central nervous system (Korsching, 1986; Whittemore and Seiger, 1987).

A role in neural differentiation and repair, in addition to a variety of other physiological activities, has also been ascribed to gangliosides (Schengrund, 1990). Gangliosides are natural components of the plasma membrane of cells in vertebrates and are mainly found in brain tissue (Fishman and Brady, 1976). GM1 (nomenclature recommended by Svennerholm, 1980) belongs to this family of compounds which, due to their neuritogenic and neuronotrophic properties (Leeden, 1984), are used therapeutically to stimulate and accelerate the repair and regeneration of damaged peripheral nerves.

TABLE 1

Hyaluronane derivatives used in the preparation of microspheres

Compound	% esterification	Esterifying alcohol
1 HYAFF 7p75	75	ethyl alcohol
2 HYAFF 7	100	ethyl alcohol
3 HYAFF 11p75	75	benzyl alcohol
4 HYAFF 11	100	benzyl alcohol

In the case of partial esters, remaining groups (25%) are present in the sodium salt form.

In recent years, an increase in research and development of bioactive substances has led to the greater availability of polypeptides and proteins with potential therapeutic applications. Consequently, much research has focussed on solving the complex problems surrounding the delivery of peptides and proteins. One of the main problems during the storage and in vivo administration of protein drugs is to avoid environmental and physiological inactivation and to maintain their biological activity.

In the present study, we describe the methods of preparation HYAFF microscpheres as carriers for both NGF and an NGF/GM1 mixture, in an attempt to design a device able to protect, carry and release protein drugs. A preliminary in vitro release study has been carried out to define, among the polymers and formulations used, the most suitable combination for further investigation.

Materials and Methods

Materials

The four hyaluronane derivatives (**1**, HYAFF 7p75; **2**, HYAFF 7; **3**, HYAFF 11p75; **4**, HYAFF 11) used in the manufacture of microspheres are shown in Table 1. They were supplied in dry powder form by Fidia S.p.A. (Abano Terme, Italy) and were prepared by treating a quaternary ammonium salt of hyaluronane with an esterifying agent in a suitable aprotic solvent at a temperature between 0 and 100 °C (Della Valle and Romeo, 1987).

GM1 was supplied by Fidia Research Laboratories (Fidia S.p.A., Italy).

m-NGF (1 mg/ml in acetate buffer solution) purified from submaxillary mouse glands (Mobley et al., 1976), affinity-purified rabbit polyclonal anti-mβNGF IgG and the monoclonal antibody αD11 (Bigon et al., 1991) were supplied by Fidia Advanced Technology Division (Fidia S.p.A., Italy).

Arlacel ® A (mannide monooleate) and mineral oil (heavy white oil) were purchased from Sigma Chemical Co. (St. Louis, MO).

All the solvents and reagents were analytical grade (Merck, Germany) and were used without further purification.

Microsphere preparation

From each HYAFF polymer three batches of microspheres were prepared: (i) blank microspheres made from the polymer only; (ii) NGF microspheres which contained NGF 0.02% w/w; (iii) NGF + GM1 microspheres which contained NGF 0.02% and GM1 20% w/w with respect to the polymer (the ratio NGF/GM1 was 1:1000 w/w, as recommended by Della Valle et al., 1990).

Microspheres of water-insoluble HYAFF (**2–4**) were produced using the solvent extraction method (Benedetti et al., 1990). An emulsion was prepared in which the internal phase was a 6% w/v polymer solution in dimethylsulphoxide (DMSO) containing the drugs, while the external phase consisted of mineral oil containing 0.5% w/v of the surfactant Arlacel® A. GM1 was mixed with DMSO after previous solubilization in water (its aqueous solubility was 100 mg/ml). The inner phase was added to the outer (their respective ratio was 1:16 v/v) with continuous stirring for 10 min (1000 rpm, RW 20 Kika-Werk instrument). Ethyl acetate, the extraction solvent, was then added to the emulsion at a ratio of 2:1 v/v. The extraction proceeded for 15 min at a reduced stirring rate (700 rpm), until the extraction was complete and the microparticles were formed. The suspension was then filtered and extensively washed with *n*-hexane. After drying under vacuum, the microspheres were examined using a scanning electron microscope (SEM 505 Philips) to confirm the morphological characteristics. In addition, a light scattering particle analyzer (Mastersizer Malvern) was used to study the size characteristics and the homogeneity of the powder, which was suspended in ethyl acetate.

Aqueous solutions of HYAFF 7p75 (**1**) in the different formulations proposed were processed in a spray drier (Büchi model 190 apparatus) under the following experimental conditions: inlet temperature, 98–120 °C; outlet temperature, 45–48 °C; flow rate, 0.3 l/h. The resulting microspheres were submitted to the same preliminary morphological analysis as described above.

Determination of GM1 content

The total amount of ganglioside incorporated into the microspheres was determined using HPLC quantitative analysis. A standard solution of GM1 was treated similarly to the microsphere samples in order to examine the possibility of drug degradation during the analysis. 10 mg samples of compounds **2–4** were dispersed separately in 15 ml of 0.02 N NaOH and stirred (100 rpm, Certomat R instrument) at 37 °C. Under these conditions the polymer was completely hydrolyzed, releasing all of the incorporated GM1.

The amount of GM1 in the HYAFF 7p75 particles was determined by first dissolving the polymer in phosphate buffer solution (pH 7.4, $\mu = 0.5$ M).

The HPLC equipment consisted of the following components (Water Associates): Wisp model 712 sample processor; model 510 two pumps; automated gradient controller; model 484 tunable absorbance UV detector. A Hewlett Packard HP3369A integrator was used to record the retention times and to determine the integrated UV absorbance peak areas.

A reversed-phase Viosfer C8 column (5 μm, 150 × 4.6 mm, Violet, Rome) was used, operating in isocratic elution mode at room temperature. The mobile phase was a mixture 70% acetonitrile and 30% phosphate buffer (5 mM, pH 7.4), with elution at a constant flow rate of 1 ml/min. The wavelength for GM1 detection was 200 nm. Solvents employed for the analysis were HPLC grade, while buffers were prepared with Milli-Q water and degassed prior to use.

In vitro GM1 release

A dissolution test was performed for all the microsphere formulations.

Accurately weighed amounts of microspheres were dispersed in phosphate buffer (pH 7.4 μ = 0.5 M) and kept at 37 °C under continuous stirring (100 rpm, Certomat R instrument). At fixed time intervals after the start of the experiment,

samples of the solutions were removed and the volume was readjusted with the buffer solution.

The quantitative determination of GM1 in the collected fractions was carried out using the HPLC method, by measuring the peak area and comparing with a calibration curve of known standards.

NGF content of determination

The NGF content of the microspheres was determined using a specific ELISA method (Soranzo et al., 1991). The NGF content of the hydrophobic formulations was determined after solubilization of the samples in DMSO, and was measured using aqueous solutions of particles of HYAFF 7p75.

Affinity-purified IgGs against mouse βNGF, able to detect only the dimeric bioactive form of NGF, were absorbed onto microtiter plates. The organic solutions were loaded in the ELISA plate wells and serially diluted 1:2 eight times, using a solution of phosphate-buffered salts, containing 1% bovine serum albumin (BSA) and 0.05% v/v of the surfactant Tween 20. Two linear dilutions of NGF standard solution were also loaded onto the plates. The second antibody αD11 was applied after 2 h. The resulting antibody complex was detected using goat anti-rat immunoglobulins (H + L chains), labelled with horseradish peroxidase (HRP) (KpL, Maryland). Enzyme activity was quantified by measuring the change in absorbance at 492 nm using a cromogen substrate (Sorin Biomedica, Italy). The absorbance values were measured using an ELISA Titertek reader (Flow Laboratories).

In vitro NGF release

Accurately weighed samples of each microsphere formulation were incubated in phosphate buffer (pH 7.4, $\mu = 0.5$ M) at 37 °C with stirring at 100 rpm (Certomat R instrument). At fixed time intervals up to 24 h, the supernatant liquid was completely removed after microsphere sedimentation and NGF was analyzed by ELISA.

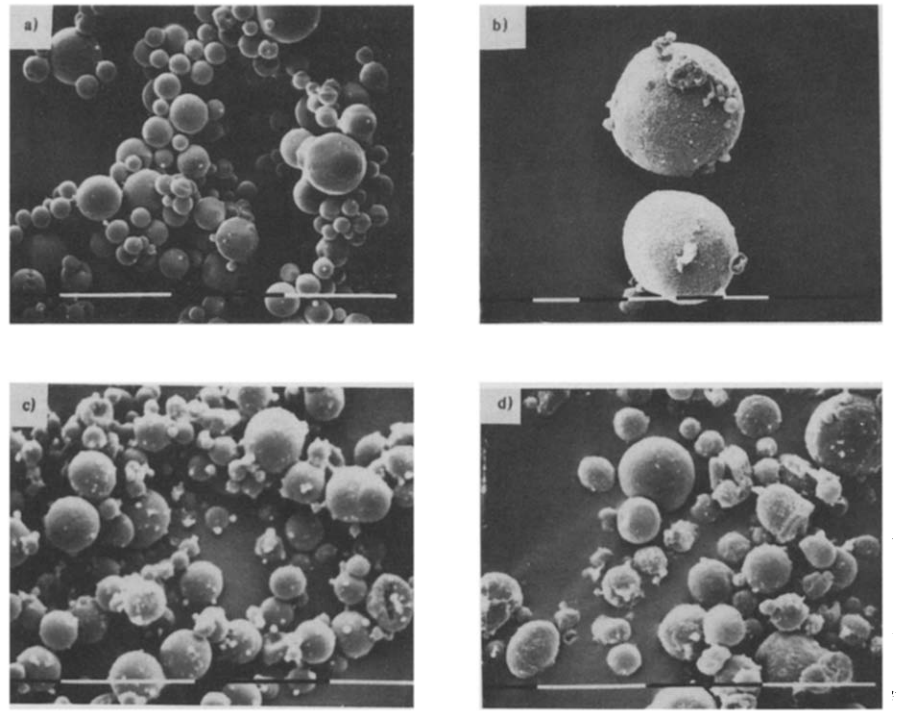

Fig. 2. Electron micrographs of microspheres loaded with NGF, whose matrices are: (a) HYAFF 7p75; (b) HYAFF 7; (c) HYAFF 11p75 and (d) HYAFF 11. White bar represents 10 μm.

After the last sample the NGF content of the microparticle pellets was also determined after DMSO solubilization. The quantity of NGF released was expressed as a percentage of the NGF content of the microspheres, determined as described above.

In order to study the extent of NGF degradation during the in vitro assay, a standard solution of the protein was incubated under the same analytical conditions and the growth factor was analyzed using the ELISA method. The values obtained were considered to be equivalent to the maximum concentrations detectable in the release test.

In addition, a test was performed in order to investigate the possibility of ganglioside interference in the ELISA determination of NGF. The ELISA results for a standard solution of NGF in phosphate buffer were compared to those for a similar solution containing NGF and GM1, at a ratio of 1 : 1000, respectively.

Results and Discussion

The SEM micrographs of the particles and their size distribution profiles, for each type of HYAFF used, are reported in Figs 2 and 3.

Microspheres of HYAFF 7p75, produced with the spray drier instrument, were the most regular in shape and dimension, being perfectly spherical, with smooth surfaces and giving rise to the narrowest size distribution curve.

Microspheres were also obtained from the other three HYAFF derivatives, as shown in Fig. 2, but some surface irregularities were evident under high magnifications. The analysis of size distributions indicated that their dimensions spanned a wider range than that of the water-soluble microspheres. The average sizes found were similar for all the samples (2–10 μm), except for the HYAFF 7 particles in which the mean diameter was considerably larger (21 μm).

The percentage of GM1 successfully loaded

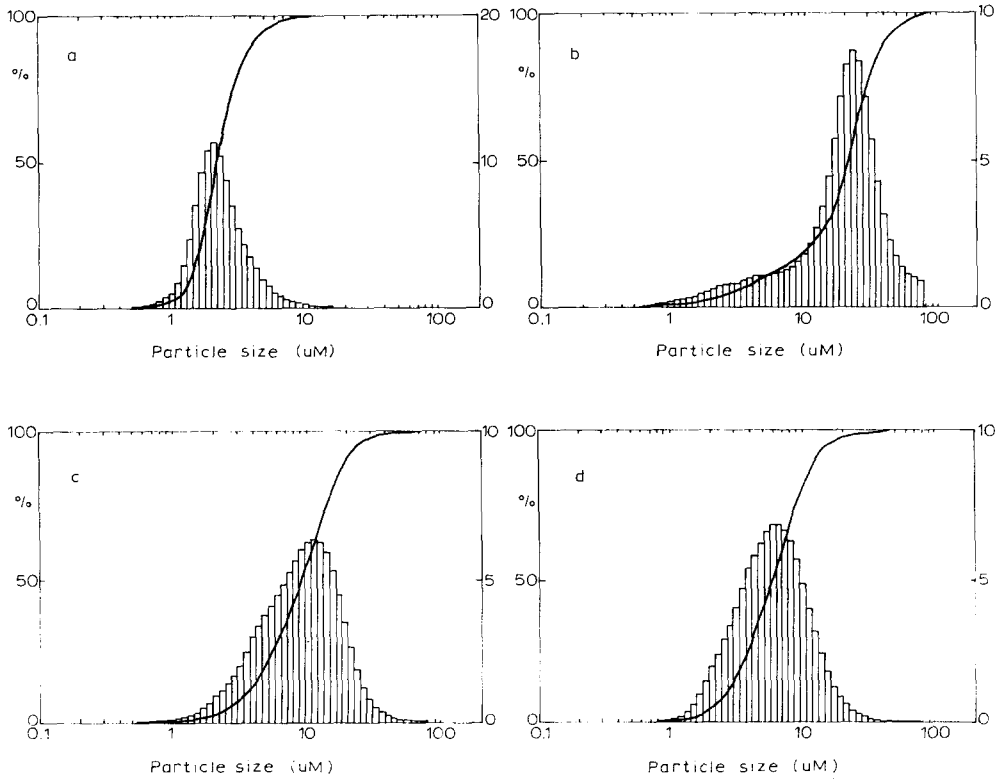

Fig. 3. Particle size distributions of microspheres loaded with NGF and GM1, whose matrices are: (a) HYAFF 7p75; (b) HYAFF 7; (c) HYAFF 11p75 and (d) HYAFF 11.

into the particles is listed in Table 2. The incomplete incorporation observed for the formulations prepared using the extraction method is due to loss of the ganglioside during extraction with ethyl acetate, as proved by HPLC analysis of the GM1 content in the organic solvent.

The molecule of GM1 is physically incorporated into the particles and, because of its high water solubility, the rate of release in aqueous media is mainly dependent on the hydration of the polymers (Hunt et al., 1990). The GM1 release profiles of the four formulations under study are shown in Fig. 4. Whereas complete recovery of ganglioside incorporated into HYAFF 7p75 microspheres was observed 1 h after dissolution, the release from the suspensions of the water-insoluble formulations reached a plateau value after 2 days. For the latter formulations, we demonstrated a small difference among the HYAFF derivatives **2–4**, which correlates with their hydrophobicity, increasing from HYAFF 11p75 to HYAFF 7 and HYAFF 11, respectively (Hunt et al., 1990). The release of GM1 was not strongly influenced by the slight difference in the microsphere dimensions, but was affected more by the hydrophobic/hydrophilic properties of the polymer matrix. It should be noted that in the control experiment no significant degradation of the standard GM1 solution was observed under the assay conditions used.

TABLE 2

Loaded amounts of GM1 (mg / 100 mg of microspheres) and NGF (μg / 100 mg of microspheres) resulting from the content evaluation analysis

Formulations	Active components	
	% NGF	% GM1
HYAFF 7p75 + NGF	53	
HYAFF 7p75 + NGF + GM1	35	100
HYAFF 7 + NGF	21	
HYAFF 7 + NGF + GM1	26	78
HYAFF 11p75 + NGF	20	
HYAFF 11p75 + NGF + GM1	25	76
HYAFF 11 + NGF	20	
HYAFF 11 + NGF + GM1	39	79

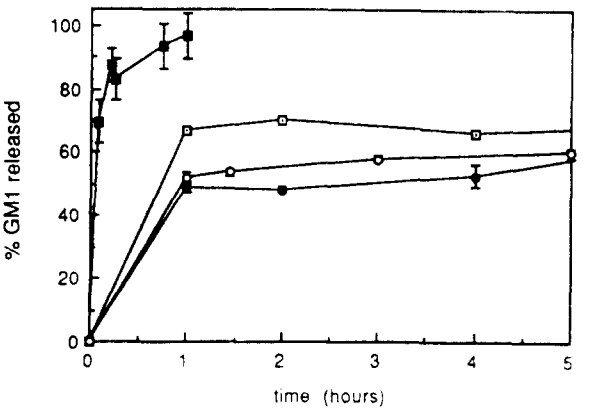

Fig. 4. In vitro GM1 released from the four microsphere formulations: (■——■) HYAFF 7p75; (○——○) HYAFF 7; (⊡——⊡) HYAFF 11p75 and (●——●) HYAFF 11. The analyses were performed in phosphate buffer (pH 7.4, μ = 0.5 M at 37 °C.

Interestingly, we recovered the polypeptide molecule of NGF both from an aqueous solution of microspheres of compound **1**, which were subjected to high temperatures during their preparation, and after DMSO solubilization of the particles from the matrices **2–4**, whose production involved contact between NGF and organic solvents. The amounts of NGF recovered from the microsphere formulations are listed in Table 2.

However, using both approaches, in the determination of NGF content, a considerable loss of protein was observed during the ELISA measurements. There are several possible explanations for this protein deficit. Firstly, the immunoassay was not able to detect NGF when present in a non-dimeric form. For example, a strong reduction (−50%) in the recorded absorbance values of standard NGF was evident after dissolution in DMSO instead of in 1% BSA solution. This behaviour may be ascribed to protein unfolding in the organic medium. Secondly, the experimental conditions required for microsphere preparation, as described above, such as the use of organic solvents and the high process temperatures, may cause some denaturation, even when these steps are performed very quickly. Thirdly, HYAFF matrices and NGF were solubilized in DMSO in glass containers. It is a well-known phenomenon (Sato et al., 1983) that denatured proteins can

adsorb onto the surface of glass and plastic materials and thus escape detection by the employed techniques. Finally, polypeptidic chains may interact with the hydrophobic HYAFF polymers and these noncovalent interactions may hinder the release of free protein into the medium.

Despite the limitations of the ELISA method, due to the low NGF concentration in the microspheres, it was the only useful assay for the dissolution test samples covering a range between 0.5 and 25 ng/ml.

In an alternative experiment HPLC was used to detect the monomeric form of NGF and NGF fragments, using high microsphere concentrations in phosphate buffer (50 ng/ml). However, this assay failed, because at this high concentration of particles, the polymers interfered with the detection of the polypeptide fragments and the protein.

A release study was also carried out using a 1% BSA solution instead of phosphate buffer, in an attempt to prevent NGF adsorption to glass surfaces, however, the recovery of the growth factor was not improved.

Despite these problems, it was remarkable that the fraction of protein determined was present in such devices in its biologically active conformation. This was shown by a PC-12 cell test (still in progress in Fidia Laboratories) as well as by the ELISA method used in the present study, which identifies only the dimeric form of NGF. Furthermore, during the period of our experiments (almost 1 year), our formulations preserved NGF from degradation: microspheres did not need particular storage precautions to be taken, but were kept at 4 °C in normal glass flacons. The prolonged stability of NGF in the microsphere devices is due to the physical entrapment of the protein in a solid structure, which provides an anhydrous environment, protection from atmospheric oxidation, and allows preservation of the intact structure of the protein which, after release, regains its activity.

A peculiar behaviour regarding NGF release was observed with respect to the presence/absence of GM1 in the same formulation and to the physico-chemical properties of the polymer employed.

In microspheres of partially esterified hyaluronane derivatives, NGF was detectable in both cases, being either the singly incorporated drug or in combination with GM1. No interference of GM1 with the ELISA determination of NGF was observed. The absorbance of standard solutions was found to be independent of the correspondence of GM1. In the case of HYAFF 7p75 derivatives, it was difficult to define a trend of release, because of the rapid solubilization of the matrix in the buffer; the total amount of protein incorporated was recovered within 1 h (data not shown).

For the formulations prepared with the other three polymeric derivatives, the protein release rate was observed to be slower.

With the HYAFF 11p75 samples, rapid initial release of NGF was found, which correlated with the rapid hydration of the polymer matrix and subsequent protein dissolution followed by the diffusion of the molecules out of the microspheres. In addition, the presence of GM1 significantly increased the rate and extent of NGF release (Fig. 5a).

The promotion of NGF release by GM1 may be due to the fact that it represents a considerable percentage of the microspheres. Consequently, as the ganglioside is solubilized in the phosphate buffer, the fraction of NGF surrounded by GM1 also diffuses in the medium. Furthermore, after solubilization and release of GM1 through microchannels in the polymer matrix (Langer, 1981), the surface area of the polymer exposed to further hydrolysis and swelling is increased, giving rise to a greater release of the protein.

Microspheres of HYAFF 7 and HYAFF 11, which have totally esterified matrices, behave in a similar way in terms of NGF delivery (Fig. 5b and c). As previously observed for GM1, they give rise to a slower and reduced release of protein, which is more prolonged than for the partially esterified devices. The release trends are well characterized only for the formulations containing GM1, as otherwise the hydration rate is very slow and modest, and only traces of NGF are detectable.

In all of the microsphere pellets obtained after sample centrifugation at the end of the release

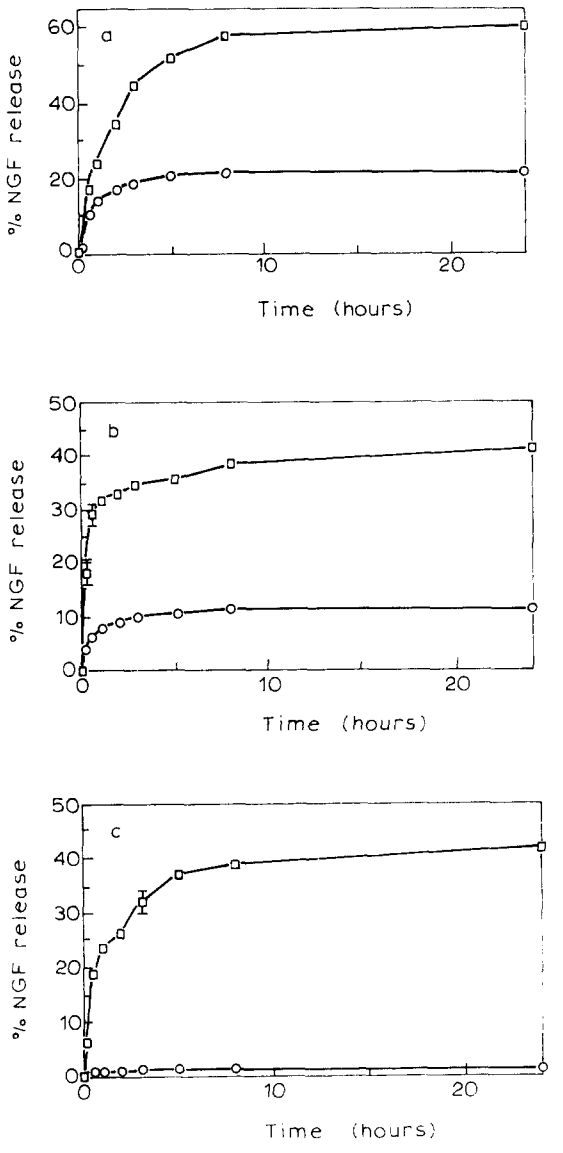

Fig. 5. In vitro NGF released from microsphere formulations, whose matrices are: (a) HYAFF 11p75; (b) HYAFF 11 and (c) HYAFF 7. (□——□) NGF + GM1 formulations; (◇——◇) NGF alone. The analyses were performed in phosphate buffer (pH 7.4, $\mu = 0.5$ M) at 37 °C.

studies, 4–11% of the total amount of NGF incorporated within the particles was recovered. This data indicates that the rate of release of the protein from the polymers, even if independent of chemical bond cleavage, is nevertheless affected by the physico-chemical protein/matrix interactions.

Conclusions

Both solvent extraction and spray drying methods were suitable techniques for the entrapment of NGF in biocompatible, biodegradable polymeric microspheres.

NGF incorporated into such devices maintained its natural conformation for a long period of time, thus avoiding the problems of storage of simple solutions of the protein.

Of the hyaluronane derivatives tested in the present study, HYAFF 7p75 was the only polymer unsuitable for the development of sustained delivery devices for polypeptide and protein drugs. For the other three matrices, we were able to obtain suitable release profiles which differed from one another according to the hydrophobic/hydrophilic properties of the polymer and to the presence or absence of the ganglioside GM1.

These results demonstrate the potential of different HA derivatives for the production of delivery devices not only for the two drugs tested, but also for other potentially active polypeptidic derivatives. Using specific derivatives of hyaluronane biopolymers and by controlling the particle dimensions, it may be possible to modify the release time of incorporated drugs.

An obstacle which has not yet been overcome is the incomplete recovery of the protein during the release tests. Modifications of the experimental conditions used in the present work are currently under investigation in order to obtain a complete protein balance.

Acknowledgements

We would like to thank Drs Julie L. Richardson and Giovanna Ferrari for reading the manuscript and providing constructive criticism.

References

Benedetti, L.M., Topp, E.M. and Stella, V.J., Microspheres of hyaluronic acid esters - fabrication methods and in vitro hydrocortisone release. *J. Controlled Release*, 13 (1990) 34–41.

Bigon, E., Soranzo, C., Minozzi, M.C., Skaper, S.D. and Callegaro, L., Large scale purification and immunological characterization of human placental nerve growth factor. *J. Neurochem. Res.*, 15 (1990) 1197–1202.

Comper, W.D. and Laurent, T.C., Physiological function of connective tissue polysaccharides. *Physiol. Rev.*, 58 (1978) 255–315.

Della Valle, F. and Romeo, A., Hyaluronic acid esters and their medical and cosmetic uses and formulations. *Eur. Patent Appl. EP 0433113*, June 1991.

Della Valle, F., Callegaro, L., Leon, A. and Romeo, A., Stabilization and maintenance of the human nerve growth factor's biological activity by use of natural gangliosides or derivatives thereof. *Eur. Patent Appl. EP 0433113*, June 1991.

Fishman, P.H. and Brady, R.O., Distribution and function of gangliosides, *Science*, 194 (1976) 906–915.

Hunt, J.A., Joshi, H.N., Stella, V.J. and Top, E.M., Diffusion and drug release in polymer films prepared from ester derivatives of hyaluronic acid. *J. Controlled Release*, 12 (1990) 159–169.

Korsching, S., The role of nerve growth factor in the CNS. *Trends Neurosci.*, 9 (1986) 570–573.

Langer, R., Polymers for the sustained release of macromolecules: their use in a single-step method of immunization. *Methods Enzymol.*, 73 (1981) 57–75.

Leeden, R.W., Biology of gangliosides: neuritogenic and neuronotrophic properties. *J. Neurosci. Res.*, 12 (1984) 147–149.

Levi-Montalcini, R., The nerve growth factor 35 years later. *Science*, 237 (1987) 1154–1162.

Matta, S.G., Yorke, G. and Roisen, F.J., Neuritogenic and metabolic effects of individual gangliosides and their interaction with nerve growth factor in cultures of neuroblastoma and pheochromocytoma. *Devel. Brain Res.*, 27 (1986) 243–252.

Mobley, W.C., Schenker, A. and Shooter, E.M., Characterization and isolation of proteolytically modified nerve growth factor. *Biochemistry*, 15 (1976) 5543–5552.

Mobley, W.C., Server, A.C,. Ishii, D.N., Riopelle, R.J. and Shooter, E.M., Nerve growth factor. *New Engl. J. Med.*, 297 (1977) 1149–1158.

Sato, S., Ebert, C.D. and Kim, S.W., Prevention of insulin self association and surface adsorption. *J. Pharm. Sci.*, 72 (1983) 228–232.

Schengrund, C.-L., The role(s) of gangliosides in neuronal differentiation and repair: a perspective. *Brain Res. Bull.*, 24 (1990) 131–141.

Soranzo, C., Martini, I., Bigon, E., Callegaro, L., Cazzola, F., Corona, G., Skaper, S.D. and Negro A., Synthesis and expression of recombinant human nerve growth factor in a mammalian cell line. In Lenfant C., Paoletti R. and Albertini A. (Eds), *Growth Factors of the Vascular and Nervous Systems. Int. Symposium on Biotechnology of Growth Factors, Milan, 1991*, Karger, Basel, 1992, pp. 72–79.

Svennerholm, L., Ganglioside designation. *Adv. Exp. Med. Biol.*, 125 (1980) 11–19.

Varma, R. and Varma, R.S., *Mucopolysaccharides – Glycosaminoglycans of Body Fluids in Health and Disease*, De Gruyter, New York, 1983, pp. 12, 107–109.

Whittemore, S.R. and Seiger, A., The expression, localization and functional significance of b-nerve growth factor in the central nervous system. *Brain Res. Rev.*, 12 (1987) 439–464.