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Lipid-based microtubular drug delivery vehicles

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Abstract

Lipid microtubules that self-assemble from a diacetylenic lipid are suitable structures for the sustained release of bioactive agents. Microtubules were loaded with agents under aqueous conditions and embedded in an agarose hydrogel for localization at areas of interest. Protein release from our microtubule–hydrogel delivery system was characterized *in vitro*, and *in vivo* biocompatibility was examined. The influences of protein molecular weight and initial loading concentration on release profile were evaluated by releasing test proteins myoglobin, albumin, and thyroglobulin. Protein molecular weight inversely affected the release rate, and loading with a higher protein concentration increased the mass but not the percent of initially loaded protein released daily. Preservation of protein activity was demonstrated by the ability of a neurotrophic factor released from the delivery system to induce neurite extension in PC12 cells. Bovine aortic smooth muscle cells co-cultured with the microtubule–hydrogel system showed no evidence of cytotoxicity and proliferated in the presence of the microtubules. Subcutaneous implantation of microtubules in rodents revealed no significant inflammatory response after 10 days. Our microtubule–hydrogel system is useful for applications where sustained release without contact between agent and organic solvents is desired. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Advances in the fields of molecular and medical science have identified numerous proteins and genetic agents with therapeutic potential. Drug delivery systems currently available to release these therapeutic agents include lipid-based systems such as liposomes [1,2] or cochleates [3], polymer-based systems such as ethylene–vinyl acetate copolymer [4–6], and

biodegradable systems such as polylactides [7,8]. However, these systems often involve exposing the therapeutic agent to organic solvents such as dichloromethane [9,10] or methylene chloride [11,12] which can easily denature the tertiary structures of sensitive proteins and genetic materials [13]. While organic solvents have been used to study proteins in partially or completely denatured states [14–16], this characteristic is not desirable for drug delivery. In this study we describe a microtubule–hydrogel system consisting of lipid microtubules embedded in agarose hydrogels for the slow release of proteins. Lipid microtubules provide the slow release com-

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ponent whereas the hydrogel localizes the microtubules at the desired site by preventing their dispersion.

Lipid microtubules made from the diacetylenic lipid 1,2-bis(tricosano-10,12-diyne)-*sn*-3-phosphocholine (DC_{8,9}PC) were initially described by Yager and Schoen [17]. Microtubules are hollow and open-ended tubules with a lumen diameter of approximately 0.5 μm [18] and walls formed by one or more lipid bilayers. They form spontaneously while passing through a phase transition temperature during a controlled cooling process. The chiral interactions between lipid molecules cause the bilayer to twist and form a tubular structure [18,19]. Since the chiral packing of the molecules is very structured, the walls are highly ordered, and release of agent from within the lumen occurs via the two ends of each microtubule [20].

Lipid microtubules have been shown to provide the sustained release of proteins such as transforming growth factor- β [21] and antifouling agents including tetracycline [20] and 2-methoxynaphthalene [22]. Rudolph et al. [23] found that microtubules have no significant effect on the *in vitro* proliferation of U937 histocytic monocytes, K52 erythroblasts, or Jurkat derivative lymphoblasts.

To enable localization of microtubules at a particular location, microtubules were embedded in SeaPrep[®] agarose hydrogel, a polysaccharide derived from red algae that melts at 50°C and gels at 7–18°C. At low concentrations (1–2% w/v), this thermoreversible hydrogel is very porous [24]. With an average pore size less than 1 μm , SeaPrep allows small molecules such as proteins to diffuse through the hydrogel without allowing the physical movement of the microtubules away from the implant site [25].

To characterize our microtubule–hydrogel system, microtubules were loaded with proteins of increasing molecular weight, myoglobin, albumin, and thyroglobulin, to determine the influence of molecular weight and initial loading concentration on the release profile of the microtubules. A PC12 cell-based bioassay for nerve growth factor (NGF) was used to assess bioactivity of growth factor released from the system. Biocompatibility was investigated *in vitro* by a bovine aortic smooth muscle cell

(BASMC) co-culture model and *in vivo* in a rodent model of subcutaneous implantation.

2. Materials and methods

2.1. Lipid microtubule fabrication

Microtubules were fabricated using a method adapted from Spargo et al. [21]. Briefly, 10 mg DC_{8,9}PC lipid (Avanti Polar Lipids, Alabaster AL, USA) were dissolved in 7 ml of 100% ethanol at 55°C, and 3 ml sterile, deionized water (55°C) were added. The solution was kept at 55°C for 6 h, cooled to 25°C at 2.5°C/h, reheated to 33°C, and again cooled to 25°C in a microprocessor-controlled refrigerated water bath (Neslab RTE-111, Neslab Instruments, Newington NH, USA). The microtubules were stored at room temperature until further use.

To evaluate length, an aliquot of microtubules was placed on a glass slide and covered with a glass coverslip to create a single layer of microtubules. The microtubules were imaged using a Nikon Eclipse TE300 light microscope (Nikon, Tokyo, Japan) connected via a Javelin CCD camera (JE7862, Javelin Systems, Torrance CA, USA) to a desktop computer running public domain imaging software, NIH Image 1.61. This software was used to trace the length of at least 250 microtubules and to capture microtubule images.

The yield of microtubules was calculated by placing an aliquot of a known dilution of microtubules on a glass slide as above. Using the light microscope, the number of microtubules in at least 10 fields of view was counted and used to calculate the number of microtubules formed from 1 mg of lipid.

At the time of usage, the cryoprotectant trehalose (Sigma, St. Louis, MO, USA) [26,27] was added to the microtubules at a final concentration of 50 mM to preserve the lipid structures during the drying process. Briefly, the microtubules were centrifuged (1500 g, 5 min), the supernatant was removed, 0.2 ml sterile deionized water was added, and the microtubules were rotary evaporated until mostly dry. The microtubules were then dried under air for 24 h. Typically, 10 mg of dry microtubules were

loaded by adding 400 μl of phosphate buffered saline (PBS, control) or a concentrated solution of protein dissolved in PBS.

2.2. Effect of protein molecular weight

Lipid microtubules were loaded with 0.45 mM myoglobin (17.8 kDa, Sigma), albumin (66.4 kDa, Sigma), or thyroglobulin (660 kDa, Sigma) by adding 200 μl of protein solution to 5 mg of dry microtubules. These proteins were chosen for the molecular weight range they represent and for their high solubility in aqueous solutions. Control microtubules were loaded with PBS. Extra-tubular protein was diluted by adding 5 ml PBS, and removed by centrifuging at 400 g for 10 min and discarding the supernatant.

To embed the microtubules in the hydrogel, the microtubules were resuspended in room temperature PBS in a total volume of 0.2 ml. A 0.4 ml aliquot of 1.5% (w/v) SeaPrep agarose hydrogel (FMC Bioproducts, Rockland ME, USA) solution equilibrated to 37°C was added to the tubule solution for a final concentration of 1% agarose. The resulting suspension was gently mixed until the microtubules were uniformly distributed throughout the hydrogel, and the mixture was gelled in a flat-bottomed vial at 4°C for 30 min. Triplicate vials were prepared for microtubules loaded with each of the following: PBS (control), 0.45 mM myoglobin, 0.45 mM albumin, and 0.45 mM thyroglobulin.

To each microtubule–hydrogel vial, 1.2 ml of PBS was added, and the vials were incubated at 37°C. Every 24 h, the PBS was removed and replaced with 1.2 ml of fresh PBS. Using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules CA, USA), the amount of protein released from the microtubule–hydrogel delivery system during each 24 h period was quantified. Absorbance readings were taken at 595 nm using a Shimadzu UV-1601 UV-spectrophotometer (Shimadzu, Kyoto, Japan) and compared to a standard curve specific to each protein. From these data, a release profile over 5 days was generated. Results represent an average of six data points from two separate experiments.

The percent release of initially loaded protein was calculated by dividing the amount released each day

by the total protein loaded. Total protein loaded was determined by multiplying the number of microtubules in the sample, the average inner volume per microtubule, and the concentration of the loading solution. To verify the total amount of protein loaded, albumin-loaded microtubules were sonicated for 3.5 min on ice to break apart the tubular structures and release entrapped protein. The protein assay was then used to determine the amount of protein actually loaded. PBS-loaded microtubules were used as a negative control.

2.3. Effect of protein loading concentration

Triplicate vials of microtubules were loaded with PBS, 0.45, 1.51, and 2.27 mM (3, 10, and 15% w/v, respectively) albumin, and release profiles were generated over a period of 10 days and analyzed by methods described in the previous section. At least two identical, independent experiments were performed and combined for analysis.

2.4. Contribution of hydrogel

To differentiate between the effect of the hydrogel and the effect of the lipid microtubules, hydrogel samples were prepared with albumin but without lipid microtubules. The mass of albumin used, 0.6, 2.0, and 3.0 mg, corresponded to the amount of albumin loaded in the microtubules for a 0.45, 1.51, and 2.27 mM albumin loading concentration, respectively. The albumin was placed in a total volume of 200 μl of PBS and mixed with 400 μl of 1.5% SeaPrep agarose hydrogel. These mixtures were made and gelled in triplicate, and release profiles were generated as described for the protein-loaded microtubule–hydrogels. Protein release was quantified every 24 h for the first week and then every 7 days for the next 3 weeks.

2.5. Post-release bioactivity assay

Dried microtubules (10 mg lipid) were loaded with mouse NGF (2.5S, Alomone Labs, Jerusalem, Israel) by hydrating the microtubules with 400 μl of 25 $\mu\text{g}/\text{ml}$ NGF solution. Unincorporated NGF was removed from the sample by three exchanges of the

supernatant with PBS after centrifugation (1500 g, 5 min). Control microtubules were loaded with PBS. Loaded microtubules were placed in 1 ml PBS and incubated at 37°C. Every 24 h, the microtubule solution was centrifuged (1500 g, 5 min), the supernatant was removed, and 1 ml fresh PBS was added. After 7 days of release, the microtubules were added to a 1.25% (w/v) SeaPrep agarose hydrogel solution to obtain a final agarose gel concentration of 1%.

NGF-primed PC12 cells were washed free of exogenous NGF using PBS and plated in a 48-well tissue culture plate, and 100 µl of hydrogel solution containing NGF-loaded lipid microtubules were added to triplicate wells. Triplicate control wells received 100 µl of hydrogel containing lipid microtubules loaded with PBS without NGF. The solutions were gelled at 4°C for 15 min. Cell growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL Products, Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco) was added (1 ml per well), and the cells were cultured at 37°C with 95% humidity and 5% CO₂.

After 48 h, neurite extension was evaluated by counting the number of cells extending neurites greater than one cell diameter in wells incubated with NGF-loaded microtubules and wells incubated with PBS-loaded microtubules. Cell morphology was examined, and images were captured using the light microscopy imaging system described earlier.

2.6. Co-culture with BASMC

Lipid microtubules (10 mg) were dried in a rotary evaporator, rehydrated with 400 µl PBS, and aliquoted into 10 samples of 40 µl each. As with protein-loaded microtubules, these microtubules were washed by adding 1 ml sterile PBS to each sample and centrifuging (7800 g, 1 min). The supernatant was removed, leaving the microtubules in 20 µl of PBS.

To each microtubule pellet, 80 µl of sterile 1.25% SeaPrep agarose solution was added. Control hydrogels were prepared by adding 80 µl of agarose solution to 20 µl sterile PBS without any microtubules. Hydrogel solutions were gelled at 4°C for 30

min. After gelation, 1 ml sterile PBS was added to each hydrogel, and they were stored at 37°C for 3 h.

BASMC were isolated as previously described [28], plated onto Costar[®] 24-well tissue culture plates (Corning Inc., Corning, NY, USA, 12,000 cells per well, passages 5–15), and cultured at 37°C, 5% CO₂, and 95% humidity in 0.5 ml cell growth medium (DMEM, 10% FBS, and 1% penicillin/streptomycin). Once the cells reached 40–60% confluence, the growth medium was replaced with fresh growth medium, and hydrogels with and without lipid microtubules were added to triplicate wells. Control wells received only fresh growth medium.

On the fourth day of culture, morphology was observed under light microscopy, and the number of BASMC in each well was determined by counting cells in three representative viewing frames using our image analysis system. Three separate experiments were performed, and the data were pooled for analysis.

2.7. Rodent implantation model

In vivo and ex vivo visualization of microtubules was enhanced by labeling with the lipophilic fluorescent dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes, Eugene, OR, USA) [29]. Eight milligrams of lipid microtubules were first loaded with 320 µl PBS, and 64 µl of 2 mg/ml DiI in ethanol was added. After a 30 min incubation, 10 ml PBS was added, the solution was centrifuged (400 g, 10 min), and the supernatant was removed. The microtubules were stored for 3 days at room temperature and then washed three times with 5 ml PBS to ensure removal of ethanol and excess dye.

Twelve-week, female Sprague-Dawley rats (250–350 g) were anaesthetized with ether, and the inferior dorsal area was shaved. NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) were observed. Subcutaneous injections of 0.2 ml were made proximal to each hind leg using a 27-gauge needle. The controls that were injected included PBS and 0.04 mg/ml DiI. The test materials injected were 0.5, 5.0, and 25.0 mg/ml microtubules labeled with DiI and 5 mg/ml unlabeled microtubules. After 10 days, the animals

were treated with a lethal dose of ether, and a skin flap from the injection site was removed. A portion of each skin flap was immediately frozen in liquid nitrogen and stored at -70°C until sections were made. Additional tissue samples were fixed in formalin (10% phosphate buffered, Fisher Scientific, Pittsburgh, PA, USA) and stored at 4°C . Frozen sections were examined using confocal scanning laser microscopy (BioRad) equipped with fluorescence to detect the DiI from the microtubules. Formalin fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) using standard histological protocols. These sections were examined to determine biocompatibility by evaluating the inflammation and fibrosis due to the implanted materials. These responses were graded on a scale of 0 to 5 with 5 being the most severe reaction.

2.8. Statistics

Data are represented as the average value \pm the standard error of the mean (S.E.M.). Results from all experiments in this study were analyzed with the Student *t*-test, and $P < 0.05$ was used to indicate statistical significance.

3. Results

3.1. Physical characteristics of microtubules

The microtubules used in these studies (Fig. 1) had an average length of $46 \pm 21 \mu\text{m}$. A typical length distribution is shown in Fig. 2. While the majority of microtubules were in the lower half of the range, some microtubules were as long as $115 \mu\text{m}$. The microtubules fabricated from 1 mg of $\text{DC}_{8,9}\text{PC}$ lipid typically numbered 2×10^8 , and the experimentally determined mass of protein loaded was within 1% of the theoretical value computed from the volume of microtubules.

3.2. Effect of protein molecular weight

When equimolar concentrations (0.45 mM) of myoglobin, albumin, and thyroglobulin (0.8, 3.0,

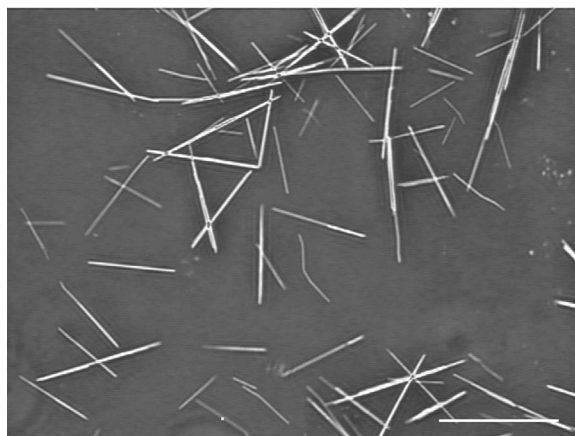


Fig. 1. Light micrograph of lipid microtubules formed from $\text{DC}_{8,9}\text{PC}$ lipid. Scale bar $50 \mu\text{m}$.

30.0% w/v, respectively) were loaded into the microtubules and their release profiles were plotted (Fig. 3), myoglobin release decreased rapidly and dropped below the detection limit of the protein assay by day 3. Albumin release declined more gradually and remained detectable for at least 5 days. Thyroglobulin release was also quantifiable for at least 5 days, and the release was not significantly different among days 2, 3, and 4.

3.3. Effect of protein loading concentration

Protein released daily from microtubules loaded with 0.45, 1.51, and 2.26 mM albumin solutions was calculated as mass released (Fig. 4) and as the percent of initially loaded protein released (Fig. 5). The mass released increased with an increase in initial loading concentration. Release from microtubules loaded with 0.45 mM was always significantly lower ($P < 0.05$) when compared to release from higher concentrations on the same day. However, the release from the 1.51 mM and 2.26 mM concentrations was only significantly different on days 1–3 ($P < 0.05$).

The percent release was not statistically different among the three albumin concentrations with the exception of 0.45 mM on days 2 and 3 and 2.26 mM on day 4 ($P < 0.05$).

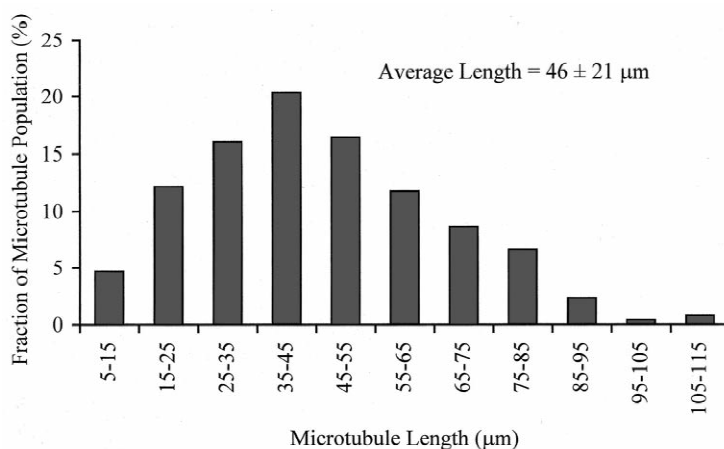


Fig. 2. Typical length distribution for lipid microtubules used in these studies as measured by light microscopy.

3.4. Contribution of hydrogel

Hydrogels loaded with albumin in the absence of microtubules released 80–100% of the loaded protein within 7 days. Fig. 6 shows the cumulative percent of albumin released from hydrogels with and without microtubules for a representative albumin loading concentration. The solid line, representing albumin release from the hydrogel, projects from day 7 to day 14 (not shown). In the case of the hydrogel, without the microtubules, 50% of the loaded protein is released within 2.2 days, whereas the micro-

tubule–hydrogel system takes 8.4 days to release 50% of the loaded protein.

3.5. Post-release bioactivity assay

In the presence of control PBS-loaded microtubules, less than 5% of the PC12 cells extended neurites (Fig. 7A). Over 90% of PC12 cells incubated with NGF-loaded microtubules extended neurites (Fig. 7B). When 7-day-old NGF-loaded microtubules were co-cultured with PC12 cells, they

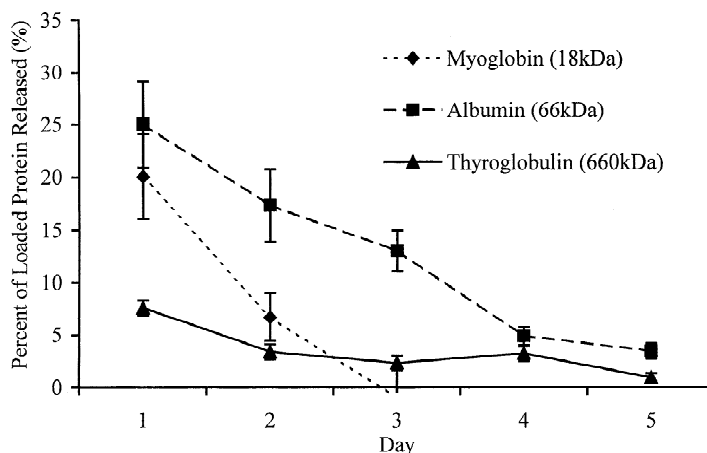


Fig. 3. Effect of protein molecular weight on release profile. Data shown are the average \pm S.E.M. ($n = 9$).

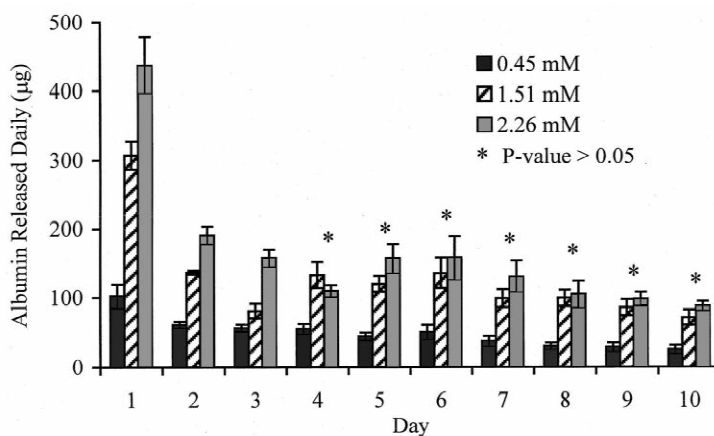


Fig. 4. Effect of protein loading concentration on mass released daily. Data are shown as the average \pm S.E.M. ($n = 6$). The mass released from microtubules loaded with 0.45 mM albumin was statistically significant with respect to release from the other two loading concentrations for each day. * $P < 0.05$ indicates no statistically significant difference between 2.26 mM albumin and 1.51 mM albumin.

released enough NGF over the next 48 h to stimulate neurite outgrowth.

3.6. Co-culture with BASMC

BASMC were incubated in the presence of hydrogels either with or without PBS-loaded lipid micro-

tubules. After 4 days of culture, there was no significant difference in the morphology or in the number of cells in control wells with only growth medium, test wells with growth medium and hydrogel, and test wells with growth medium and PBS-loaded microtubules embedded in the hydrogel (Fig. 8).

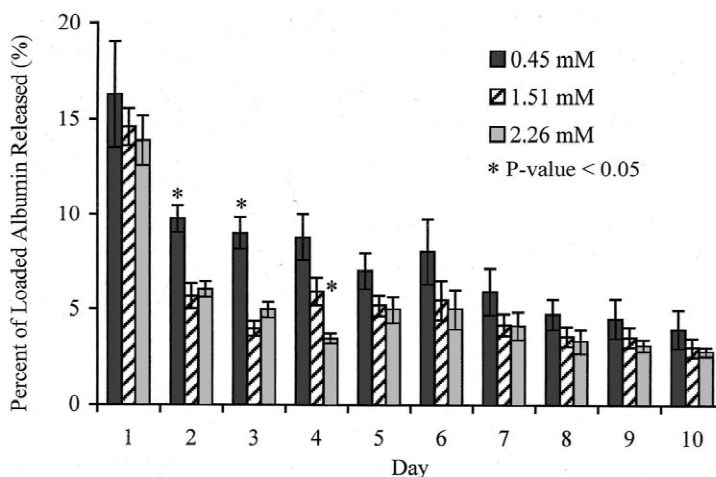


Fig. 5. Effect of protein loading concentration on percent of protein released daily. Release in terms of percentage of the total protein initially loaded did not depend upon the albumin concentration of the loading solution. Data are shown as the average \pm S.E.M. ($n = 6$). * $P < 0.05$ indicates a significant difference between release from the concentration the * is over and release from the two other concentrations for the same day.

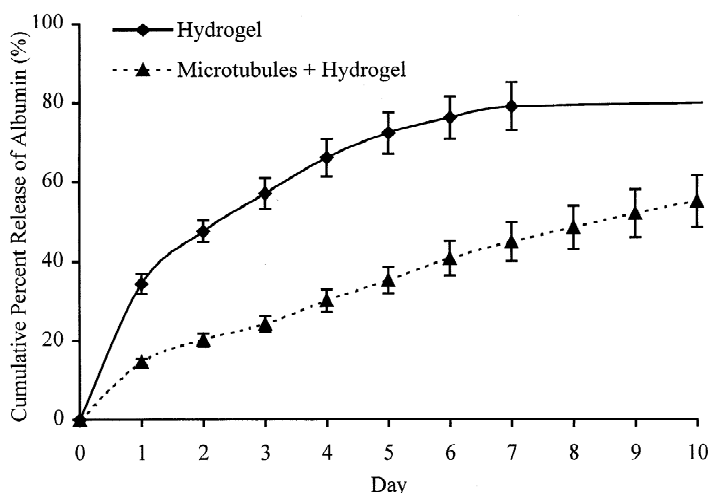


Fig. 6. Cumulative percent release of albumin from either hydrogel loaded with 2 mg albumin or the microtubule–hydrogel delivery system loaded with 2 mg albumin (corresponds to a 1.51 mM albumin loading concentration). Data shown are average \pm S.E.M.

3.7. Rodent implantation model

Frozen tissue sections from DiI-labeled microtubule implant sites revealed an increased amount of fluorescence at the implantation site when compared to the PBS injection or the microtubules without DiI (data not shown). H&E staining showed very few inflammatory cells for all implantation sites. The extent of inflammation and fibrosis at the implant sites is summarized in Table 1. Increasing the microtubule concentration (from 0.5 to 5.0 to 25.0 mg/ml) did not change either of these reactions.

4. Discussion

To be effective, a drug delivery system should: (1) allow drug loading without jeopardizing drug integrity, (2) provide predictable and prolonged release, (3) retain drug bioactivity after release, and (4) not elicit an inflammatory response at the implantation site. The results from this study of our lipid microtubule–hydrogel delivery system indicate that it fulfills these objectives.

Microtubules were successfully fabricated and safely loaded with protein. Although the microtubules were formed in 70% ethanol solution, all of the ethanol was removed by drying the microtubules prior to protein loading. As the microtubules rehy-

drate, capillary action draws the protein solution into the lumen of the tubules thereby loading the protein into the microtubules. With the microtubule–hydrogel delivery system, the protein to be released is dissolved in aqueous buffer, and no harmful organic solvents are involved.

Release from the microtubule–hydrogel system was predictable as seen by the effects of molecular weight and loading concentration on the release profile. The release curves generated by loading equivalent molar amounts of myoglobin, albumin, and thyroglobulin indicated that the molecular weight affected the percent release. The smaller myoglobin molecule had a higher diffusivity and a faster release rate. The thyroglobulin-loaded microtubules showed a more sustained, constant release due to the higher molecular weight of thyroglobulin. This trend will be useful in predicting release for future applications, particularly for globular proteins.

The initial protein loading concentration directly affected the mass of protein released daily (Fig. 4). Increasing the mass loaded caused a greater mass of protein to be released each day. The difference in mass released was not significant between 1.51 mM and 2.26 mM after 4–10 days. We hypothesize that this is due to the small difference between the two loading concentrations. Whereas there is over 300% increase between 0.45 mM and 1.51 mM (where the difference in release is significantly different at every

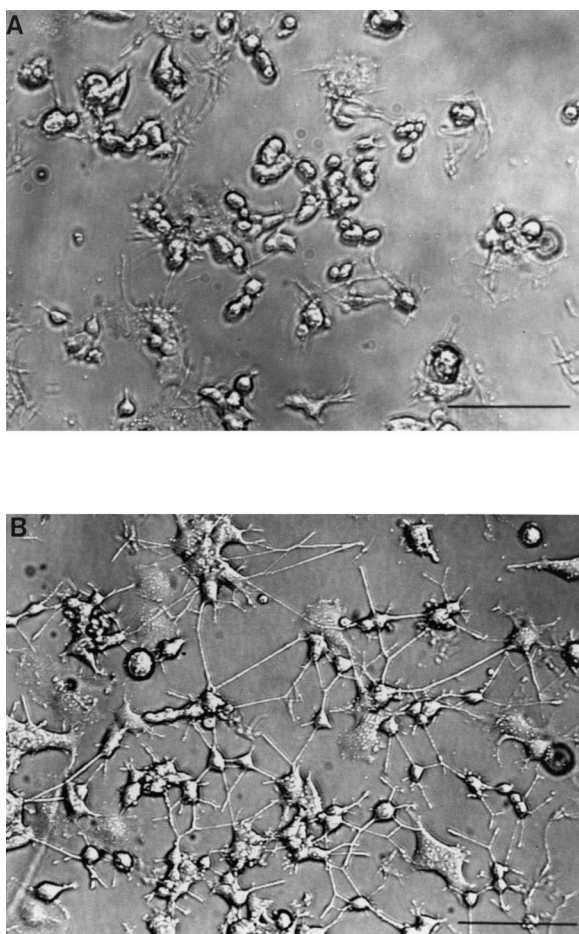


Fig. 7. PC12 cells incubated with lipid microtubules loaded with (A) PBS and (B) NGF. PBS-loaded microtubules produced very little (<5%) neurite outgrowth (see A). Microtubules loaded with NGF were able to deliver sufficient NGF on their seventh day of release at 37°C to induce PC12 cells to extend neurites (see B). Scale bar 100 μm .

time point), there is only a 50% increase between 1.51 mM and 2.26 mM. These concentrations were chosen to correspond to 3, 10, and 15% (w/v) albumin in PBS, stopping near the limit of albumin aqueous solubility at 37°C.

For the three albumin concentrations loaded, the percent of the loaded protein released was independent of the amount of protein initially loaded. As seen in Fig. 5, microtubules loaded with various concentrations of albumin had similar release profiles when release was expressed as a percentage of the

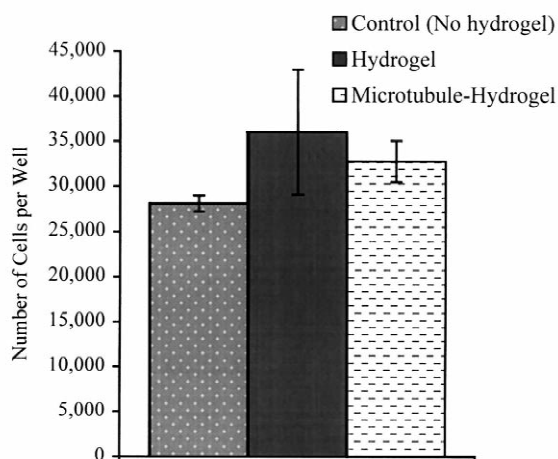


Fig. 8. Effect of SeaPrep agarose hydrogel blocks with and without lipid microtubules on BASMC proliferation. The initial plating density was 12,000 cells per well. No statistically significant difference existed between the three conditions ($P < 0.05$). Data are graphed as the number of cells per well after 4 days and the bar graphs are the average \pm S.E.M. ($n = 9$).

total albumin loaded. This data suggests that once a release profile has been generated for a given protein loading concentration, the amount of protein to load for other applications can be calculated. Studies are underway to develop a mathematical model to predict the results observed and to allow us to calculate the theoretical release of any protein.

In addition, our microtubule–hydrogel delivery system enables sustained release, a characteristic crucial to an effective delivery system. Other studies in our laboratory have demonstrated release of albumin from microtubules that was quantifiable for at least 30 days (data not shown).

In the delivery system, the hydrogel is included as a carrier to localize the microtubules, while the microtubules provide the agent reservoirs. To differentiate between the effects of the hydrogel and the effects of the tubules, we loaded hydrogels with albumin in the absence of the tubules and found that the hydrogel contributes minimally to sustained release. Therefore, the majority of the sustained release demonstrated by the microtubule–hydrogel system is dependent upon the microtubules and not the hydrogel carrier system.

We have shown that agents such as NGF released from this microtubule–hydrogel system remain via-

Table 1

In vivo reaction to subcutaneous microtubule injections using a rodent implantation model of inflammation

Sample injected	Inflammatory reaction ^a (scale 0–5)	Fibrosis ^a (scale 0–5)
Control (no microtubules)	0	0
DiI solution (0.04 mg/ml DiI)	2	2
Microtubules (5 mg/ml lipid)	1	0
Microtubules with DiI (0.5 mg/ml lipid)	1	1
Microtubules with DiI (5 mg/ml lipid)	1	1
Microtubules with DiI (25 mg/ml lipid)	1	1

^a Scale goes from 0 to 5, with 5 being the most severe reaction.

ble and biologically active. One growth factor bioassay tests for the presence of NGF using PC12 cells [30–32]. PC12 cells require NGF to extend processes [33], and our bioassay exploiting this suggests that physiologically relevant amounts of NGF can be released. Even on the seventh day of release, the microtubule–hydrogel delivery system was able to provide sufficient amounts of active NGF to initiate neurite outgrowth. In contrast, PBS-loaded microtubules produced no significant change in PC12 phenotype.

The lack of any adverse effects on proliferation and morphology of BASMC cultured in the presence of hydrogel with and without PBS-loaded microtubules indicates the benign properties of the hydrogel as well as the microtubule–hydrogel delivery system. Both the NGF bioassay and the BASMC co-culture experiments suggest that microtubules embedded in a hydrogel are not cytotoxic and are safe for use in tissue culture experiments. In addition, the small size of lipid microtubules prevents them from physically obstructing cell growth and neurite extension.

The rodent model using subcutaneous implantation indicated a minimal inflammatory response to microtubules after 10 days of implantation. Histological staining (H&E) of paraffin-embedded sections from these same locations was used to evaluate biocompatibility. The level of inflammatory cell reaction and fibrosis seen in our study when compared to cases of severe inflammation [34,35] was very minimal, and there was no significant difference between the reaction to the control saline injection and the microtubule injections. Thus, our microtubule–hydrogel delivery system appears to be

biocompatible, and future tests including biodistribution assays are underway.

Several parameters of our microtubule–hydrogel delivery system can be easily changed to alter release profiles. These parameters include the average microtubule length, the concentration of microtubules used, the loading concentration of the agent to be released, and the porosity of the hydrogel in which the microtubules are embedded. With options such as these, this system will be able to provide very versatile release for a range of applications.

5. Conclusions

In this study, we have characterized protein release and shown that biological agents can be loaded into the microtubule–hydrogel delivery system and still retain their biological function when released. We further investigated biocompatibility by looking at the response of BASMC to lipid microtubules embedded in a hydrogel and the inflammation and fibrosis resulting from subcutaneous microtubule injections in a rodent implantation model. These results suggest that lipid microtubules can be used in conjunction with agarose hydrogel to create a delivery system that is controllable, predictable, and biocompatible.

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References

- [1] T.M. Allen, Liposomes. Opportunities in drug delivery, *Drugs* 54 (Suppl. 4) (1997) 8–14.
- [2] G. Gregoriadis, A.T. Florence, Liposomes in drug delivery. Clinical, diagnostic and ophthalmic potential, *Drugs* 45 (1) (1993) 15–28.
- [3] R. Santangelo, P. Paderu, G. Delmas, Z.W. Chen, R. Manino, L. Zarif, D.S. Perlin, Efficacy of oral cochleate-amphtericin B in a mouse model of systemic candidiasis, *Antimicrob. Agents Chemother.* 44 (9) (2000) 2356–2360.
- [4] T.T. Hsu, R. Langer, Polymers for the controlled release of macromolecules: effect of molecular weight of ethylene-vinyl acetate copolymer, *J. Biomed. Mater. Res.* 19 (4) (1985) 445–460.
- [5] J.K. Sherwood, R.B. Dause, W.M. Saltzman, Controlled antibody delivery systems, *Biotechnology* 10 (11) (1992) 1446–1449.
- [6] M.V. Sefton, L.R. Brown, R.S. Langer, Ethylene-vinyl acetate copolymer microspheres for controlled release of macromolecules, *J. Pharm. Sci.* 73 (12) (1984) 1859–1861.
- [7] J.L. Cleland, Protein delivery from biodegradable microspheres, *Pharm. Biotechnol.* 10 (1997) 1–43.
- [8] V.R. Sinha, Bioabsorbable polymers for implantable therapeutic systems, *Drug Dev. Ind. Pharm.* 24 (12) (1999) 1129–1138.
- [9] E.R. Edelman, D.H. Adams, M.J. Karnovsky, Effect of controlled adventitial heparin delivery on smooth muscle cell proliferation following endothelial injury, *Proc. Natl. Acad. Sci. USA* 87 (10) (1990) 3773–3777.
- [10] R.L. Cleek, A.A. Rege, L.A. Denner, S.G. Eskin, A.G. Mikos, Inhibition of smooth muscle cell growth in vitro by an antisense oligodeoxynucleotide released from poly(DL-lactic-co-glycolic acid) microparticles, *J. Biomed. Mater. Res.* 35 (4) (1997) 525–530.
- [11] T.L. Wyatt, W.M. Saltzman, Protein delivery from nondegradable polymer matrices, *Pharm. Biotechnol.* 10 (1997) 119–137.
- [12] A.J.S. Jones, J.L. Cleland, Technical and regulatory hurdles in delivery aspects of macromolecular drugs, *J. Control. Release* 41 (1996) 147–155.
- [13] R.S. Raghuvanshi, S. Goyal, O. Singh, A.K. Panda, Stabilization of dichloromethane-induced protein denaturation during microencapsulation, *Pharm. Dev. Technol.* 3 (2) (1998) 269–276.
- [14] M.M. Harding, D.H. Williams, D.N. Woolfson, Characterization of a partially denatured state of a protein by two-dimensional NMR: reduction of the hydrophobic interactions in ubiquitin, *Biochemistry* 30 (12) (1991) 3120–3128.
- [15] S. Bhattacharjya, P. Balaram, Effects of organic solvents on protein structures: observation of a structured helical core in hen egg-white lysozyme in aqueous dimethylsulfoxide, *Proteins* 29 (4) (1997) 492–507.
- [16] P. Fan, C. Bracken, J. Baum, Structural characterization of monellin in the alcohol-denatured state by NMR: evidence for beta-sheet to alpha-helix conversion, *Biochemistry* 32 (6) (1993) 1573–1582.
- [17] P. Yager, P.E. Schoen, Formation of tubules by a polymerizable surfactant, *Mol. Cryst. Liq. Cryst.* 106 (1984) 371–381.
- [18] J.M. Schnur, Lipid tubules: a paradigm for molecularly engineered structures, *Science* 262 (1993) 1669–1676.
- [19] M.S. Spector, J.V. Selinger, J.M. Schnur, Thermodynamics of phospholipid tubules in alcohol/water solutions, *J. Am. Chem. Soc.* 119 (1997) 8533–8539.
- [20] R. Price, M. Patchan, Controlled release from cylindrical microstructures, *J. Microencapsul.* 8 (1991) 301–306.
- [21] B.J. Spargo, R.O. Cliff, F.M. Rollwagen, A.S. Rudolph, Controlled release of transforming growth factor-beta from lipid-based microcylinders, *J. Microencapsul.* 12 (3) (1995) 247–254.
- [22] J.M. Schnur, R. Price, A.S. Rudolph, Biologically engineering microstructures: controlled release applications, *J. Control. Release* 28 (1994) 3–13.
- [23] A.S. Rudolph, G. Stilwell, R.O. Cliff, B. Kahn, B.J. Spargo, F. Rollwagen, R.L. Monroy, Biocompatibility of lipid microcylinders: effect on cell growth and antigen presentation in culture, *Biomaterials* 13 (15) (1992) 1085–1092.
- [24] G.P. Dillon, X. Yu, A. Sridharan, J.P. Ranieri, R.V. Bellamkonda, The influence of physical structure and charge on neurite extension in a 3D hydrogel scaffold, *J. Biomater. Sci. Polym. Ed.* 9 (10) (1998) 1049–1069.
- [25] R. Bellamkonda, J.P. Ranieri, N. Bouche, P. Aebischer, Hydrogel-based three-dimensional matrix for neural cells, *J. Biomed. Mater. Res.* 29 (5) (1995) 663–671.
- [26] J.H. Crowe, L.M. Crowe, J.F. Carpenter, A.S. Rudolph, C.A. Wistrom, B.J. Spargo, T.J. Anchordoguy, Interactions of sugars with membranes, *Biochim. Biophys. Acta* 947 (2) (1988) 367–384.
- [27] A.S. Rudolph, J.H. Crowe, Membrane stabilization during freezing: the role of two natural cryoprotectants, trehalose and proline, *Cryobiology* 22 (4) (1985) 367–377.
- [28] L.T. Goodnough, M.E. Kleinhenz, G.H. Goldsmith Jr., N.P. Ziats, A.L. Robertson Jr., Bovine aortic endothelial cells elaborate an inhibitor of the generation of lipopolysaccharide-stimulated human blood monocyte procoagulant activity, *J. Clin. Invest.* 74 (1) (1984) 75–81.
- [29] A.L. Plant, D.M. Benson, G.L. Trusty, Probing the structure of diacetylenic phospholipid tubules with fluorescent lipophiles, *Biophys. J.* 57 (5) (1990) 925–933.
- [30] C.J. Robinson, R. Stammers, An in vitro bioassay for nerve growth factor based on 24-hour survival of PC-12 cells, *Growth Factors* 10 (3) (1994) 193–196.
- [31] L.A. Greene, A quantitative bioassay for nerve growth factor activity employing a clonal pheochromocytoma cell line, *Brain Res.* 133 (1977) 350–353.
- [32] S.M. Scott, R. Tarris, D. Eveleth, H. Mansfield, M.E. Weichsel Jr., D.A. Fisher, Bioassay detection of mouse nerve

- growth factor (mNGF) in the brain of adult mice, *J. Neurosci. Res.* 6 (5) (1981) 653–658.
- [33] A.S. Tischler, L.A. Greene, Nerve growth factor-induced process formation by cultured rat pheochromocytoma cells, *Nature* 258 (5533) (1975) 341–342.
- [34] J.M. Anderson, In vivo biocompatibility of implantable delivery systems and biomaterials, *Eur. J. Pharm. Biopharm.* 40 (1994) 1–8.
- [35] J.E. Babensee, J.M. Anderson, L.V. McIntire, A.G. Mikos, Host response to tissue engineered devices, *Adv. Drug Deliv. Rev.* 33 (1998) 111–139.