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Developing a cross-linked hyaluronic acid microparticle system for protein encapsulation

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Title (English)

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Abstract

The aim of this study was to develop a microparticle system optimal for protein drug delivery. The protein was first encapsulated in liposome nanoparticles and thereafter in cross-linked hyaluronic acid microparticles. Characterization of these particles showed this method to be milder on protein stability than conventional Microencapsulation methods. Although release kinetics, protein loading and the biocompatibility remain to be optimized, this microparticle system appears to be a promising delivery vehicle for proteins.

Keywords

Drug-delivery, microparticle, liposome, hyaluronic acid, protein stability, biocompatibility

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Sammanfattning

Proteinläkemedel är mycket potenta och specifika läkemedel. Normalt ges dessa till patienter genom regelbundna injektioner, eftersom då dessa mediciner ges oralt bryts de ned av enzymer som finns i magsäcken. Injektioner är relativt dyra och kräver oftast närvaro av sakkunnig vårdpersonal. Därför är proteiner bra kandidater för ett alternativt leveranssystem. Ett sådant system kan till exempel vara inkapsling av proteiner i mikrosfärer uppgjorda av ett polymer. Det är viktigt att dessa mikropartiklar är biokompatibla, vilket betyder att de inte igenkänns av immunsystemets som främmande material. Det är också betydelsefullt att proteinet inte skadas under inkapslingsprocessen.

I det här projektet har ett system av mikropartiklar utvecklats. Principen är att proteiner först innesluts av ett fettsyrehölje (liposom), och därefter i tvär-bunden hyaluronsyra (HAX). Liposomen fungerar som ett bra skydd för protein stabiliteten och hyaluronsyra är ett mycket biokompatibelt material. Därför är det troligt att ett sådant partikelsystem skulle kunna t ex sprutas in i patienternas blod och gradvis frisätta protein läkemedel utan att orsaka en inflammatorisk process i vävnader där det applicerats.

Karakteriseringen av dessa så kallade LipHAX partiklar har visat att de fungerar bra som skydd för proteiner. En rad andra egenskaper hos systemet måste optimeras, som till exempel hur mycket protein som finns inkapslat och hur mycket och hur fort proteinet släpps ut från partiklarna.

Examensarbete 20p i Molekylär Bioteknikprogrammet

Uppsala universitet april 2006

“I’ll believe it when I see it”
-Prof. Nikolai Piskunov

To my father who always challenges me

INDEX

CHAPTER 1: INTRODUCTION

1.1 Protein pharmaceuticals

Protein pharmaceuticals are highly specific and potent therapeutic drugs. In the modern day's expansion of the biotechnological industry new recombinant protein drugs are constantly created, optimized and mass-produced. The efficient and safe delivery of therapeutic proteins is the key to commercial success [1].

Peptides and proteins, such as insulin and vasopressin, are usually administered by injection. When given orally they are degraded by the proteolytic enzymes in the gastrointestinal tract, or they cannot cross the intestinal mucosa because of their hydrophilicity and large molecular size. Most protein products are delivered intravenously, intramuscularly or subcutaneously. Due to the short half-lives of peptide/protein drugs, frequent injections are required. This is a costly method and requires assistance from a trained health care professional [1]. For these reasons protein pharmaceuticals are ideal candidates for a controlled delivery system, e.g. microencapsulation. However, there are many obstacles in developing such delivery systems because proteins are easily destroyed in the process.

1.2 Microencapsulation

Microencapsulation has proven to be a good method for long-term drug delivery as well as a way for controlling the release kinetics for many smaller drugs. However, optimizing this method for large molecules such as peptides and proteins has proven to be a complicated task, mainly due to their delicate physical and chemical properties. Most proteins are sensitive to the changes in the environment and often loose their biological activity due to degradation or aggregation within the microparticles during the manufacturing process and during the release period [2-4]. Controlling the release of the protein is also difficult [5, 6].

Microencapsulation, using conventional polymeric systems (e.g., poly lactic-co-glycolic acid and encapsulation methods (e.g., double emulsion method), has showed limited success largely due to denaturation of encapsulated proteins during the encapsulation process and within the hydrophobic polymer environment. The proteins become unstable upon prolonged exposure to stressful conditions, such as a large water/organic solvent (w/o) interfacial area, high mechanical stress, acidic and hydrophobic microenvironments, or elevated temperature [2, 7-10].

1.3 Cross-linked hyaluronic acid

Hyaluronic acid (HA) is a naturally occurring linear polysaccharide, comprised of β-1,4 linked D-glucoronic acid and β-1,3 N-acetyl-D-glucosamine disaccharide units, with a number of properties, such as excellent biocompatibility, hydrophilicity, and viscosity, which make it appealing for biomedical applications including post-surgical adhesion preventions, tissue engineering, and controlled drug delivery [11].

Using cross-linked hyaluronic acid (HAX) microparticles to encapsulate protein drugs was presumed to have a number of potential advantages. Firstly, we anticipated that this method would be milder than the conventional methods since the preparation of HAX microparticles does not introduce any organic solvents, avoiding therefore a number of stability issues that arise at w/o interface [3]. Secondly, due to biocompatibility of HA we hope to minimize the inflammatory reactions that were shown to occur for PLGA in vivo [12].

1.4 Liposomes

Phospholipid vesicles (liposomes) are one of the most studied types of drug-carriers in the recent time, although due to their instability they are not ideal for long term drug delivery [13, 14]. However for purposes like reducing toxicity and enhancing cellular uptake, liposomes act as good carrier vehicles for proteins [15]. Our hypothesis was that encapsulating the proteins in liposome nanoparticles prior to encapsulation in HAX could improve particle formation.

1.5 LipHAX microparticles

The main goal of this project was developing a method of microencapsulation optimal for protein stability. It was suggested that the stability would be improved using a novel encapsulation method which involves encapsulation of the protein in liposome nanoparticles and thereafter in cross-linked hyaluronic acid (LipHAX microparticles). This method of preparing microparticles has several potential advantages. First, encapsulation in liposomes reduces contact between protein and polymer minimized interactions that could hinder efficient cross-linking of HA derivatives, as well as protects the protein from other stresses during the different steps of microencapsulation. Second, preparation of LipHAX microparticles does not involve the unfavorable water/organic solvent environment and the mechanical stress of this method is also considered to be milder that conventional microencapsulation methods.

This system was developed on three fairly different proteins used as model drugs: albumin, beta-glucosidase and tissue plasminogen activator (tPA) to validate these expectations. The in *vitro* protein stability and release kinetics were examined and the impact of the new microencapsulation method on these properties was taken into account. The biocompatibility of the LipHAX particles was investigated *in vivo*.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

Hyaluronic acids (HA, sodium salt) molecular weight of 1.3MD and 490kD were purchased from Genzyme Corporation (Cambridge, MA, USA) and HA with molecular weight of 50kD was obtained from Lifecore biomedical (Chaska, MN, USA). Adipic dihydrazide (ADH), 1-Ethyl-3-[3-(dimethylamino) propyl] carvodiimide (EDC), ethylene glycerol, sodium periodate were obtained from Aldrich (Milwaukkee, WI, USA). 1-Hydroxybenzotriazole (HOBt) was purchased from Alfa Aesar (Ward Hill, MA, USA). Cholesterol, 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Albumin, fluorescein isothiocyanate albumin (FITC-albumin), beta-glucosidase from almonds, and p-nitrophenyl-beta-D-glucopyranoside, (P-NBDG) were obtained from SIGMA (St. Louis, MO, USA), Tissue Plasminogen Activator (tPA) from Cathflo^{TM} Activase (Alteplase, Genentech, Inc. South San Francisco, CA, USA). Poly(lactic-*co*-glycolic acid) (PLGA) (lactic acid to glycolic acid ratio = 50:50, high intrinsic viscosity) was acquired from Birmingham Polymers (Birmingham, AL, USA). Mineral oil and Span 80 (Sorbitan monouleate) were purchased from SIGMA. Laemmli sample 20X, 12% HCl SDS-PAGE, B-mercaptoethanol were acquired from BIORAD. The bicinchoninic acid (BCA) and microBCA assay agents were obtained from Pierce.

2.2 Preparation of cross-linkable hyaluronic acids

In situ cross-linkable HA derivatives were synthesized according to a previously developed method [11, 16] (Figure 1 The illustration was adapted from [11]). Briefly, HA-adipic dihydrazide (HA-ADH) was prepared by reacting HA (medium MW unless specified otherwise) with a 30-fold molar excess of adipic dihydrazide in the presence of 1-ethyl-3-carbodiimide (EDC) and 1-hydroxybenzotriazole (HOBt) at pH 6.8 and room temperature. The product was purified by exhaustive dialysis and ethanol precipitation. HA-aldehyde (HA-CHO) was prepared by reacting HA (high MW unless specified otherwise) with an equimolar sodium periodate for 2 hours at room temperature in the dark. The reaction was

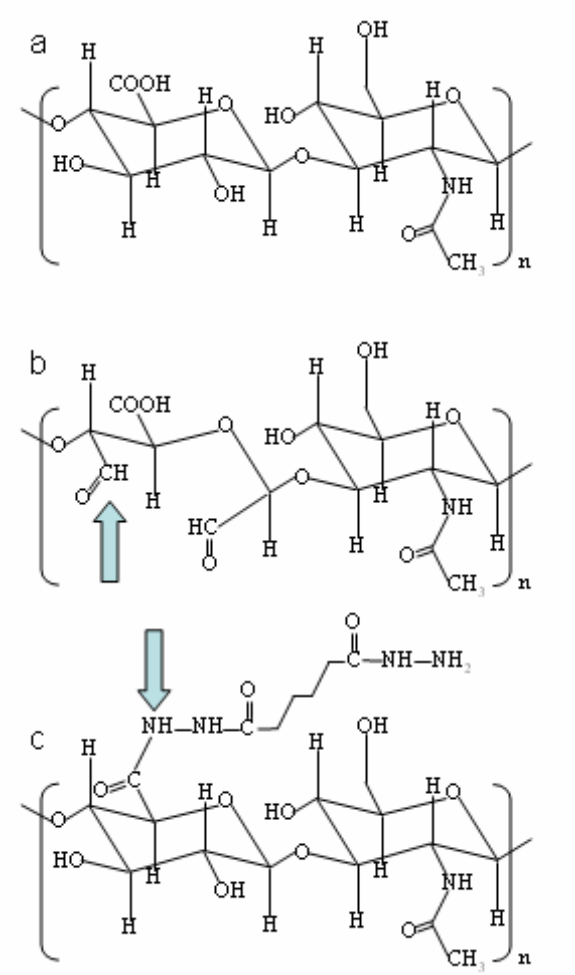

Figure 1: Hyaluronic acid. (A) Unmodified. (B) Periodate oxidation (to HA–CHO). (C) Hydrazide modification (to HA–ADH). Arrows indicate site of modification.

terminated by adding ethylene glycol. The product was purified by exhaustive dialysis. The purified products were lyophilized and stored at 4°C.

2.3 Preparation of microparticles

2.3.1 Cross-linked HA microparticles (HAX)

Encapsulation of protein in cross-linked HA microparticles (HAX) was explored by sequentially adding solutions of HA-ADH (490kD, 20mg/ml), protein of varying concentrations, and HA-CHO (1.36MD, 20mg/ml) in different volume ratios (**Table 1**, Appendix) to a continuous phase which consists of 25 g of mineral oil and 0.1 g of Span 80. The mixture was homogenized for 5 minutes to produce a water-in-oil emulsion at 1500 rpm (Silverson L4RT-4 homogeniser) with a medium size rod. The emulsion was stirred at 40ºC overnight to evaporate water from dispersed phase. The microparticles were washed with isopropyl alcohol six times, followed by evaporation of the residual isopropyl alcohol.

2.3.1.1 Optimizing the HA-ADH vs. HA-CHO ratio in HAX

The encapsulation was performed with HA-ADH (490 kD, 20 mg/ml) to HA-CHO (1.36 MD, 20 mg/ml) weight ratios of 2, 1 and 0.5. The protein concentration was kept constant at 1 mg/ml of FITC albumin in distilled water (**Table 1**, Appendix).

2.3.1.2 Investigating protein-HA interaction

The encapsulation was performed with different concentrations of albumin: 10 mg/ml albumin mixture (2.5 mg/ml FITC albumin and 7.5 mg/ml albumin), 2.5 mg/ml FITC albumin, 1 mg/ml FITC albumin to establish if the protein-HA ratio affected particle formation. The encapsulation was also done with beta-glucosidase in concentrations of 2.5 mg/ml and 1 mg/ml to see if the protein itself influenced encapsulation (**Table 1**, Appendix).

Presence of interaction between the protein and HA-ADH and/or HA-CHO was studied by means of sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Both reduced and non-reduced samples were analyzed to see interaction might occur in proteins natural conformation. For the reducing SDS-PAGE samples with twice the volume of loading buffer (Laemmli sample 20X in beta-mercaptoethanol) were boiled at 110°C for 5 min. For non-reducing SDS-PAGE 150 µl sample with 50ml sample buffer A (2.5ml 1M Tris-HCl pH 6.8, 1g SDS, 2.0ml 20mM EDTA) were heated at 50°C for 15min, after which 20 µl of sample buffer B (0.625ml 1M Tris-HCl pH 6.8, 0.01g Bromophenol blue, 7ml Glycerol 2.375ml distilled water). In each case 15µg of sample was loaded in each well on a 12% SDS-PAGE gel and electrophoresis was performed for 1h at 120 V. The gel was thereafter stained and distained with Coomassie Brilliant Blue Staining and Distaining Solutions (Biorad).

2.3.2 Liposomes

Liposomes were prepared by lipid film hydration. A thin film consisting of DOPC 100 mg (127 µmol) and cholesterol 25 mg (64.6 µmol) was formed in a round bottom flask. The film was hydrated with albumin solution (20 ml, containing 180 mg albumin and 20 mg model drug). Large multilamellar vesicles (LMV) were obtained by vortex-mixing the flask, repeated freezing-thawing cycles [17], and sonication. The LMV were then extruded through

polycarbonate filters with pore size 3, 1 and 0.4 µm to obtain small unilamellar vesicles (SUV)[1]. Non-encapsulated protein was removed by ultra-filtration through a 500 kD membrane.

2.3.3 Liposome-HAX composite particles (LipHAX)

Encapsulation of liposome in cross-linked HA microparticles (LipHAX) was done by sequentially adding 1.0 ml HA-ADH (490 kD, 20 mg/ml), 0.5 ml liposome (12.45 mg/ml) and 0.5 ml HA-CHO (1.36 MD, 20 mg/ml) in distilled water to a continuous phase which consists of 25 g of mineral oil and 0.1 g of Span 80. The mixture was homogenized for 5 minutes to produce a water-in-oil emulsion at 1500 rpm (Silverson L4RT-4 homogenizer) with a medium size rod. The emulsion was stirred at 40ºC overnight to evaporate water from dispersed phase. The microparticles were purified by exhaustive washing with isopropyl alcohol, followed by evaporation of the residual isopropyl alcohol.

In order to obtain highly cross-linked HAX matrix, LipHAX microparticles were also prepared with 2.0 ml low Mw HA-ADH (50 kD, 70 mg/ml), 100 mg liposome, 1ml HA-CHO (1.36 MD, 70 mg/ml). The continuous phase consisted of 50 g mineral oil and 0.2 g of Span 80 (**Table 1**, Appendix). The mixture was homogenized for 5 minutes at a faster speed of 9000 rpm to create a more uniform particle size distribution. The overnight evaporation of water and purification was done as described above.

2.3.4 PLGA water/oil/water microspheres with protein

For comparison, PLGA microparticles were prepared by a double emulsion-solvent evaporation method from literature [18] with minor modifications. To keep the protein concentrations comparable with the LipHAX particles, 150mg PLGA dissolved in 3.75 ml dichloromethane was sonicated for 2 min at 40% amplitude with 0.375 ml 10mg/ml tPA with albumin (1:9 ratio). The solution was placed into 25ml 1% polyvinyl alcohol (PVA) (6kD) and homogenized for 2 min at 9000 rpm. The homogenized mixture was poured into a larger beaker containing 50 ml water and a stir bar. After 2h the remaining dichloromethane was evaporated by means of a Rotovapor. The microparticles were purified by exhaustive washing with distilled water and lyophilized.

2.4 Characterization of microparticles

2.4.1 Protein content

2.4.1.1 Liposomes

Liposome samples were lyophilized in aliquots of known volume and resuspended in 1ml PBS. The liposomes were then disrupted, releasing the protein by sonication for 1 min with 1s-1s pulse and 40% amplitude. The solution was thereafter centrifuged at 10,000 rpm for 20 min to remove any debris. The protein content was determined by enzyme-linked immunosorbent assay (ELISA) for human tPA total antigen (Innovative Research, Southfield, MI, USA). A standard curve was created for known tPA concentrations and the amount of encapsulated protein in the liposomes was calculated analytically from this curve.

[1] For FITC liposome in batch 5 the LMV were then extruded through polycarbonate filters with pore size 3, 1, 0.4 and 0.2 µm to obtain small unilamellar vesicles therefore obtaining smaller particle size as presented in Table 2

2.4.1.2 LipHAX microparticles

It was assumed that 100% of liposome was encapsulated and therefore the mount of protein was directly proportional to the amount of liposome used for the formulation.

2.4.1.3 PLGA microspheres

The amount of tPA encapsulated in PLGA microparticles was estimated to be as the amount used for production of PLGA particles minus the amount detected in the three washes that was detected with human tPA total antigen ELISA.

2.4.2 Protein activity

2.4.2.1 Liposomes

Protein activity and total antigen were measured for beta-glucosidase and tPA, respectively, in order to determine how these proteins react in extreme treatments during protein encapsulation. To simulate each condition of the liposome preparation process, 0.2 ml samples of 0.5 mg/ml beta-glucosidase in distilled water were subjected to the repeated freezing and thawing (n=4), heating to 40°C for 1h (n=4), 2h (n=4) and 4h (n=4). Untreated protein, stored in water solution at 4°C, was used as a control for this study. Beta-glucosidase activity was quantified through a reaction that involves hydrolysis of p-nitrophenyl-beta-D-glucopyranoside (P-NPBG). A volume of 10 μl of the each beta-glucosidase sample was added to 1 ml of 2 mM P-NPBG in 0.05 M sodium acetate buffer pH 5.0. The enzyme and P-NPBG were allowed to react for 10 minutes stirring. The reaction was then stopped by adding 2ml of 1M sodium bicarbonate pH 9.0 and absorbance was read at 400 nm. The total amount of beta-glucosidase in the samples was detected by BCA Protein Assay Kit (Pierce) and the final data is presented as a quote of protein activity and the total amount of protein.

Similar experiment was performed with 0.5 mg/ml tPA, dissolved in distilled water with protective albumin (9.5 mg/ml). Samples with 35 μl of protein solution were subjected to freezing-thawing, heating as described previously for beta-glucosidase (n=4 for each treatment and time point). The amount of released tPA was quantified by human tPA total antigen ELISA (Innovative Research).

2.4.2.2 LipHAX microparticles

Once inside the liposome, the protein is assumed to be protected from all external stress, as was previously shown for other drugs. No extra activity study was conducted for protein encapsulated in LipHAX.

2.4.2.3 PLGA microspheres

To imitate conditions of the double emulsion method, 0.2 ml samples with 0.5 mg/ml beta-glucosidase in distilled water (n=4) were sonicated for 2 min at 40% amplitude with a tenfold of dichloromethane on ice, after which to each sample 0.8 ml distilled water was added. Samples were centrifuged at 4000 rpm for 5 min. The protein was extracted with the aqueous phase. Beta-glucosidase activity was quantified through a reaction that involves hydrolysis of (P-NPBG) and the total amount of beta-glucosidase in the samples was detected by BCA Protein Assay Kit (Pierce) as described in section 2.4.2.1. The final data is presented as a quote of protein activity and the total amount of protein.

Equivalent experiment was performed with 0.5 mg/ml tPA, dissolved in distilled water with protective albumin (9.5 mg/ml). Samples with 35 μl of protein solution (n=4) were subjected to

sonication for 2 min at 40 % amplitude with a tenfold of dichloromethane on ice, after which to each sample 0.965 ml distilled water was added. Samples were centrifuged at 4000 rpm for 5 min. The protein was extracted with the aqueous phase. The amount of released tPA was quantified by human tPA total antigen ELISA (Innovative Research).

2.4.3 Morphology, size distribution

2.4.3.1 Liposomes

Liposomes were stained with phosphotangstic acid pH 6.8 and imaged with transmission electron microscope (TEM) using 62kV accelerating voltage. Particle size was measured by light scattering using Brookhaven ZetaPALS.

2.4.3.2 LipHAX microparticles

The size of LipHAX microparticles was measured by a Beckman-Coulter Multisizer III and confirmed by scanning electron microscopy (SEM). Lyophilized LipHAX particles were attached to specimen stubs using double-coated/etch tape and sputter coated with gold-palladium in presence of argon gas using DESK II cold sputter unit (Denton vacuum, LLC. Moorestown, NJ). The particles were imaged with a JSM 6320 FEG scanning electron microscope using 3.0 kV accelerating voltage at 5.0 mm working distance.

2.4.3.3 PLGA microspheres

The size of PLGA microspheres was measured by a Beckman-Coulter Multisizer III.

2.4.4 Release kinetics

The release of tPA from LipHAX (batch LH6 see Table 1, Appendix) was studied *in vitro* and compared with the release from PLGA microparticles. Samples of 2-4 mg of microparticles were resuspended in 1ml PBS and incubated at 37°C for six days. With predetermined intervals, samples containing liposomes or microparticles were centrifuged at 8000 rpm for 5min, 0.8ml of the supernatant removed and replaced with new PBS. The released tPA was quantified by ELISA for human tPA total antigen (Innovative Reasearch).

2.4.5 Cytotoxicity

Human mesothelial cells (ATCC, CRL-9444) were cultured in Medium 199, containing Earle's salts, L-glutamine, and 2.2 g/L sodium bicarbonate and supplemented with 3.3 nM epidermal growth factor, 400 nM hydrocortisone, 870 nM insulin, 20 mM HEPES, and 10% fetal bovine serum. Mesothelial cells were seeded into 24-well plates at a density of 50,000 cells per well in 1 ml of culture medium and incubated overnight at 37°C. Blank LipHAX particles (LH40) were sterilized by UV light for 2 hours, resuspended in Saline and put on cells at concentrations 0.5mg/ml, 0.05mg/ml and 0.005mg/ml. After 1, 3, and 5 days of incubation in presence of LipHAX particles, cell viability was assessed with an MTT assay kit (Promega CellTiter 96 Non-Radioactive Cell Proliferation Assay). Results were normalized to the absorbance of non-treated control cells (% normalized cell viability = 100 × Absorbance for cells grown in the presence of a sample in medium/absorbance for cells grown in medium).

2.4.6 In vivo biocompatibility

Animals were cared for in compliance with protocols approved my Massachusetts Institute of Technology Committee on Animal Care, in conformity with the “Principles of Laboratory Animal Care” (NIH publication #85-23, revised 1985). Male SV129 mice weighing 20-35 grams,

obtained from Charles River Laboratories (Wilmington, MA, USA) were housed in groups and kept in a 6am-6pm light-dark cycle.

Animals were anesthetized with ketamine 50 mg/kg SQ and xylazine 10 mg/kg SQ. When they were asleep, their abdomen and thorax was shaved, and prepped in a sterile manner with 70% (v/v) isopropanol in water. Next, the injected anesthetics were supplemented with 1% isoflurane in 100% oxygen. A 0.5 cm skin incision was made in the skin 0.5 cm above the costal margin, and was tunnel caudad beneath the skin until a point 0.5 cm below the costal margin. There, the peritoneum was nicked with a 22G needle (<1 mm incision), and a 24 gauge angiocath was be introduced, through which 1.0 ml of the particle formulations was injected. The skin was closed with 1 or 2 stitches. Animals were euthanized with carbon dioxide after one week or one month after injection. The abdominal contents were examined for presence of peritoneal adhesions and particulate residue, photographed with a digital camera and processed for histology. An adhesion was defined as an abnormal connection between intra-abdominal contents that could not be disrupted by gentle separation with blunt probe. Microparticles attached to an intra-abdominal surface but not causing apposition of two surfaces were not counted as adhesions. Residue was defined as any mass that was at least partially composed of microparticles.

The materials used for injections were blank LipHAX microparticles (batches LH43-47, Table 1, Appendix), blank LipHAX hydrogel and blank LipHAX hydrogel pulverized microparticles. The blank LipHAX microparticles were sterilized by gamma irradiation (1.5 Mrad). Particles were, thereafter, resuspended in sterile PBS and injected at concentrations 50 mg/ml or 25 mg/ml (n=4 per group). After 1 week or 1 month, animals were sacrificed and the peritoneal cavity was examined.. The LipHAX hydrogel was prepared by dissolving 133 mg HA-ADH (50 kD) and 66 mg HA-CHO (1.3 MD), sterilized by UV for 2 hours, in 2.5 ml blank liposome (19.16 mg/ml) solution, sterilized by filtration through a 0.2 μm filter. The two solutions were then injected simultaneously by dual microdispensers at a final volume of 0.5 ml (=50 mg) per animal, 1 week (n=4). The blank LipHAX hydrogel pulverized microparticles in a similar fashion, but instead of injecting directly into the animal, the mixture was allowed to form a gel in a 50ml Falcon tube, after which the gel was lyophilized and pulverized using a pestle and mortar. Particles were sterilized by gamma irradiation (1.5 Mrad), resuspended in sterile PBS at concentration 50mg/ml, and then injected to the peritoneum as described above. Animals were sacrificed after 1 week, and tissue reactions were observed (n=4).

2.4.7 Cytokine measurement

Mouse peritoneal macrophages were seeded in 24-well plates at a density of 150,000 cells per well in 1 ml DMEM. After overnight incubation, one of the following materials was added to four wells: blank liposome (LH4), cross-linked HA (HAX) hydrogel, blank LipHAX or liposome cross-linked in HAX hydrogel. The concentration of each added material was kept constant at 2 mg/ml. As a positive control cells were incubated with 100 μl of PBS and for the negative control 100 μl lipopolysaccharide solution (final concentration in the culture medium: 0.2 μg/ml, 0.5 μg/ml and 1.0 ug/ml) was added to cells.

Blank LipHAX (4.9 μm) was sterilized by gamma irradiation (1.5 Mrad) and thereafter resuspended in PBS. Blank liposomes (354 nm) in water solution, HA-ADH (30kD) and HA-CHO (1.3MD) were sterilized by UV for two hours. The hydrogel was prepared by dissolving 26.6 mg HA-ADH and 13.3 mg HA-CHO in 0.5 ml distilled water and transferred simultaneously to a sterile syringe by dual microdispensers where the mixture was allowed to harden before cylindrical gels were added 35 μl/well. The liposome cross-linked in a hydrogel

was prepared in a similar manner with the exception that HA-ADH and HA-CHO were each dissolved in 0.5 ml blank liposome (19.2 mg/ml in water) solution.

After 36 hours, the culture media were centrifuged at 2000 rpm for 3 min to separate supernatants. Concentrations of mouse TNF-α and MIP-2 in the supernatants were determined by enzyme immunometric assay kits (Quantikine, R&D systems, Minneapolis, MN). The minimum detectable dose of each cytokine is as follows: TNF-α, 13.0 pg/ml; MIP-2, 1.5 pg/ml.

2.4.8 Particle uptake by macrophages

Mouse peritoneal macrophages (ATCC, CRL-2457) were cultured as a suspension in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and supplemented with 5 mM HEPES and 5% fetal bovine serum (ATCC).

Neutralized BD Cell-tak adhesive solution (BD Biosciences) (1 mg/ml) was prepared by mixing 150 μl of 1.45 mg/ml BD Cell-tak adhesive stock solution with 50 μl of 2 M sterile sodium carbonate solution. After adding 20 μl of neutralized BD Cell-Tak adhesive solution to the center of 22 mm * 22 mm coverslips in the laminar flow hood, the liquid was evenly distributed over the surface using the edge of a second coverslip held at a 45° angle. The coverslips were allowed to air dry for 5 min and stored at 4°C.

Macrophages were seeded at density of 100,000 cells in 3 ml culture medium in a 6 well plate. After an overnight incubation, cells were incubated for another night with 300 μl of 20 mg/ml FITC-albumin labeled LipHAX (LH6), sterilized by UV for 2 hours and resuspended in PBS. Dye solution was prepared by dissolving CellTracker Red CMTPX (Invitrogen) in high-quality DMSO to 10mM. The solution was then diluted with serum-free medium to make 5 μM and warmed to 37 °C prior to use. Cells were centrifuged at 1200 rpm for 3 min, cell media removed. Cell pellets were resuspended in 3 ml of dye solution and incubated at 37°C for 30 min. Cells were once again centrifuged, supernatant removed and 3 ml of fresh, pre-warmed culture-medium added. Cells were incubated at 37°C for 30 min. Coverslips were rinsed with distilled water and 20 μl of cell suspension were added covering as much area as possible. Cells were permitted to attach for 5 min before the excess medium was removed by suction. The cells were then washed with PBS, fixed with 0.5ml 3.7% formaldehyde in PBS for 15min at room temperature, and washed with PBS. Next, one drop of Vectashield H-1000 was added directly to the cell prep. Coverslips were mounted and excess mounting medium was removed. Cells were imaged with Zeiss LSM510 Laser scanning confocal microscope (Carl Zeiss, Inc.).

CHAPTER 3: RESULTS

3.1 Preparation of microparticles

3.1.1 Cross-linked HA microparticles (HAX)

HAX microparticles were produced as described in section 2.3.1. The results of different formulations are described in **Table 1**, Appendix. Production of HAX proved to be difficult due to aggregation and precipitation of material in the mineral oil. Further experiments and modifications were done to investigate and improve this method.

3.1.1.1 Optimizing the HA-ADH vs. HA-CHO ratio in HAX

Optimizing the HA-ADH vs. HA-CHO ratio with constant protein concentration gave the best yield of 38% in batch H31 at polymer ratio 2:1 (**Table 1**, Appendix). The other HAX batches showed a high level of aggregation and precipitation. This suggested that excess of HA-ADH is favorable for particle formation and that there is a possibility of interaction between one of the cross-linking components with the protein, disrupting the particle formation. Varying protein concentration in batches H4 and H5 showed that increased protein concentration disrupts its encapsulation. Encapsulating of beta-glucosidase instead of albumin proved to be similar. For batch H61 particles formed but the yield was very small (10%). The oil contained aggregates. Batch H62 with larger concentration of beta-glucosidase consisted of mostly aggregates.

3.1.1.2 Investigating protein-HA interaction

The interaction between the protein and the polymers HA-ADH and HA-CHO, was investigated by performing SDS-PAGE with albumin and various concentrations of either polymer. If the protein interacted with either polymer, the amount of albumin (67kD) that would appear on the SDS-PAGE gel would decrease with the increasing concentration of the polymer.

SDS-PAGE with both reduced samples (Figure 2a) and non-reduced samples (Figure 2b) showed no interaction between albumin and either one of the polymers. The insignificant extra bands appearing on this gel in rows B, E, F and G (Figure 2b) are assumed to be artifacts due to the non-reduced sample preparation. Therefore we conclude that there is no apparent chemical interaction between the protein and the cross-linkable components. However, the increase in protein concentration impairs with particle formation.

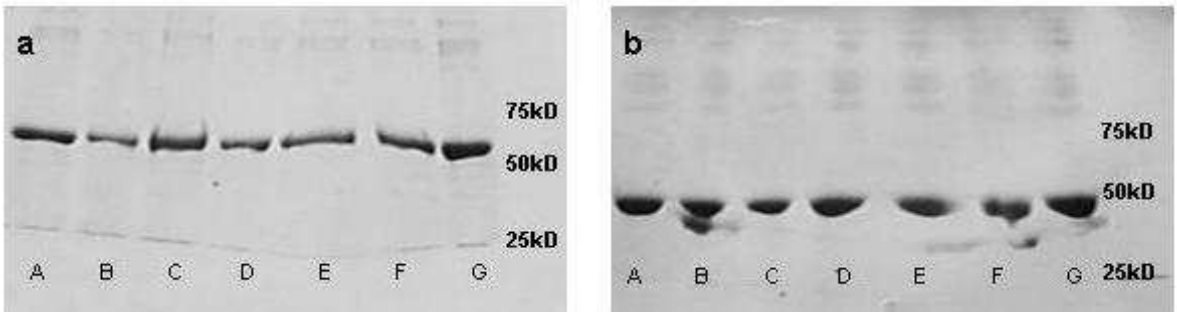

Figure 2: a)Reducing SDS-PAGE; b) Non-Reducing SDS-PAGE; Samples contain: A) HA-ADH (490kD, 20mg/ml): albumin (10mg/ml) volume ratio 1:1; B) HA-ADH (490kD, 20mg/ml): albumin (10mg/ml) volume ratio 2:1; C) HA-ADH (490kD, 20mg/ml): albumin (10mg/ml) volume ratio 4:1; D) HA-ADH (490kD, 20mg/ml): albumin (10mg/ml) volume ratio 1:1; E) HA-CHO (490kD, 20mg/ml): albumin (10mg/ml) volume ratio 2:1; F) HA-CHO (490kD, 20mg/ml): albumin (10mg/ml) volume ratio 4:1; G) albumin (10mg/ml).

3.1.2 Liposomes

In order to better protect the protein, as well as avoid any possible interaction with the polymer, the protein was encapsulated in liposome nanoparticles prior to encapsulation in HAX microparticles. Liposomes were produced with FITC albumin, beta-glucosidase, or tPA according to the method described in section 2.3.2 with yield between 56 and 77% (**Table 2**, Appendix). The yield for liposome with beta-glucosidase (L2) was smaller than that for liposome with FITC albumin (L1), 56% vs. 77%

3.1.3 Liposome-HAX composite particles (LipHAX)

The liposomes were used for production of LipHAX particles as described in section 2.3.2. The LipHAX formulations are presented in **Table 4**, Appendix. Unlike HAX particles, there was almost no aggregation during LipHAX particle production. The final product was a slightly yellow powder, which resuspended well in PBS. The particle yield was in general better for LipHAX particles that for HAX particles. Batches LH1a (72%) and LH1b (54%) gave clearly higher yield than same formulations without the liposome in batches H31 (38%) and H32 (18%). Once again HA-ADH/HA-CHO ratio 2:1 gave the highest particle yield, while ratio 1:2 gave no particles at all. To determine if this, in fact, was not caused by protein interaction with eg. HA-ADH, which would make cross-linking more difficult, batch LH2 (**Table 4**, Appendix) was prepared with no liposome and no protein and HA-ADH/HA-CHO ratio 1:2. Despite the absence of protein, no particles formed, which leads us to the conclusion that cross-linking in general works better in excess of HA-ADH.

3.1.4 PLGA microspheres

PLGA microspheres were prepared according method described in section 2.3.4 with a yield of 65%. The final product was a dry powder white powder that easily resuspended in PBS.

3.2 Characterization of microparticles

3.2.1 Protein content

The theoretical loading efficiency (TLE) of the protein for every type of particle was calculated as the quote between the mass of loaded tPA and the sum of masses of polymers/lipids and proteins used for that particular formation. The loading efficiency was then measured experimentally (ELE) by determining the amount of encapsulated protein and dividing it by the amount of formed particles. The total tPA encapsulation efficiency (EE) was calculated as the ratio between the theoretical and the experimental values (ELE/TLE). These results are presented in **Table 3**, Appendix.

3.2.1.1 Liposomes

The liposome protein content was determined according to the method described in section 2.4.1.1. A standard was created by with known concentrations of tPA. Protein content was calculated analytically from this curve to be approximately 0.028 mg/ml tPA in liposome and the encapsulations efficiency was 26 % (**Table 3**, Appendix).

3.2.1.2 LipHAX microparticles

Assuming that once the protein is encapsulated in liposome it stays there, the encapsulation efficiency was calculated to be 100%. Calculations are presented in **Table 3**, Appendix.

3.2.1.3 PLGA microparticles

The tPA protein content in PLGA microspheres was estimated to be the amount of tPA used for production of PLGA particles minus the amount detected in the three washes with human tPA total antigen ELISA. Very small amount of tPA were detected in the washes (0.59 μg) which would indicate a 153% encapsulation efficiency. However, this result is not fully trusted since a large amount of protein could have been lost in the first water phase prior to the washes (see section 2.3.4) which was not measured in this study. To be fully reassured over the encapsulation efficiency it is important find a better way to measure total protein in PLGA.

3.2.2 Protein activity

3.2.2.1 Liposomes

To ensure that encapsulation of the protein inside liposomes does not harm the protein, causing protein denaturation, an activity study was conducted. The effect of different steps during liposome preparation on beta-glucosidase and tPA was investigated according to methods described in section 2.4.2.1.

The enzymatic activity of beta-glucosidase divided by the total amount of protein in the sample is presented in Figure 3. Figure 4 shows the presence of total tPA antigen. The data was normalized by the amount of active protein in distilled water at 4°C. The study shows that beta-glucosidase is fairly stable it 40°C. The activity however drastically decreased during the freezing/thawing procedure. A possible explanation for this was that due to the fact that thawing was done at temperature closer to 60°C the protein might have been partially degraded. It appeared that these treatments had similar results on tPA as described in Figure 4. The protein appeared to be more active at 40°C rather than at 4°C, but due to a large standard deviation, these results cannot be fully trusted.

3.2.2.2 LipHAX microparticles

Once encapsulated inside the liposome the protein is protected from external stress. No additional activity study was performed for LipHAX particles.

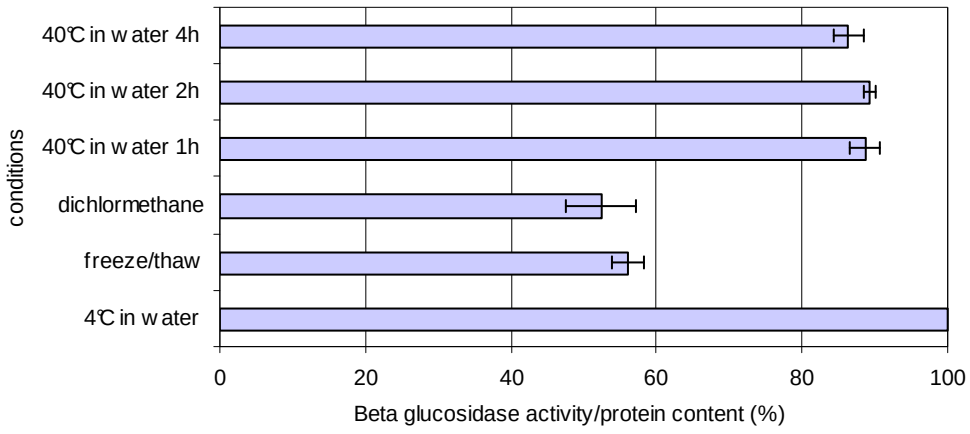

Figure 3: Beta-glucosidase stability in stress conditions during liposome preparation.

3.2.2.3 PLGA microparticle

To compare the LipHAX particle preparation with a conventional microencapsulation method, beta-glucosidase and tPA were exposed to dichloromethane treatment (section 2.4.2.3), mimicking the double emulsion-solvent evaporation method. The enzymatic activity of beta-glucosidase divided by the total amount of protein in the sample is presented in Figure 3.

Figure 4 shows the presence total tPA antigen. There was a substantial decrease in protein activity as well as a decrease in total amount of beta-glucosidase (data not shown here) after the treatment with dichloromethane imitating the double emulsion method. This confirms that some protein is denatured from direct exposure to an organic solvent, as well as from conformation changes induced by the water/organic solvent interface.

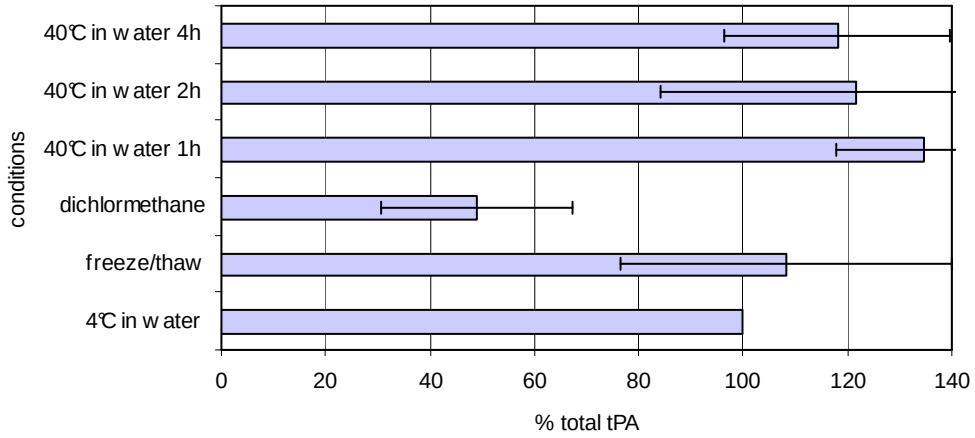

Figure 4: tPA stability in stress conditions during liposome preparation.

3.2.3 Size distribution and morphology

3.2.3.1 Liposome

The size of the nanoparticles after ultra-filtration varied between 206nm and 560nm. For some of the formulations the particle size increased after the ultra-filtration, which suggested presence of aggregation. The size and the morphology of liposomes were investigated by TEM.

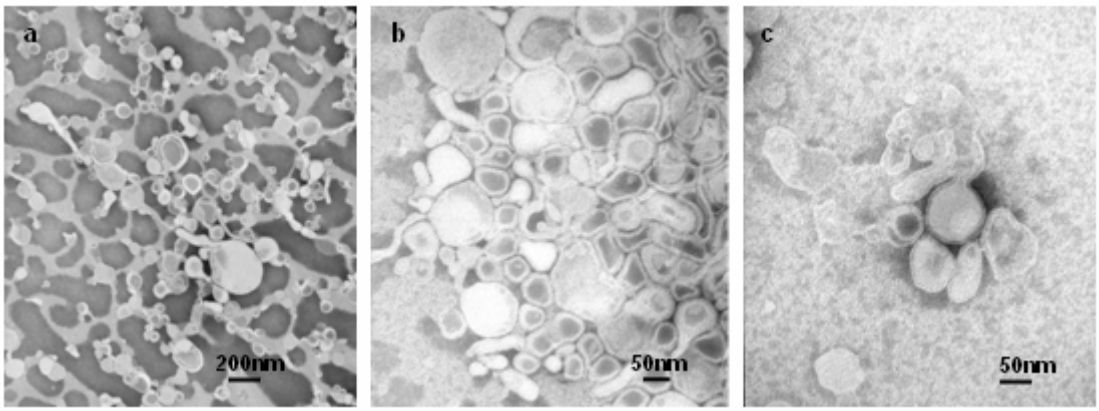

Figure 5: TEM of liposomes with FITC albumin (batch L1) after extrusion. Liposome concentration, 12.46 mg/ml, particle size 50-200 nm imaged at magnifications a) x30, b) x100 and c) x150.

Figure 5 shows that liposomes are generally spherical and particle size varies between 50 and 200 nm.

3.2.3.2 LipHAX

The average size of HAX particles and LipHAX particles in batches LH1-LH40 was 20 µm. The particle size for batches LH41-LH47 was approximately 5 µm, and 3 µm for batches LH5 and LH6. This means that increasing the homogenization speed from 1500 rpm to 9000 rpm makes the particle size smaller and more uniform. Batches LH5 and LH6 were made with smaller liposome than the other batches, which may explain the smaller particle size.

The particle size was confirmed in scanning electron micrographs (Figure 6). Morphologically LipHAX particles appear to be spherical with a size distribution of 5-15 µm for particles prepared with homogenization speed of 1500 rpm, and 0.5-5.0 µm for particles with homogenization speed of 9000 rpm. Particles also seem to remain intact after sterilization with gamma irradiation (1 Mrad) as demonstrated in (Figure 6d).

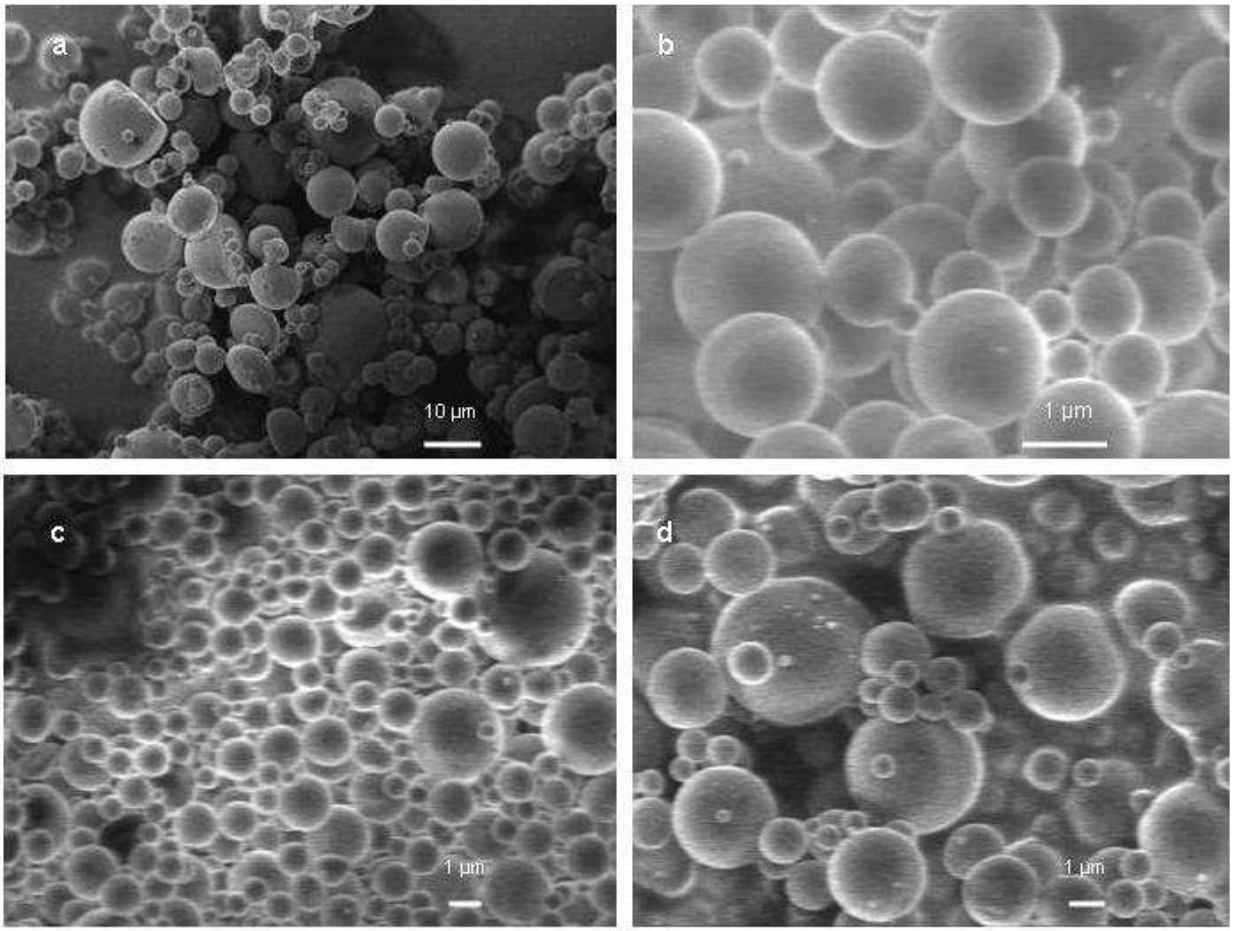

Figure 6: Scanning electron micrographs, 3.0kV, WD=5.0 mm. (a) LipHAX with FITC albumin (LH1) homogenized at 1500rpm, size 5-15µm, magnification x1100; (b, c, d) blank LipHAX (LH43) homogenized at 9000rpm, 0.5-5 µm: (b) magnification x16000, (c) magnification x6000, (d) particles after 1Mrad gamma irradiation magnification x10000.

3.2.3.3 PLGA

The average size of PLGA was determined by means of a coulter counter (Beckman-Coulter Multisizer III) microparticles was measured to be 5 µm.

3.2.4 Release kinetics

The release of tPA from LipHAX and PLGA microparticles studied in vitro (2.4.3.3). The results are presented in Figure 7. There seems to be presence of a burst release for both LipHAX and PLGA systems. It is worth noting that while estimation of tPA encapsulated in PLGA microparticles were fairly high, tPA release from particles was insignificant. The release kinetics for both types of particle formulation needs to be improved.

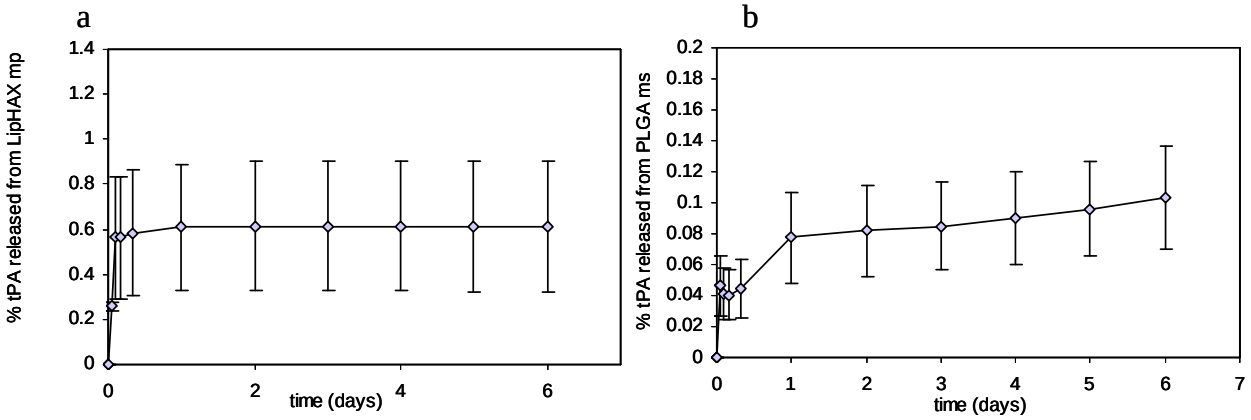

Figure 7: a) Release of tPA from LipHAX microparticles during period of 6 days; b) Release of tPA from PLGA microspheres during a period of 6 days.

3.2.5 Cytotoxicity

Before testing LipHAX *in vivo* the cytotoxicity of the particles was assessed *in vitro* using cultures of human mesothelial cells. The MTT assay showed no reduction in the viability of human mesothelial cells when exposed to 0,005 mg/ml 0.05 mg/ml, 0.5 mg/ml of blank LipHAX particles when incubated with these concentrations for 1, 3 and 5 days (Figure 8).

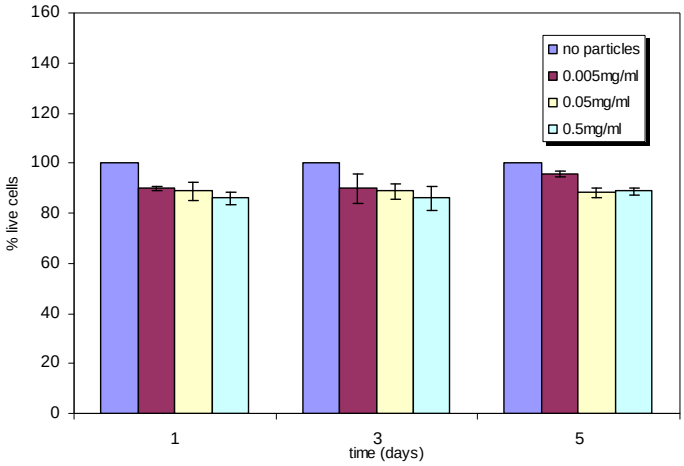

Figure 8: Results of the cytotoxicity study when incubated human mesothelial cells were incubated with 0.005mg/ml, 0.05mg/ml and 0.5mg/ml of blank LipHAX.

3.2.6 In vivo biocompatibility

Laparotomies were performed with a number of different formulations to determine the biocompatibility of the LipHAX particles and their ingredients in the peritoneum (Table 5). Injections of 50 mg blank LipHAX (LH1-7) in 1 ml PBS (1 week, n=4) gave 100% adhesions and 100% residue. Blank LipHAX particles 25mg in 1ml PBS were injected (1week, n=4) to determine if the particle concentration had an effect on biocompatibility. Once again 100% adhesions as well as residue were observed. The experiment was repeated with 25 mg in 1ml PBS (1 month, n=4) to assess if the adhesions and the residue would disappear with time. The results gave 100% adhesions and residue. In order to determine if these results were inflicted by the particle composition or their texture two additional formulations were tested. To achieve this injections of LipHAX hydrogel 50 mg per animal (n=4, 8.5 days) and LipHAX hydrogel pulverized particles 50 mg in 1ml PBS (n=4, 8.5 days) were performed as described in section 2.4.6. With LipHAX hydrogel adhesions were observed in three out of four mice while only two out of four mice had adhesions in the case of LipHAX hydrogel pulverized particles. Slight increase of spleen sizes was noted in 25 mg LipHAX mp, 50 mg LipHAX hydrogel and pulverized particles (average normal spleen area = 0.7 cm^2), but the histology of this organ appeared to be normal (not shown here). The digital pictures of the animal studies and the histology results are presented in Figure 9 and Figure 10.

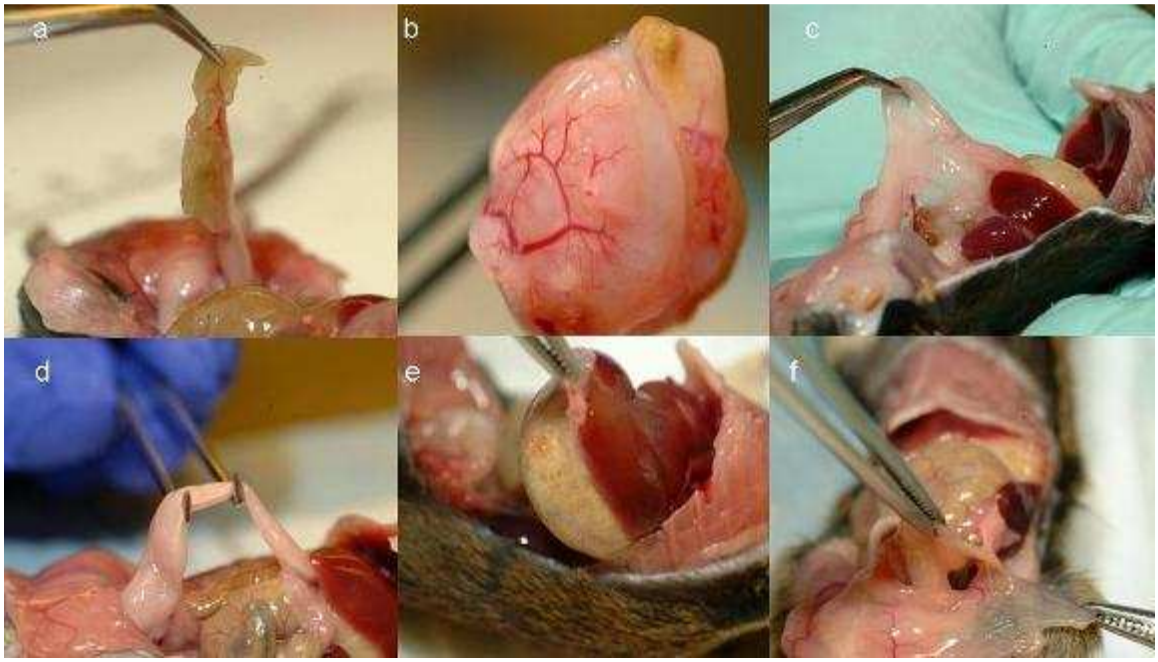

Figure 9: ***In vivo*** **test of LipHAX** a) 50mg LipHAX mp, 1 week, material adherent to corpus adiposum; b) 50mg LipHAX mp, 1week, vascular ingrowth, bowel, corpus adiposum and material; c) 25 mg LipHAX mp, 1week, adhesion involving corpus adiposum, stomach, spleen, material**;** d) 25 mg LipHAX mp, 4 weeks, adhesion involving corpus adiposum, liver and stomach; e) 50 mg pulverized LipHAX hydrogel mp, 1 week, liver adherent to stomach f) 50mg LipHAX hydrogel gel, 1week, adhesion between corpus adiposum and abdominal wall.

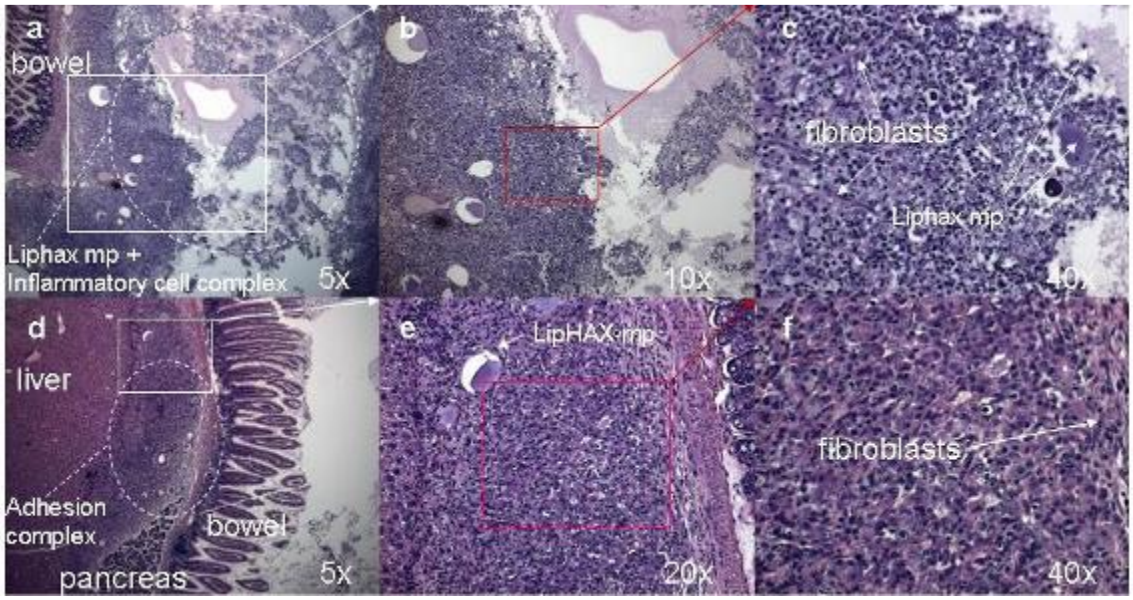

Figure 10: Histology of areas with adhesions from two *in vivo* experiments: (a-c) LipHAX mp, 50 mg 1 week (corpus adiposum, bowel, material) magnifications 5x, 10x, 40x; LipHAX mp, 25 mg 1week (adhesion complex involving liver, peritoneum, bowel).

3.2.7 Cytokine measurements

It has previously been suggested that adhesions, as those observed in the *in vivo* study, form because the applied material stimulates the release of pro-inflammatory cytokines, [19]. To test if this theory applies in this case, we investigated if presence of the materials used in this study induced the peritoneal macrophages to secrete TNF-α and macrophage inflammatory protein 2 (MIP-2). TNF-α stimulates endothelial cells and macrophages leading to a variety of inflammatory events. Macrophage inflammatory protein 2 (MIP-2), a murine functional homologue [20] of interleukin (IL)-8 [4], a chemokine which attracts and activates neutrophils. The results of the cytokine secretion study are presented in Figure 11.

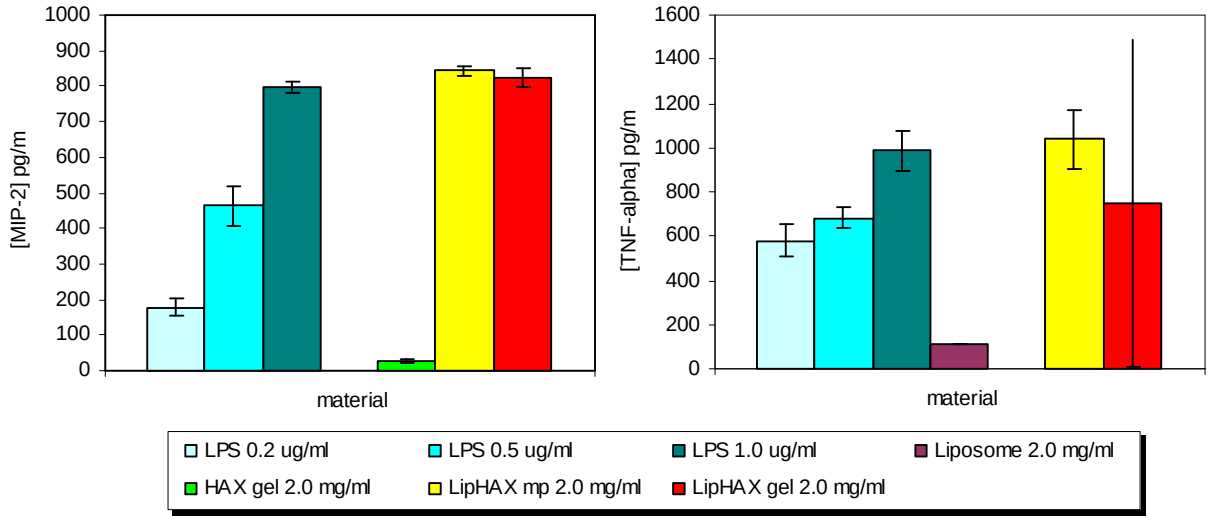

Figure 11 Secretion of MIP-2 and TNF-alpha in mouse mesothelial cells in response of different materials.

Liposome and HAX hydrogel appear to cause almost no cytokine expression. LipHAX particles showed MIP-2 and TNF-α concentrations equivalent to the secretion from macrophages exposed to 1 µg/ml of LPS, a known toxin. This led to a question weather there were other impurities other than liposome or the polymer itself that caused the inflammation, such as mineral oil and Span 80.

Therefore cytokine level was studied from cells incubated with HAX gel containing liposomes (LipoHAX gel). The result was the same as for LipHAX particles in case of MIP-2. Due to high error (50%) valid conclusion was not made for TNF-α production.

3.2.8 Particle uptake by macrophages

Macrophages were incubated with LipHAX particles marked with FITC-albumin or PLGA microparticles containing FITC-albumin overnight. For confocal microscopy, macrophages were stained with CellTracker Red CMTPX (Invitrogen) according to the protocol. The results of this experiments are presented in Figure 12.

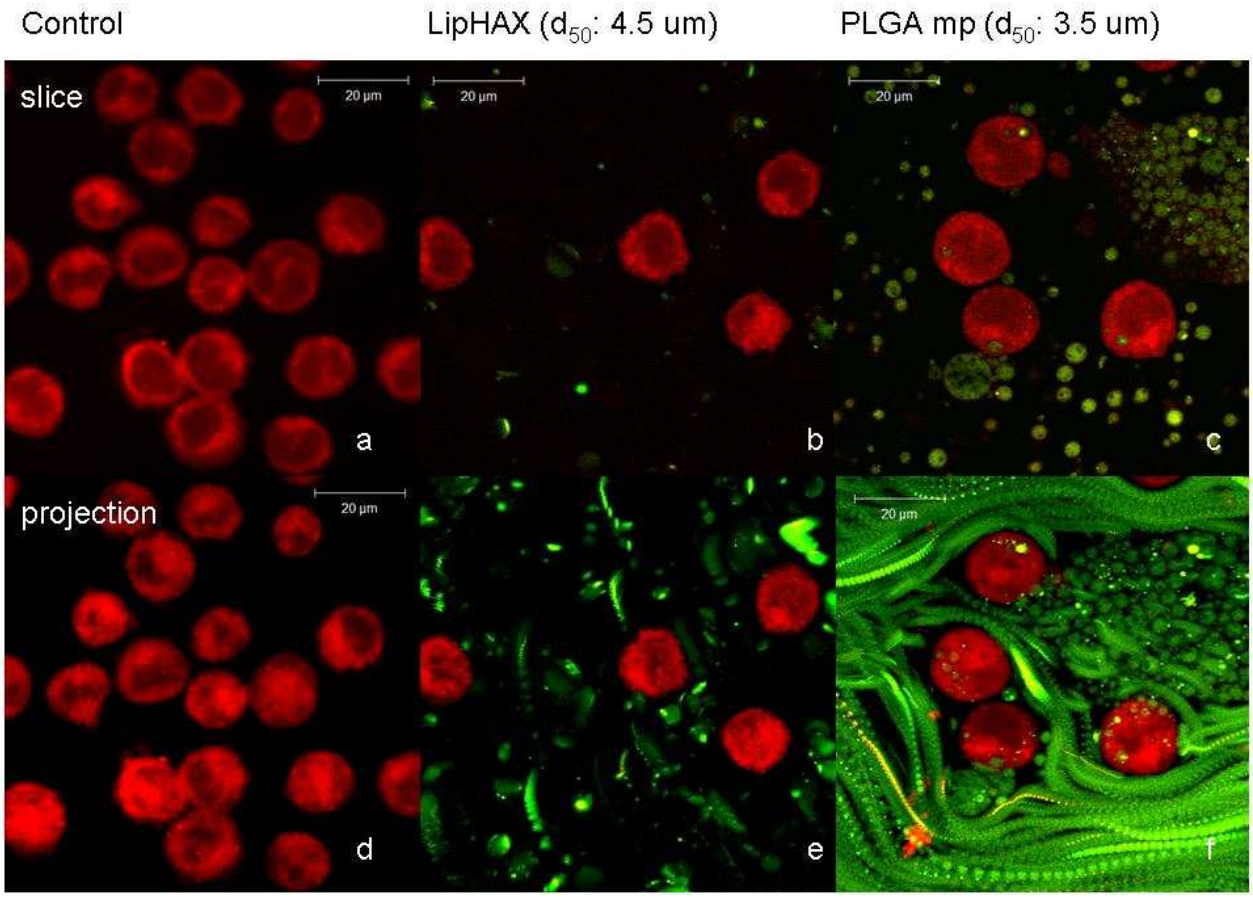

Figure 12: Results of confocal microscopy. Mouse peritoneal macrophages (red) a) incubated with LipHAX microparticles with FITC albumin (green) or c) PLGA microspheres with FITC albumin (green); From projection images it is visible that e)LipHAX simply pass by the cells while f) some of the PLGA are actually being taken up by the macrophages (the green dots that are not moving).

While LipHAX particles were small enough to be taken up by macrophages (although they tended to swell and some of them aggregated), they remain outside of the cells (Figure 12 b, e). This is not the case with PLGA particles, which can be found inside the cells (Figure 12 c, f). This is an interesting observation when it comes to biocompatibility of the particles. It shows that there is a high possibility that it is not the LipHAX particles themselves but the possible soluble material released from these that cause the inflammatory effects described in previous sections.

CHAPTER 4: DISCUSSION

As described earlier the greatest difficulty with many conventional microencapsulation methods is the degradation and aggregation of the encapsulated protein drug. This occurs in the presence of harsh conditions during particle preparation, such as to w/o interfaces, hydrophobic organic solvents and mechanical stress. The purpose of this work was to develop a system that would be gentle on the protein and have good biocompatibility.

Three different proteins were chosen as model drugs for this project: albumin, beta-glucosidase and tissue plasminogen activator (tPA). Albumin performs many functions including maintaining the "osmotic pressure" that causes fluid to remain within the blood stream instead of leaking out into the tissues. When it is labeled by fluorescein isothiocyanate (FITC), albumin can be easily detected through fluorescence and can be used for imaging of microparticles. Beta-glucosidase catalyzes the hydrolysis of glucosides and is fairly sensitive to environmental changes. This made it a good candidate for protein activity study. Cathflo™ Activase® [Alteplase] is a tissue plasminogen activator produced by recombinant DNA technology. Tissue plasminogen activator (tPA) is a thrombolytic agent which is used to dissolve blood clots in patients having a heart attack or stroke and is a good example of an actual therapeutic drug.

Hyaluronic acid (HA) was chosen as the appropriate polymer for protein encapsulation with its well documented and appealing characteristics such as biocompatibility, hydrophilicity and viscosity. We anticipated the preparation method of cross-linked hyaluronic acid (HAX) microparticles would be generally milder on the protein than conventional methods. Encapsulation in HAX does not introduce organic solvents and therefore avoids a number of stability issues.

Attempts to encapsulate protein inside cross-linked hyaluronic acid with different volume ratios and concentrations of protein and polymer showed a number of things. First, it appears that an excess of HA-ADH favorable for cross-linking. The concentration of the free protein is also important particle formation. Too much protein makes cross-linking less successful.

To avoid any interaction between the protein and the polymer, but most importantly to protect the protein from any stressful conditions during particle preparation, a novel formulation was suggested. It involved encapsulating the protein inside liposomes and then inside cross-linked hyaluronic acid. Liposomes themselves are not optimal for long term drug delivery, but they are known to be a good protective shield from mechanical and chemical stress.

The effects of the different steps of liposome encapsulation on protein activity were viewed in this study and showed that encapsulation in liposome does not cause degradation or loss of the protein as at the w/o interphase for PLGA. It appears that for both beta-glucosidase and tPA the process of the encapsulation in liposome is gentler on the stability of the protein than the double emulsion method that is used to make PLGA particles. However the encapsulation efficiency and the release kinetics remain to be optimized for this formulation.

There was an extensive *in vivo* tissue reaction to LipHAX particles, which was quite at first quite surprising, given that *in vitro* the particles showed no signs of cytotoxicity. The presence of multiple adhesions in different areas of the abdominal cavity and increased vascular growth are some of the symptoms that usually appear in the peritoneum after injury. However, since there was no injury inflicted on the test subjects, it means that the large inflammatory process was initiated in response to the material. In order to determine what exactly caused the inflammation, a number of experiments were conducted.

From the confocal data, described in Figure 12, it appeared that LipHAX particles were not taken up by macrophages like PLGA, despite the small size. This suggests that particles

themselves do not induce macrophage activation, which would result in extensive injury and subsequent adhesion formation. On the other hand, it is worth noting that LipHAX particles did induce high levels of pro-inflammatory cyto- and chemokines such as TNF-α and MIP-2 in macrophages whereas macrophages incubated with liposome or hydrogel separately did not induce the same level of cytokine activation. This suggests that the problem may lie in the soluble components released from LipHAX, such as mineral oil or surfactant (Span 80), remaining from particle production, or stray non-cross-linked hyaluronic acid. In fact low molecular weight soluble HA is known to sometimes induce cytokine expression [21]. To fully understand this, it would be necessary to measure e.g. MIP-2 levels in macrophages incubated with HAX particles in order to see the effect of possible remains as well as macrophages incubated with HA-ADH and HA-CHO separately to see if these non-cross-linked materials can cause pro-inflammatory activation.

In order to determine how the texture of the material affected biocompatibility, a LipHAX hydrogel as well as LipHAX hydrogel pulverized microparticles were tested *in vivo*. Results showed a lower number of adhesions 75% and 50% respectively, which could suggest that the last two formulations are more biocompatible than LipHAX microparticles. Therefore, if it is possible to minimize the amount of residual mineral oil and surfactant in LipHAX formulation, this microparticle system could still be a promising delivery vehicle.

CHAPTER 5: CONCLUSION

The main goal of this project was developing a method of microencapsulation optimal for protein stability. A particle system involving liposome and cross-linked hyaluronic acid particles (LipHAX) was studied. Characterization of this system suggested that this encapsulation method is gentler on the protein in terms of stability than conventional microencapsulation methods. However, there are a number of factors left to consider and improve, such as release kinetics, encapsulation efficiency and biocompatibility. Further investigations will show if this LipHAX microparticle system can be used as an alternative way to deliver protein drugs.

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REFERENCES

1. Wang, C.H. and Y.Y. Huang, *Encapsulating protein into preformed liposomes by ethanol-destabilized method.* Artificial Cells Blood Substitutes And Immobilization Biotechnology, 2003. **31**(3): p. 303-312.
2. Schwendeman, S.P., *Recent advances in the stabilization of proteins encapsulated in injectable PLGA delivery systems.* Critical Reviews In Therapeutic Drug Carrier Systems, 2002. **19**(1): p. 73-98.
3. Yeo, Y. and K. Park, *A new microencapsulation method using an ultrasonic atomizer based on interfacial solvent exchange.* Journal Of Controlled Release, 2004. **100**(3): p. 379-388.
4. Lee, J., et al., *Chemokine Binding And Activities Mediated By The Mouse Il-8 Receptor.* Journal Of Immunology, 1995. **155**(4): p. 2158-2164.
5. Hora, M.S., et al., *Release Of Human Serum-Albumin From Poly(Lactide-Co-Glycolide) Microspheres.* Pharmaceutical Research, 1990. **7**(11): p. 1190-1194.
6. Yeo, Y. and K.N. Park, *Control of encapsulation efficiency and initial burst in polymeric microparticle systems.* Archives Of Pharmacal Research, 2004. **27**(1): p. 1-12.
7. Perez, C., et al., *Recent trends in stabilizing protein structure upon encapsulation and release from bioerodible polymers.* Journal Of Pharmacy And Pharmacology, 2002. **54**(3): p. 301-313.
8. Putney, S.D. and P.A. Burke, *Improving protein therapeutics with sustained-release formulations.* Nature Biotechnology, 1998. **16**(2): p. 153-157.

9. van de Weert, M., W.E. Hennink, and W. Jiskoot, *Protein instability in poly(lactic-co-glycolic acid) microparticles.* Pharmaceutical Research, 2000. **17**(10): p. 1159-1167.
10. Yeo, Y. and K. Park, *Characterization of reservoir-type microcapsules made by the solvent exchange method.* Aaps Pharmscitech, 2004. **5**(4).
11. Jia, X.Q., et al., *Prolongation of sciatic nerve blockade by in situ cross-linked hyaluronic acid.* Biomaterials, 2004. **25**(19): p. 4797-4804.
12. D. S. Kohane, J.Y.T., Y. Yeo, R. Padera, M. Shubina, and R. Langer, *Biodegradable polymeric microspheres and nanospheres for drug delivery in the peritoneum.* Biomed. Mater. Res., 2006. **A**.
13. Dhoot, N.O. and M.A. Wheatley, *Microencapsulated Liposomes in controlled drug delivery: Strategies to modulate drug release and eliminate the burst effect.* Journal Of Pharmaceutical Sciences, 2003. **92**(3): p. 679-689.
14. Tarek, M.F., Peter M. Fong, Amit Goyal and W. Mark Saltzman, *Targeted for drug delivery.* Materials today, 2005. **8**(8): p. 18-26.
15. Yuyama, Y., et al., *Potential usage of thermosensitive liposomes for site-specific delivery of cytokines.* Cancer Letters, 2000. **155**(1): p. 71-77.
16. Bulpitt, P. and D. Aeschlimann, *New strategy for chemical modification of hyaluronic acid: Preparation of functionalized derivatives and their use in the formation of novel biocompatible hydrogels.* Journal Of Biomedical Materials Research, 1999. **47**(2): p. 152-169.
17. Sou, K., et al., *Effective encapsulation of proteins into size-controlled phospholipid vesicles using freeze-thawing and extrusion.* Biotechnology Progress, 2003. **19**(5): p. 1547-1552.
18. Cohen, S., et al., *Controlled Delivery Systems For Proteins Based On Poly(Lactic Glycolic Acid) Microspheres.* Pharmaceutical Research, 1991. **8**(6): p. 713-720.
19. Yeo, Y., J.A. Burdick, C.B. Highley, R. Marini, R. Langer, D. S. Kohane, *Peritoneal application of chitosan and UV-cross-linkable chitosan.* Journal of Biomedical Materials Research, accepted 2006.
20. Coico, R., G. Sunshine, and E. Benjamini, *Immunology: a short course*. 5th ed. 2003, Hoboken, N.J.: Wiley-Liss. xxv, 361 p.
21. Mascarenhas, M.M., et al., *Low molecular weight hyaluronan from stretched lung enhances interleukin-8 expression.* American Journal Of Respiratory Cell And Molecular Biology, 2004. **30**(1): p. 51-60.

ABBREVIATIONS

ADH	Adipic dihydrazide
BCA	Bicinchoninic acid
DMEM	Dulbecco's modified eagles medium
DOPC	1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine
EDC	Ethyl-3-[3-(dimethylamino) propyl] carvodiimide
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
HA	Hyaluronic acid
HA-ADH	HA-adipic dihydrazide
HA-CHO	HA-aldehyde
HAX	Cross-linked hyaluronic acid
HOBt	1-Hydroxybenzotriazole
LipHAX	Liposome cross-linked hyaluronic acid composite particles
LMV	Large multilamellar vesicles
LPS	Lipopolysaccharide
MIP-2	Macrophage-inflammatory protein 2
Mp	Microparticle
Ms	Microsphere
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate Buffered Saline
P-NBDG	P-nitrophenyl-beta-D-glucopyranoside
PLGA	Poly lactic-co-glycolic acid
PVA	Polyvinyl alcohol
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide
SEM	Scanning electron microscopy
SUV	Small unilamellar vesicles
TEM	Transmission electron microscope
TNF-α	Tumor necrosis factor alpha
tPA	Tissue plasminogen activator
w/o	Water/organic solvent

APPENDIX

Table 1: Results of HAX formulations.

HAX batch	HA-ADH	HA-CHO	Protein		Homogenization speed (rpm)	Mineral oil (g)	Span 80 (g)	Yield (%)
H1	490kD 20mg	1.36MD 20mg	10mg/ml 0.5ml	FITC albumin / albumin[2]	1500	25g	0.1g	0
H2	50kD 70mg	1.36MD 70mg	10mg/ml 0.5ml	FITC albumin / albumin	1500	25g	0.1g	0
H31	490kD 20mg	1.36MD 10mg	1mg/ml 0.5ml	FITC albumin	1500	25g	0.1g	38
H32	490kD 20mg	1.36MD 20mg	1mg/ml 0.5ml	FITC albumin	1500	25g	0.1g	18
H33	490kD 10mg	1.36MD 20mg	1mg/ml 0.5ml	FITC albumin	1500	25g	0.1g	15
H4	490kD 20mg	1.36MD 10mg	10mg/ml 0.5ml	FITC albumin / albumin	1500	25g	0.1g	0
H5	490kD 20mg	1.36MD 10mg	2.5mg/ml 0.5ml	FITC albumin	1500	25g	0.1g	0
H61	490kD 20mg	1.36MD 10mg	1mg/ml, 0.5ml	β-glucosidase	1500	25g	0.1g	10
H62	490kD 20mg	1.36MD 10mg	2.5mg/ml 0.5ml	β-glucosidase	1500	25g	0.1g	0

Table 2: Liposome formulations: yield and particle size.

Liposome batch	model drug	Formulation				Liposome nanoparticles			
		DOPC (mg)	Cholesterol (mg)	Albumin (mg)	Model drug (mg)	concentration (mg/ml)	volume (ml)	Yield (%)	Size[3] (nm)
L1	FITC albumin	100	25	180	20	12.46	20	77	*(289)* 463.8
L2	Beta-glucosidase	100	25	190	10	9.1	20	56	560
L30	Blank	50	12.5	95	---	3.5	10	22	322
L31[4]	Blank	100	25	180	---	10.2	20	65	387.9
L32	Blank	100	25	180	---	19.16	20	63	354
L33	Blank	100	25	180	---				
L4	FITC albumin	200	50	380	20	28	50	61	*(195)* 206
L5	tPA	23.5	5.9	44.7	2.352[5]	11.6	11	69	*(258)* 264

[2] FITC albumin/ albumin in 25:75 ratio, FITC albumin as the model drug

[3] The values indicated in *italic* were measured pre ultra-filtration; the other values indicate the particle size post ultra-filtration.

[4] Batches LH31-L4 are double batches

[5] The liposomes were prepared with 112mg of alteplase, which contains 2.353mg tPA

Table 3: Encapsulation efficiency in Liposome, LipHAX and PLGA.

Liposomes

DOPC (mg)	cholesterol (mg)	albumin (mg)	Alteplase (mg)	tPA (mg) in Alteplase	measured tPA (mg/ml) in liposome	% TLE	% ELE	**% EE**
23.5	5.9	44.7	112	2.352	0.028	1.3	0.32	**25.7**

LipHAX microparticles

HA-A (50kD) (mg)	HA-B (1.3MD) (mg)	liposome (mg)	tPA (mg) loaded	% TLE	% eLE not measured	**% EE assumed**
28	14	20	0.065	1.0E-01	----	**100**

PLGA microspheres

PLGA (mg)	albumin (mg)	tPA loaded	tPA (mg) lost in 3 washes	% TLE	% ELE	**% EE**
150	3.5	0.55	5.9E-04	0.36	0.55	**154**

%TLE = theoretical FITC-alb/liposomes % w/w
%ELE=FITC-alb/liposome %w/w
%EE=ELE/TLE

Table 4: LipHAX formulation, yield and size.

LipHAX batch	Model drug	HA-ADH	HA-CHO	Batch[6]	Liposome Volume (ml)	Liposome Concentration (mg/ml)	Mineral Oil (g)	Span 80 (g)	Homo-genization speed (rpm)	Yield (%)	Size (μm)
LH1a	FITC albumin	490kD 20mg	1.36MD 10mg	L1 6.2mg	0.5	12.46	25	0.1	1500	72	~20
LH1b	FITC albumin	490kD 20mg	1.36MD 20mg	L1 6.2mg	0.5	12.46	25	0.1	1500	54	~20
LH1c	FITC albumin	490kD 10mg	1.36MD 20mg	L1 6.2mg	0.5	12.46	25	0.1	1500	0	---
LH2	DW	490kD 10mg	1.36MD 20mg	---	0.5	-----	25	0.1	1500	0	---
LH3	Beta-glucosidase	490kD 20mg	1.36MD 10mg	L2 38mg	4.2	9.1	25	0.1	1500	26	~20
LH40	Blank	490kD 20mg	1.36MD 10mg	L3b0 19mg	5.46	3.5	25	0.1	1500	23	~20
LH41	Blank	490kD 20mg	1.36MD 10mg	L3b1 38mg	3.7	10.2	25	0.1	1500	25	~20
LH42	Blank	490kD 40mg	1.36MD 20mg	L3b2 76mg	7.4	10.2	50	0.2	9000	20	---
LH43	Blank	50kD 140mg	1.36MD 70mg	L3b 100mg	5.2	19.16	50	0.2	9000	32	---
LH44	Blank	50kD 140mg	1.36MD 70mg	L3b 100mg	5.2	19.16	50	0.2	9000	41	4.93
LH45	Blank	50kD 140mg	1.36MD 70mg	L3b 100mg	5.2	19.16	50	0.2	9000	33	---
LH46	Blank	50kD 140mg	1.36MD 70mg	L3b 100mg	5.2	19.16	50	0.2	9000	65	----
LH47	Blank	50kD 140mg	1.36MD 70mg	L3b 100mg	5.2	19.16	50	0.2	9000	40	4.90
LH5	FITC albumin	50kD 28mg	1.36MD 14mg	L4 20mg	0.71	28	25	0.1	9000	10	2.96
LH6	tPA	50kD 28mg	1.36MD 14mg	L5 20mg	1.72	11.6	25	0.1	9000	20	2.77

[6] See Table 2 for batch description

Table 5: Biocompatability in mouse model.

	Liphax mp	Liphax mp	Liphax mp	Liphax hydrogel pulverized microparticles	Liphax hydrogel
Total mass (mg)	50	25	25	50	50
Liposome (mg)	16.7	8.4	8.4	16.7	16.7
HA-A (50kDa) (mg)	23.3	11.7	11.7	23.3	23.3
HA-B (1.3 kDmg)	11.7	5.8	5.8	11.7	11.7
days to dissection	7	7	28	8.5	8.5
number of mice (n)	4	4	4	4	4
adhesion	4	4	4	2	3
residue	4	4	4	?	?
spleen size (cm^2) include stdev from the raw data	0.77	not available	0.98	0.99	0.97
adhesion (%)	100	100	100	50	75
residue (%)	100	100	100	?	?