Hyaluronan-Chitosan Polyelectrolytic Complex as a Platform for Delivery of Cytotoxic Agents

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Abstract

In the presence of water, hyaluronic acid (MW = 900k) will form a polyelectrolytic complex (PEC) with chitosan (MW = 600k) to create a network of nanoscale fibers. These fibers impart viscoelastic properties to the hydrogel while providing an excellent substrate for cell adhesion and growth. We are currently investigating the application of this material as a delivery system for cytotoxic agents. Specifically, conjugates of paclitaxel and hyaluronic acid (MW = 17k) have been synthesized to enhance the solubility of the paclitaxel while providing an additional mechanism for delivery via the CD44 receptor pathway. This conjugate is combined with the other macromolecules during the complexation process and may either become entrapped between the PEC fibers or make electrolytic complexes with the chitosan.

We hypothesize that this system will provide multiple mechanisms for drug delivery. First, free conjugate will diffuse out of the material into the surrounding tissue. As the material degrades, the conjugate will be released for long term delivery. Finally, since low molecular weight hyaluronic acid acts as a chemoattractant for many carcinomas, these cells will be directed into the material from the release of the degradation products where they will encounter the residual cytotoxic agents.

Background

Hyaluronic acid (HY) and chitin are naturally occurring biopolymers found in the extracellular matrix of mammalian tissue and in crustacean exoskeletons, respectively. Chitosan (CT) is formed from the deacetylation of chitin and rendered soluble by protonation of 70% of available amines (see Figure 1 for structures). At pH of 5.1, hyaluronic acid (pKa = 3.5) and chitosan (pKa = 6.2) become ionized in solution. In this state, the biopolymers self-assemble into nanofibers forming a polyelectrolytic complex (PEC). It is a malleable hydrogel identified by the acronym HCP-h.[25] The overall properties of the material are dictated by: a) the polymer molecular weights, b) solution pH, c) degree of deacetylation of chitosan.

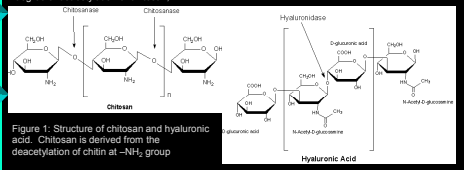

Figure 1: Structure of chitosan and hyaluronic acid. Chitosan is derived from the deacetylation of chitin at $-NH_2$ group

Cells of numerous malignancies, including adenocarcinoma of the breast, overexpress hyaluronic acid as well as its principal cell surface receptors, the receptor for hyaluronic acid mediated motility (RHAMM) and CD44.[18,27-32] A delivery vehicle based on HY might create a microenvironment favorable for migration of tumor cells into proximity with the drug. Besides the traditional modes of drug delivery (initial burst followed by sustained release), this delivery vehicle has the additional benefit of attracting malignant carcinomas into the loaded HCP-h material.

Loading of the HCP-h material with a drug is accomplished by hydrating the dry blend of CT and HY with an aqueous drug solution. Prior to the formation of PEC, This mixture can be injected to the site of a tumor in order to provide regional delivery of the cytotoxic agent. Systemic administration of cytotoxic agents is accompanied by a number of inherent difficulties, such as multiple organ toxicity and reduced concentrations of drugs reaching primary tumor sites.[20,21] High concentration, regional delivery of cytotoxic agents directly to primary tumor tissue offers a means to eliminate problems of systemic delivery while dramatically increasing concentrations of active drug at the diseased site.[22-24]

Paclitaxel (Taxol®) is currently produced for chemotherapy by semi-synthesis from 10-deacetylbaccatin as a cytotoxic agent for treatment of numerous malignant tumors including malignant melanoma and adenocarcinoma of the breast.[2-4] It is a diterpene produced by endophytic fungi residing in a variety of plants including *Taxus brevifolia* (pacific yew tree).[1] By binding to ß-tubulin in microtubule assemblies, paclitaxel and docetaxel, its more potent analog, promote formation and stabilization of microtubules. This results in disruption of centrosome organization (docetaxel more than paclitaxel), arrest of cell cycle at G2/M and induction of apoptosis.[3-5]

While paclitaxel is highly soluble in organic substances (n-octanol > 5mg/ml), it is poorly soluble in water (phosphate buffered saline = 1ug/ml).[7] Paclitaxel's poor aqueous solubility requires an excipient of polyoxyethylated castor oil and anhydrous alcohol in a 50:50 mixture, Cremaphor EL (CrEL), for its intravenous or intraperitoneal administration.[3,7] CrEL produces a number of adverse effects, chiefly hypersensitivity reactions in 10% of patients, with 2-4% of patients experiencing anaphylaxis or severe hypersensitivity episodes on first exposure. Consequently, all patients receiving taxanes are premedicated with corticosteroids and antihistamines.[8-10] Various efforts to circumvent CrEL toxicity focus on conjugation of paclitaxel with amino acids or low molecular weight hyaluronan, reformulation with albumin or encapsulation within liposomes.[11-18] Joining HY to paclitaxel results in a hydrophilic formulation capable of interacting with specific overexpressed receptors and upregulated metabolic pathways in cells of many malignant tumors.[19]

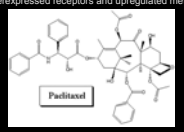

Figure 2: Structure of paclitaxel. HY is conjugated at the 2' hydroxyl group

Materials and Methods

Conjugate Synthesis

HY-Paclitaxel conjugate was synthesized using methods described by Luo and Prestwich.[17] An aqueous solution of excess adipic dihydrazide (ADH; Aldrich) was reacted with hyaluronan-sodium salt (Lifecore Biomedical;17 kDa), mediated by a carbodiimide (EDCI; Aldrich). The reaction was maintained at pH 4.7 for one hour, adjusted to pH 7.0, and the HY-ADH product purified by dialysis.

Succinic anhydride (Aldrich) was reacted with paclitaxel's 2'-hydroxyl group (Hauser Pharmaceuticals) at room temperature for 72 hours in CH_2Cl. The resulting paclitaxel-hemisuccinate was purified through silica gel and added to N-hydroxysuccinimido diphenyl phosphate (SDPP), independently synthesized from N-hydroxysuccinimide (NHS; Aldrich)) and diphenylphosphoryl chloride (Aldrich). The resulting paclitaxel-NHS ester was conjugated with HY-ADH to form HY-paclitaxel, purified by dialysis and lyophilized. Sixty milligrams HY-paclitaxel lyophilizate was hydrated with 5 ml Type 1A water (Dako) over 24 hours and centrifuged to remove undissolved particles. The supernatant was sterilized by passage through a 0.22µm filter. A concentration of 185ug/ml paclitaxel in solution was identified by scanning UV-VIS spectroscopy.

HCP-h Preparation

Under locally sterile conditions, 0.1% solutions of filter sterilized CT (pH=5.1; NovaMatrix) and acid form hyaluronan (HY+, pH=4.4) were lyophilized separately, subjected to mechanical particle size reduction and blended at a mass ratio of 1 part HY+ to 1.44 parts CT. The degree of deacetylation of CT is 85%. 35mg of HCP-h dry blend, 59% CT and 41% HY (by mass), were exposed to 500µL paclitaxel-HY conjugate solution (185µg paclitaxel / ml) in a 1.0cc tuberculin syringe and allowed to equilibrate under refrigeration for 24 hours. HCP-h dry blend imbibed 400uL of this solution leaving 100µL unabsorbed.

Cell Culture

Malignant melanoma cells, type C8161 were cultured in separate 6 well plates under Dulbecco's Modified Eagles Medium (DMEM) with natal calf serum (10% v/v) and gentamycin (0.02% v/v), at 37ºC under 5% CO2 atmosphere until they reached confluence at 48 hours. 250 µl of drug loaded hydrogel, charged with 46.25 µg paclitaxel-HY conjugate, was placed in one well while 150 µl hydrogel, charged with 27.75 µg paclitaxel-HY conjugate, was placed in another and cultured for 72 hours more at 37ºC. Control and experimental wells were then treated with propidium iodide (PI) and calcein-acetoxymethylester (calcein-AM) fluorescent stains to estimate the proportion of viable vs. non-viable cells. Except where noted all materials were obtained from Sigma Chemicals.

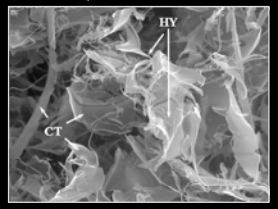

Figure 3: SEM of dry chitosan (CT) and dry hyaluronan (HY) leaflets following mechanical mixing to form the HCP-h dry blend. Orig. Mag: 200x

Figure 4: Insoluble fibers formed of HY-CT polyelectrolytic complexes (PECs) being drawn upward through unreacted HY and CT solution. Stereoscope, Orig. Mag: 50x.

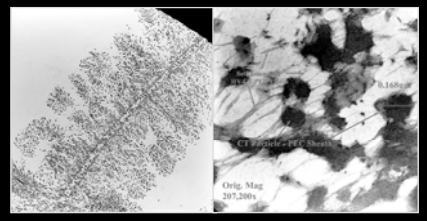

Figure 5: (Left) TEM of early HCP-h formulation revealing a highly ordered arrangement of HY-CT-PEC fibers. (Right) TEM image of specimen shown in left figure at high magnification. Note individual CT particles surrounded by HY-CT-PEC membrane and interconnecting network of HY-CT-PEC fibers at the nanometer scale. Orig. Mag: 207,200x

Control Wells - Vital Cell vs. Non-vital Cell Area Percent

Control Well Image	Control Well 1 Image 1	Control Well 1 Image 2	Control Well 1 Image 3	Control Well 1 Image 4	Control Well 2 Image 5	Control Well 2 Image 6	Control Well 2 Image 7	Control Well 2 Image 8	Average
Area Percent Vital Cell (Calcein-AM)	0.6305	0.2024	0.9040	0.8503	0.5151	0.7242	1.0494	0.1878	0.6330
Area Percent Non-vital Cell (Propidium Iodide)	0.0212	0.0425	0.1016	0.1144	0.1961	0.1752	0.2262	0.0766	0.1193
Ratio Vital Cell vs. Non-vital Cell Area Percent	29.7406	4.7624	8.8977	7.4327	2.6268	4.1336	4.6393	2.4517	5.3060

Table 1: Histomorphometric data of control wells showing a preponderance of vital vs. non-vital cells.

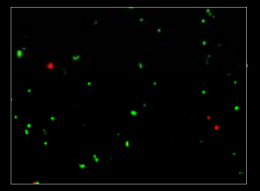

Figure 6: Typical control well image showing 0.9 area-percent vital cells vs. 0.1 area-percent non-vital cells. Orig. Mag: 10x.

Experimental Wells - Vital Cell vs. Non-vital Cell Area Percent

	250µL HCP-h Image 1	250µL HCP-h Image 2	250µL HCP-h Image 3	250µL HCP-h Image 4	250µL HCP-h Average	150µL HCP-h Image 1	150µL HCP-h Image 2	150µL HCP-h Image 3	150µL HCP-h Image 4	150µL HCP-h Average
Area Percent Vital Cell (Calcein-AM)	0.5357	0.5806	0.4056	0.4040	0.4815	0.3710	0.6760	0.5738	0.9149	0.6340
Area Percent Non-vital Cell (Propidium Iodide)	0.1473	0.4268	0.0666	0.1148	0.1889	0.5532	0.7493	0.6209	0.6383	0.6404
Ratio Vital Cell vs. Non-vital Cell Area Percent	3.6368	1.3604	6.0901	3.5192	2.5490	0.6706	0.9022	0.9241	1.4333	0.9900

Table 2: Histomorphometric data of the experimental wells treated with 250µL and 150µL HCP-h "cocoons". Non-vital cells predominate in sample treated with 150µL HCP-h / paclitaxel system.

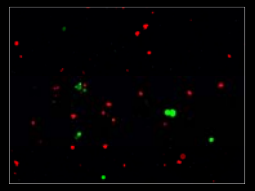

Figure 7: Image typical of experimental cells exposed to 150µL HCP-h "cocoon".

Results and Discussion

The theoretical ratio of 18% conjugation between HY carboxylate and paclitaxel was predicted. The current preparation was analyzed by UV / VIS spectrophotometry (Hitachi-U2001 scanning UV / VIS spectrophotometer with matched 10cm quartz cells) and determined to have 15.7% of HY carboxylate groups occupied by paclitaxel. Quantitation was determined by comparing absorbance of known paclitaxel standard vs. HY-paclitaxel conjugate sample at 227 nm.

Figure 3 shows CT leaflets juxtaposed to distinct HY particles in dry blend. Such positioning facilitates self-assembly of HY-CT polyelectrolytic complex (PEC) fibers, which begin forming immediately upon hydration of the dry blend (Figure 4) and contribute to mechanical properties of the fully formed HCP-h drug delivery device.

Cells from both experimental wells and from two control wells were trypsinized, centrifuged together with their media and resuspended in 2.5 ml PBS containing calcein-AM (10µL/ml) and propidium iodide (5µL/ml) to identify vital vs non-vital cells. Samples of each well were deposited on separate glass slides for observation by a Nikon Eclipse TE2000-S microscope at fitted with a monochrome digital camera (Diagnostic Instruments, Inc., Model No.: 11.0). Four images were acquired from each of the experimental samples and nine from the control samples. All were brought to color by ImageJ (National Institutes of Health) and analyzed for vital and non-vital cell area-percent comparisons by histomorphometry using Media Cybernetics' Image Pro-Plus 4.0 (Figs 6 and 7). Tables 1 and 2 summarize data from all images.

The drug delivery platform described here is a colloid in which insoluble, polyelectrolytic complex, fibers of hyaluronan and chitosan function as colloidal particles and water of hydration becomes the dispersing medium. Within minutes of hydration, these self-assembled PEC combinations produce an internal network of insoluble fibers, nanometers in thickness, sufficient to retain a large volume of water while providing the hydrogel with mechanical properties and stable geometry (Figures 3-5).[37,38] Figure 5 demonstrates transmission electron microscopy (TEM) images of the present HCP-h formulation showing collections of HY-CT-PEC fibers of nanometer proportions.

Acid form hyaluronan (HY+) was used for HCP-h colloid formation because dissociation of glucuronic acid's carboxylate in the acid form is substantially greater and more rapid than that of its salt. This facilitates formation of hydrogel material (ie. microscopic PEC fibers of HY and CT), but increases potential for HY autohydrolysis, especially following lyophilization.[39] Consequently, HY+ employed in the current formulation carried a lower weight average molecular weight (Mw = 350,000) and a much larger polydispersity (ρ = 32) than was originally intended. Since autohydrolysis proceeds in HY+ solutions even when frozen, and is exacerbated by lyophilization, it is important to employ newly produced HY+ material of the highest weight average molecular weight obtainable. We have not noticed any evidence of spontaneous molecular weight degradation for chitosan.

The proof of concept exercise reported here suggests that HCP-h material can function as a drug delivery platform. Area percent ratios for vital cells vs. non-vital cells decline when paclitaxel charged HCP-h is administered to the culture. Comparison of viable to non-viable cell area percent ratios from 150µL and 250µL cocoons reveals an inverse relationship of HCP-h volume and lethal efficacy. This counterintuitive finding may result from uneven distribution of HY-paclitaxel hydrating solution within HCP-h material as evidenced by its 80% hydration efficiency. Another possibility may reside in non-uniform distribution of dry CT and HY particles during dry blending, such that a disproportionately large mass of CT would be found in the 250µL "cocoon". This would favor PEC interactions between chitosan and the HY component of HY-paclitaxel conjugates, thus retarding drug release from this sample. Nevertheless, the average of non-vital cell area percentages from experimental cultures are greater than the average observed in control wells.

HCP-h may be employed, without accoutrements, as a three-dimensional cell culture system providing a microenvironment more closely resembling the cells' native, *in vivo*, circumstances. Since HCP-h retains significant volumes of water, we also conceive of it as a means for rehydrating the degenerating nucleus pulposus.[40-43] Additionally, cargos such as growth factors, morphogens, pluripotent cells and other therapeutic agents may be incorporated in the HCP-h system, making it useful in a variety of clinical applications. HCP-h may provide a continuum joining the basic science of cell biology with clinical applications in tissue engineering, regenerative medicine and disease control.

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References available upon request

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