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Evaluation of hyaluronic acid–protein conjugates for polymer masked–unmasked protein therapy

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ABSTRACT

Bioresponsive polymers may effectively be utilized to enhance the circulation time and stability of biologically active proteins and peptides, while reducing their immunogenicity and toxicity. Recently, dextrin-epidermal growth factor (EGF) conjugates, which make use of the Polymer-masked UnMasked Protein Therapy (PUMPT) concept, have been developed and shown potential as modulators of impaired wound healing. This study investigated the potential of PUMPT using hyaluronic acid (HA) conjugates to mask activity and enhance protein stability, while allowing restoration of biological activity following triggered degradation. HA fragments (Mw ~90,000 g/mol), obtained by acid hydrolysis of Rooster comb HA, were conjugated to trypsin as a model enzyme or to EGF as a model growth factor. Conjugates contained 2.45 and 0.98% (w/w) trypsin or EGF, respectively, and contained <5% free protein. HA conjugation did not significantly alter trypsin's activity. However, incubation of the conjugate with physiological concentrations of HAase increased its activity to ~145% ($p < 0.001$) that of the free enzyme. In contrast, when HA–EGF conjugates were tested in vitro, no effect on cell proliferation was seen, even in the presence of HAase. HA conjugates did not display typical masking/unmasking behavior, HA–trypsin conjugates exhibited ~52% greater stability in the presence of elastase, compared to free trypsin, demonstrating the potential of HA conjugates for further development as modulators of tissue repair.

1. Introduction

The potential of PEGylated protein and peptide conjugates has been realized (e.g. Neulasta®, Oncaspar® and Somavert®), establishing their routine use in a number of human diseases, including cancer, viral infections and rheumatoid arthritis (Duncan, 2003, 2006). However, while the 'first generation' polymer–protein conjugates utilized poly(ethylene) glycol (PEG), a non-biodegradable polymer, it is now evident that repeated administration of non-biodegradable polymers may be associated with potential problems including vacuolization, lysosomal storage diseases and, at high concentrations, may induce other pathological metabolic changes (Bendele et al., 1998; Chi et al., 2006; Miyasaki, 1975). Consequently, in an attempt to avoid these problems, people are increasingly using biodegradable polymers as components of polymer therapeutics. Duncan et al. (2008) have described a concept termed Polymer masked-UnMasked Protein Therapy (PUMPT), which uses biodegradable polymers to mask and unmask the biological activity of proteins and peptides. The hypothesis is that conjugation of a biodegradable polymer to a biologically active protein can mask activity and enhance stability in the bloodstream, while subsequent regeneration of activity can be achieved by triggered degradation of the polymer at the target site. This concept has been described using biologically inert dextrin as the model polymer, and has been successfully tested for a variety of biological proteins and peptides, including trypsin, melanocyte-stimulating hormone (MSH) (Duncan et al., 2008), phospholipase A_2 (PLA_2) (Ferguson and Duncan, 2009) and epidermal growth factor (EGF) (Hardwicke et al., 2008).

Hyaluronic acid (HA) has also demonstrated its potential as a component of PUMPT, having been conjugated to trypsin and ribonuclease A (RNase A) (Gilbert and Duncan, 2006; Gilbert, 2007). HA is a naturally occurring polysaccharide composed of N-acetylglucosamine and glucoronic acid sugar units that are polymerized into large macromolecules of several million Daltons (Almond, 2007). Unlike dextrin, HA has inherent biological properties, including antimicrobial activity, wound healing and tissue regeneration (Chen and Abatangelo, 1999; Price et al., 2007), which has led to its use in a number of medical fields, including osteoarthritis and cutaneous wound healing (Leach and Schmidt, 2004). The clinical safety of HA is, consequently, extremely well defined.

In the body, HA is enzymatically degraded by hyaluronidase (HAase) to smaller oligosaccharides (Kogan et al., 2007). This

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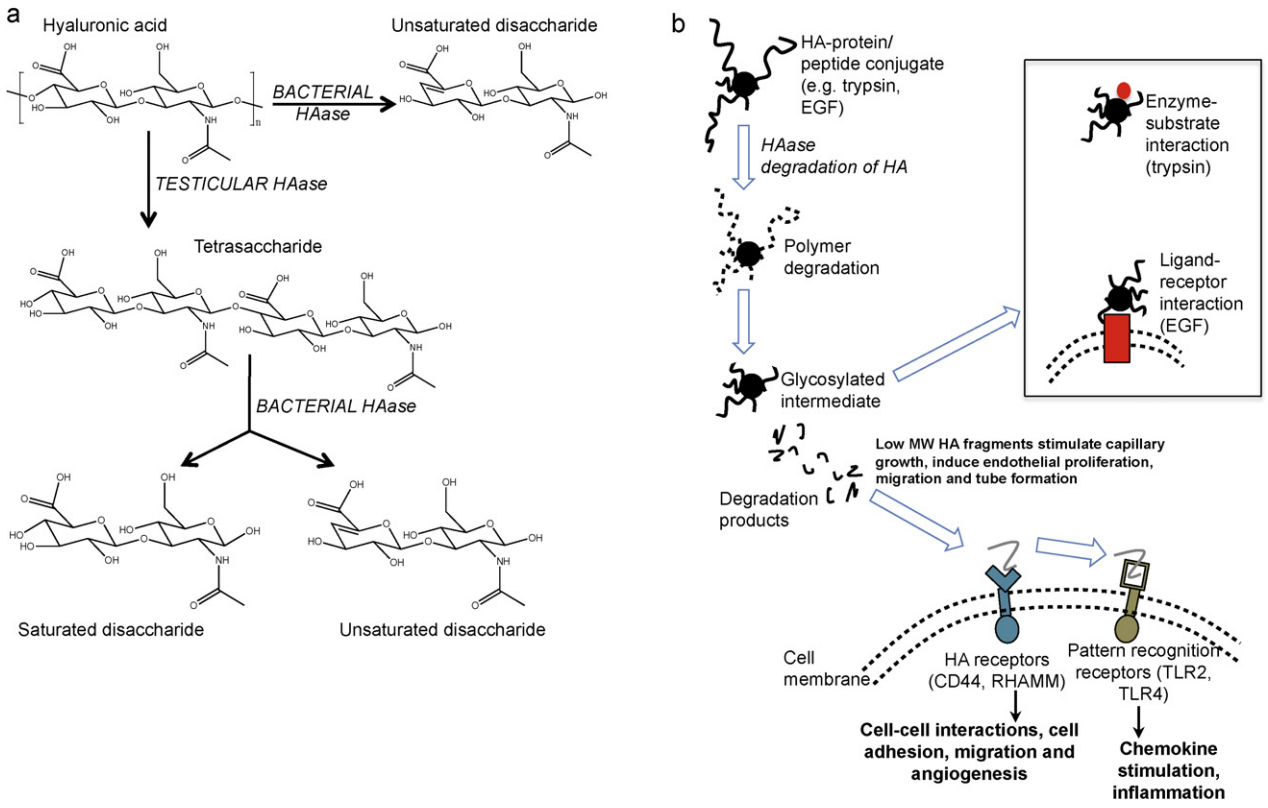

Fig. 1. Degradation of HA by HAase. Panel (a) shows the enzymatic depolymerization of HA by mammalian testicular and bacterial HAase, and panel (b) shows a schematic representation of the proposed mechanism of action of HA conjugates.

enzyme is present both in mammalian serum and intracellularly; and is secreted by bacteria. Differences exist in the mechanisms by which prokaryotic and eukaryotic HAases cleave HA, resulting in distinct differences in specificity and reaction products (Ludowieg et al., 1961; Stern et al., 2007). Mammalian testicular HAases hydrolyze the endo-β-n-acetylhexosaminic bonds of HA, chondroitin and chondroitin sulfate to yield oligosaccharides with a high prevalence of tetrasaccharides. Bacterial HAases are β-endoglycosidases that specifically cleave the β(1→4) bond of HA by β-elimination and the introduction of a saturated bond to yield disaccharides. These pathways are shown in Fig. 1a.

The aim of this study was to investigate the potential of HA as a component of PUMPT for potential use in tissue repair. It was hypothesized that conjugating HA to a complementary protein or peptide could enhance their biological effects to promote healing of chronic wounds (summarized in Fig. 1b). In these studies, the ability of three HAases (bovine, sheep and *Streptomyces hyaluroniticus*) to degrade HA was first assessed. Subsequently, the capability of HA to mask enzyme activity was measured using trypsin as a model enzyme and EGF as a model receptor binding ligand (with potential application is tissue repair). The biological activity of trypsin conjugates was assessed ±HAase using N-benzoyl-L-arginine-p-nitroanilide (L-BAPNA) as a substrate. The biological activity of the HA–EGF conjugate was measured using a cell proliferation assay with HEp2 cells.

2. Materials and methods

2.1. Materials and cells

HA from Rooster comb, HAase from sheep testes, bovine testes and *Streptomyces hyalurolyticus*, trypsin, elastase from human leukocytes, 1-ethyl-3-(3-dimethylaminopropyl carbodiimide hydrochloride) (EDC), copper (II) sulfate pentahydrate 4% (w/v) solution, bovine serum albumin (BSA), bicinchoninic acid solution (BCA), TRIZMA hydrochloride (Tris HCl), tissue culture grade dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), trypan blue and optical grade DMSO were all from Sigma–Aldrich (Poole, UK). Sodium acid phosphate, sodium phosphate, sodium chloride, N-hydroxysulfosuccinimide (sulfo-NHS) were from Fisher Scientific (Loughborough, UK). Pullulan gel filtration standards (Mw = 5900–853,000 g/mol) were from Polymer Laboratories (U.K.). Unless otherwise stated, all chemicals were of analytical grade. All solvents were of general reagent grade (unless stated) and were from Fisher Scientific (Loughborough, UK).

Human epidermoid carcinoma (HEp2) (ATCC No.: CCL-23) and Eagle's minimum essential media (EMEM) with L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate, non-essential amino acids and sodium pyruvate were purchased from LGC Promochem (Teddington, UK). Cells were screened and found to be free of mycoplasma contamination before use. Fetal calf serum (FCS) and 0.05% (w/v) trypsin–0.53 mM EDTA were obtained from Invitrogen Life Technologies (Paisley, UK).

2.2. Synthesis and characterization of HA–trypsin conjugates

First, HA (~1,400,000 g/mol) was dissolved in HCl (1 M, 37 °C) under stirring for 90 min. Subsequently, this mixture was cooled and neutralized using NaOH (10 M). This solution was transferred into a dialysis membrane (molecular weight cut-off 10,000 g/mol) and dialyzed against 4× 5 L ddH_2O. The solution was freeze-dried to yield degraded HA product as a white solid (~60% yield) that was characterized by FTIR (Avatar 360 ESP spectrophotometer with EZ OMNIC ESP 5.2 software; Thermo Nicolet, Loughborough, UK) to confirm identity, and by GPC (TSK G5000PW_{XL} and G4000PW_{XL}

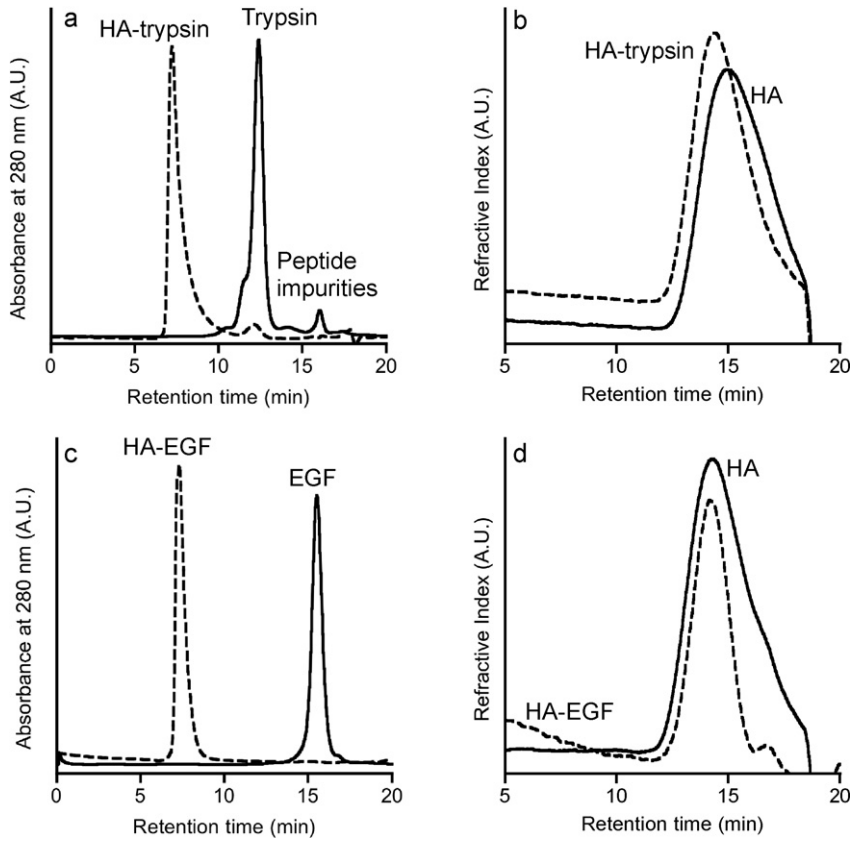

Fig. 2. Characterisation of HA–trypsin and HA–EGF conjugates. Panels (a) and (b) show FPLC of trypsin (—), reaction mixture (- - -) and purified HA–trypsin conjugate (- - -) (V_0 = void volume (7.7 mL)), and GPC of HA (—) and purified HA–trypsin conjugate, respectively (V_0 = void volume (13.5 mL)). Panels (c) and (d) show FPLC of EGF (—), reaction mixture (- - -) and purified HA–EGF conjugate (- - -), and GPC of HA (—) and purified HA–EGF conjugate, respectively.

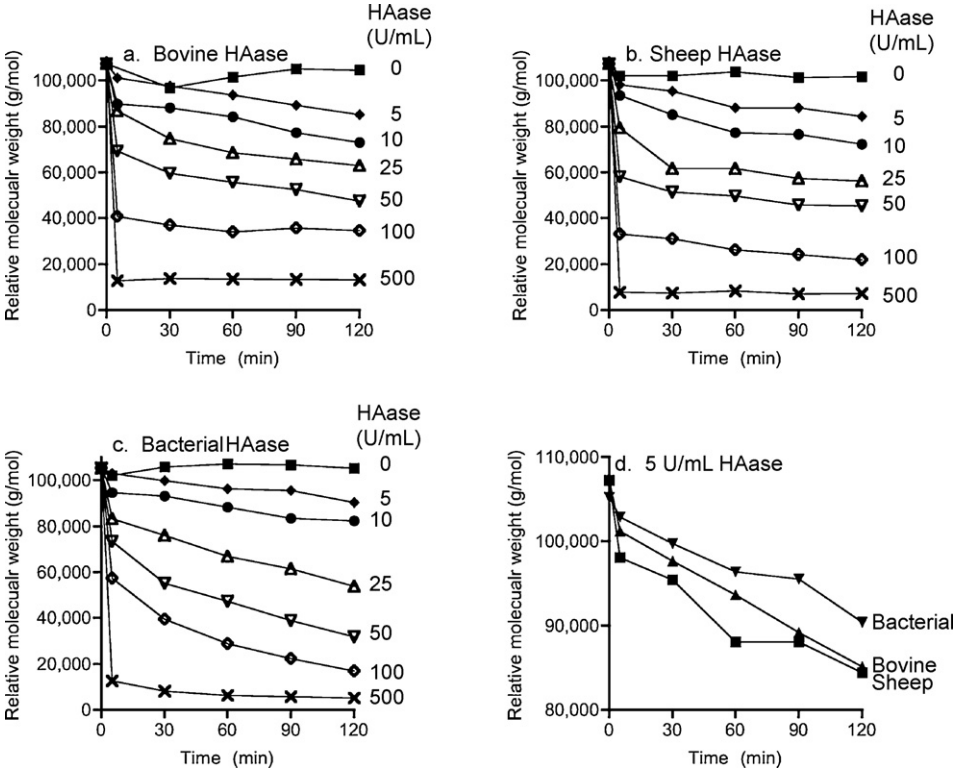

Fig. 3. Degradation of HA by (a) bovine HAase, (b) sheep HAase and (c) bacterial HAase. Panel (d) shows a comparison of HA degradation by HAases at a physiological concentration (5 U/mL).

Table 1
Characteristics of HA conjugates.

	M_w (g/mol)	Protein content (%, w/w) (molar ratio of HA:protein)	Free protein (%)	Activity (% ± SD)	K_m (mM)	V_{max} (mM/min)	K_{cat} (s^{-1})
Trypsin	23,400[a]	100	100	100	0.555	0.0224	2.91
HA–trypsin conjugate	115,500[b]	2.45 (10.9:1)	4.1	93.75 ± 8.23	0.369	0.0169	2.20
HA–trypsin conjugate + HAase	97,000[b]	–	9.27	145.08 ± 32.93	0.244	0.0214	2.79
EGF	6500[a]	–	–	–	–	–	–
HA–EGF conjugate	91,000[b]	0.98 (7.6:1)	<1	–	–	–	–

[a] M_w supplied by Sigma–Aldrich.
[b] M_w estimated by GPC using pullulan standards.

columns (Polymer Laboratories, Church Stretton, UK) in series, mobile phase PBS (0.1 M, pH 7.4), flow rate of 1 mL/min) to measure its approximate molecular weight and polydispersity (compared to pullulan standards). Samples for GPC were prepared in PBS (3 mg/mL) and the eluate was monitored using a differential refractometer (Gilson 153). PL Caliber Instrument software, version 7.0.4, from Polymer Laboratories (Church Stretton, UK) was used for data analysis.

Degraded HA was then conjugated to trypsin using methods optimized from those previously described (Duncan et al., 2008). Briefly, degraded HA (~100,000 g/mol, 200 mg, 2×10^{-6} mol) was dissolved under stirring in distilled water (dH_2O) (2 mL) in a 10 mL round-bottomed flask. To this, EDC (27.1 mg, 1.41×10^{-4} mol) was added, and the mixture allowed to dissolve for 10 min. Next, sulfo-NHS (30.7 mg, 1.41×10^{-4} mol) was added and the mixture left stirring for 40 min. Subsequently, trypsin (23.8 mg, 2×10^{-5} mol) dissolved in dH_2O (1 mL) was added, followed by NaOH (0.5 M) dropwise to raise the pH to 8.0. The reaction mixture was left stirring overnight (18 h). The conjugate was then purified from the reaction mixture by FPLC (ÄKTA FPLC; Amersham Pharmacia Biotech, UK) using a pre-packed Superdex 75 10/300 GL column with a UV detector and data analysis using Unicorn 3.20 software (Amersham Pharmacia Biotech, UK). Samples of the reaction mixture (1 mL) were injected into a 1 mL loop using 0.1 M phosphate buffer with 0.15 M sodium chloride, pH 7.4 at 0.5 mL/min as a mobile phase. Fractions (1 mL) were collected, desalted using Vivaspin tubes (10,000 g/mol cut-off) and assayed for protein content (BCA assay) before pooling fractions 6–9 containing conjugate. The final conjugate was lyophilized and stored at −20 °C.

HA conjugates were characterized by GPC (same system as used previously) to determine their approximate molecular weight (against pullulan standards), FPLC was used to assess purity (against protein standards), and the total protein content of the conjugate was determined by the BCA assay using trypsin or EGF standards. The FPLC system described above for purification was used again for final conjugate characterization. Samples (100 μL) were injected

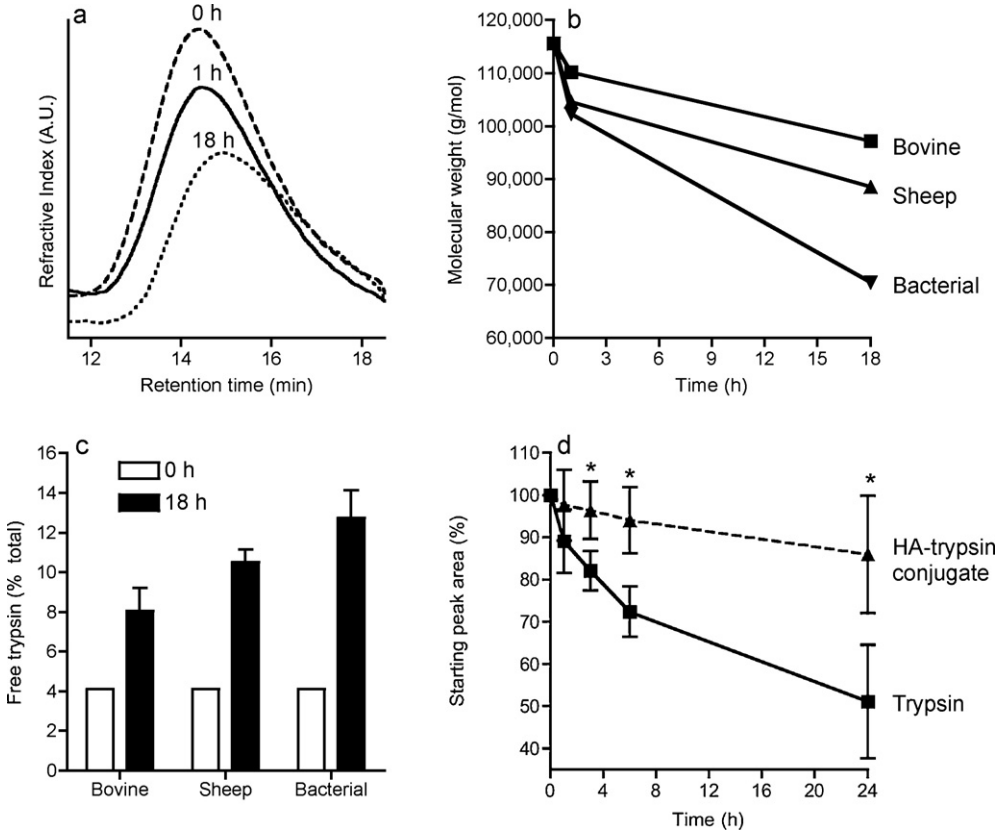

Fig. 4. Effect of addition of HAase on trypsin release and stability of HA–trypsin conjugate in the presence of neutrophil eleastase. Panel (a) shows a typical GPC elution profile of HA–trypsin conjugate, exposed to HAase (5 U/mL) for 0 h (- - -), 1 h (—) and 18 h (···). Panel (b) shows the change in relative molecular weight (GPC analysis) of HA–trypsin conjugate in the presence of bovine, sheep and bacterial HAase (5 U/mL). Panel (c) shows the release of free trypsin (quantified using FPLC) during incubation of HA–trypsin (3 mg/mL) with HAase (5 U/mL, 37 °C) expressed as percentage free trypsin. Data show data as mean ± SD, $n = 3$. Panel (d) shows the stability (using FPLC) of trypsin and HA–trypsin conjugate during exposure to elastase (0.45 U/mL).

into a 100 μL loop with a 0.1 M phosphate buffer with 0.15 M sodium chloride, pH 7.4 at 0.5 mL/min. The molecular weight was estimated relative to protein standards.

2.3. Degradation of HA and HA–trypsin conjugate by HAase

2.3.1. GPC analysis

HA and HA–trypsin (all at 3 mg/mL) were dissolved in Tris buffer, HAase from bovine testes, sheep testes and *S. hyalurolyticus* added (0–500 U/mL), thoroughly mixed and incubated at 37 °C for up to 18 h. GPC was used to estimate Mw, Mn and polydispersity as before.

2.3.2. FPLC analysis

Samples (100 μL) of HA–trypsin (with or without exposure to HAase) were prepared as above. Samples were analyzed by FPLC. The peak area corresponding to the HA–trypsin conjugate and free trypsin was determined and the data expressed as % conjugate with time.

2.4. Measurement of trypsin biological activity ± HAase

Enzyme activity was measured using a colorimetric assay with L-BAPNA (adapted from Johnson et al., 2002). First, native trypsin or HA–trypsin conjugate was dissolved in Tris buffer (equivalent to 0.1 mg/mL, pH 8.2) and a solution of L-BAPNA (7 mg/mL in DMSO) was prepared. All samples were prepared fresh before use and placed on ice throughout the experiments. L-BAPNA solution (2–10 μL) was pipetted into a 96-well plate and made up to 194 μL with Tris buffer (final L-BAPNA concentration 0.161–0.805 mM). Blank wells were also prepared containing L-BAPNA (0.161–0.805 mM) in Tris buffer. Plates were incubated at 37 °C for 5 min prior to starting the experiment. To start the assay, trypsin (6 μL of 0.1 mg/mL to make up to 200 μL) was added to the sample wells, and the rate of NAp release at 400 nm was monitored over 10 min at 37 °C. Results were expressed as rate of NAp release (±SEM, $n = 9$) over time, where ε_{NAp} was taken to be 8800 L/mol/cm (Johnson et al., 2002). The data was analyzed using the Hanes–Woolfe plot. Significance of the data was assessed using ANOVA and Bonferroni post hoc test.

The activity of HA–trypsin conjugate was also determined following incubation of the conjugate with bovine HAase. HA–trypsin conjugates (0.1 mg/mL trypsin equiv.) were first incubated with HAase (5, 10, 25 U/mL) for 18 h at 37 °C in Tris buffer (pH 8.2).

2.5. HA–trypsin conjugate stability in the presence of elastase

Trypsin and HA–trypsin (both 3 mg/mL) were each dissolved in Tris buffer (pH 8.2). Human leukocyte elastase (0.45 U/mL) was added to the samples and incubated at 37 °C for up to 24 h. Samples (200 μL) were taken at various time points and snap frozen in liquid nitrogen. At the end of the experiment, samples were defrosted and analyzed by FPLC as described above. The area of HA–trypsin and free trypsin peaks was calculated and results expressed as percentage of the starting peak area.

2.6. Synthesis and purification of HA–EGF conjugates

HA was degraded to ~90,000 g/mol and characterized as described previously. Degraded HA was conjugated to EGF using methods optimized from those previously described (Hardwicke et al., 2008). Briefly, degraded HA (23.7 mg, 3.0×10^{-7} mol) was dissolved under stirring in ddH_2O (0.5 mL) in a 10 mL round-bottomed flask. To this, EDC (3.21 mg, 1.67×10^{-5} mol) was added, and the mixture allowed to dissolve for 10 min. Next, sulfo-NHS (3.63 mg, 1.67×10^{-5} mol) was added and the mixture left stirring for 40 min. Subsequently, EGF (2 mg, 3.0×10^{-7} mol) dissolved in ddH_2O (0.5 mL) was added, followed by NaOH (0.5 M) dropwise to raise the pH to 8.0. The reaction mixture was left stirring overnight (18 h). The conjugate was then purified from the reaction mixture by FPLC using the same methods as for HA–trypsin conjugates. Total EGF content was determined using the BCA assay (with an EGF standard curve), and purity was assessed using analytical FPLC.

2.7. Cell proliferation of HEp2 cells incubated with HA, EGF and HA–EGF

The MTT assay was used to assess cell proliferation (72 h incubation) in HEp2 cell lines. Cells were seeded into sterile 96-well microtitre plates (2.5×10^4 cells/mL) in 0.1 mL/well of EMEM media containing heat-inactivated FCS (10%, (v/v)). They were allowed to adhere for 24 h. The medium was then removed and test compounds (0.2 μm filter-sterilized) were added. To study the effect of HA, HA–EGF conjugate, HA + EGF and EGF on cell proliferation, complete media (+FCS) was simply supplemented with a range of concentrations of each (0–1000 mg/mL HA, 0–500 ng/mL EGF equiv. and 0–500 ng/mL EGF, respectively). The activity of HA–EGF was also determined in the presence of HAase. Here, media containing HA–EGF conjugate was supplemented with HAase (bovine, 5 U/mL) and the protocol followed as described previously. Each day, MTT (20 μL of a 5 mg/mL solution in PBS) was added to the wells of one column and the cells were incubated for a further 5 h. The medium was then removed and the precipitated formazan crystals solubilized by addition of optical grade DMSO (100 μL) over 30 min. Absorbance was measured at 540 nm using a microtiter plate reader. Cell viability was expressed as a percentage of the viability of untreated control cells.

2.8. Statistical analysis

Data were expressed as mean ± the error, calculated as either standard deviation (SD) or standard error of the mean (SEM). Statistical significance was set at $p < 0.05$ (indicated by *). Where only two groups were compared, Student's *t*-test for a small sample size was used. In situations where more than two groups were compared evaluation of significance was achieved using a one-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests that correct for multiple comparisons. All statistical calculations were performed using GraphPad Prism, version 4.0c for Macintosh, 2005.

3. Results

3.1. Synthesis and characterization of HA conjugates

GPC confirmed a reproducible rate of degradation over time at 60 °C to achieve HA fractions with an average molecular weight (Mw) of 87,000 ± 11,500 g/mol, with negligible change in polydispersity (2.43 ± 0.3).

Using the HA intermediates, HA–trypsin conjugates were prepared and their characteristics are summarized in Table 1. The trypsin content was typically 2.2–2.7% (w/w). FPLC analysis of pure HA–trypsin conjugate showed a small amount of free trypsin (<5%) (Fig. 2a). GPC using pullulan standards was used to estimate the apparent molecular weight of the conjugates (Fig. 2b), although it should be noted that this is not an absolute value, but an estimation of hydrodynamic volume. The apparent molecular weight (Mw) of the conjugates was typically ~120,000 g/mol.

HA–EGF conjugate was also synthesized using the HA intermediates, and after FPLC fractionation, FPLC (Fig. 2c) and GPC (Fig. 2d) analysis confirmed conjugate synthesis and absence of free EGF (<1%). The total EGF content was typically 0.91–1.05% (w/w).

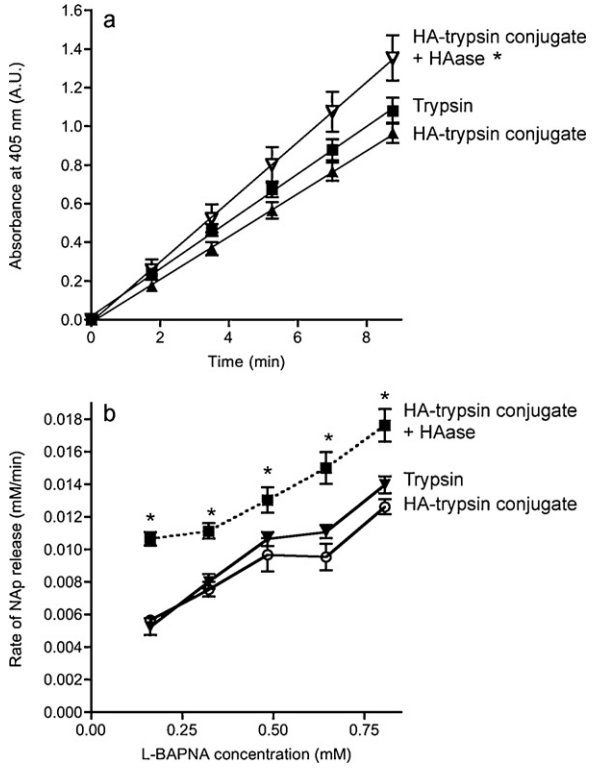

Fig. 5. Trypsin activity measured using L-BAPNA as substrate. Panel (a) shows the UV absorbance (400 nm) of NAp release from L-BAPNA by trypsin, HA–trypsin conjugate and 'unmasked' HA–trypsin conjugate (1.28×10^{-4} mM trypsin equiv.) over 9 min, and panel (b) shows concentration-dependent rate of NAp release by free trypsin, HA–trypsin conjugate and unmasked HA–trypsin conjugate. Data show activity as mean ± SEM, $n = 9$. *Significance $p < 0.001$ compared to free trypsin control.

3.2. Degradation of HA and HA–trypsin conjugate by HAase

GPC analysis of HA degradation by HAase revealed a decrease in molecular weight with time in a concentration-dependent manner (Fig. 3). While HA degraded rapidly in the presence of sheep and bovine HAase, bacterial HAase led to a more gradual degradation. At physiological concentrations of HAase (5 U/mL), HA degradation was most pronounced for mammalian HAases and a gradual decrease in HA size was observed in all cases.

Analysis of HAase-mediated degradation of HA–trypsin conjugate by GPC also showed a reduction in molecular weight over time (Fig. 4a, b). When degraded HA–trypsin conjugate was analyzed by FPLC, a peak corresponding to free trypsin appeared, which increased in intensity with time (Fig. 4c). In parallel, the peak corresponding to HA–trypsin conjugate decreased over the incubation time course. In these experiments, bacterial HAase was capable of releasing the most free trypsin (~12.5%) (Fig. 4c), and this HAase also induced the greatest reduction in molecular weight after 18 h incubation (Fig. 4b).

3.3. Measurement of trypsin biological activity ± HAase

Here, the activity of the HA–trypsin conjugate was unchanged compared to an equivalent concentration of native trypsin (Fig. 5). The rate constants, K_m, V_{max}, and K_{cat}, calculated for free and bound trypsin demonstrated reductions for HA–trypsin conjugates. While the K_{cat} was only partially reduced following conjugation (2.91 and 2.20 s^{-1}, for trypsin and HA–trypsin, respectively), V_{max} was reduced from 0.0224 to 0.0169 mM/min and K_m also decreased (0.555 and 0.369 mM for trypsin and HA–trypsin, respectively).

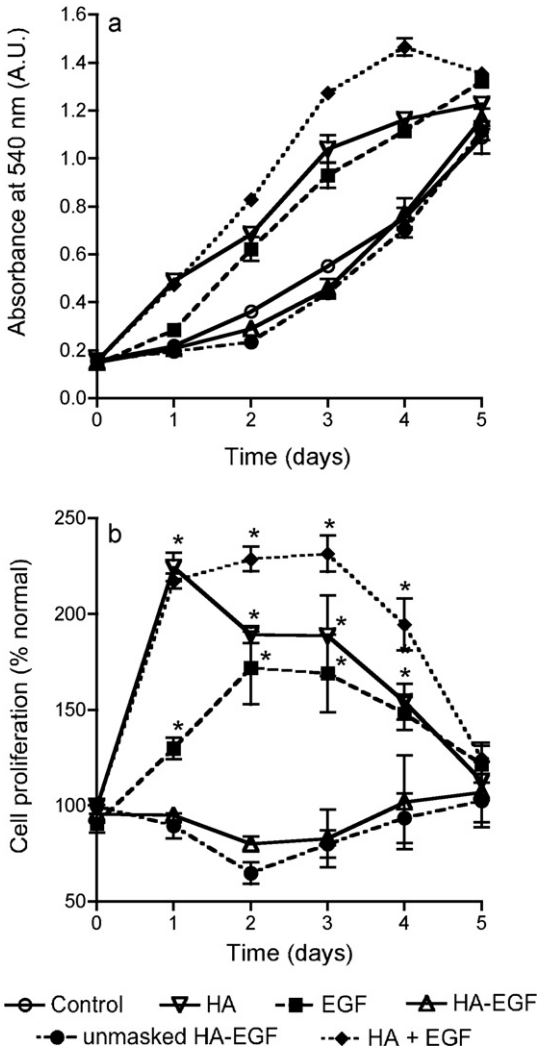

Fig. 6. Proliferation of HEp2 cells. Panel (a) shows HEp2 growth curves in the presence of serum-containing media (control), EGF, HA–EGF conjugate, HA–EGF conjugate + HAase, HA + EGF or HA, over 5 days. Panel (b) shows growth of HEp2 cells compared to untreated (control) cells. Data represent mean ± SEM, $n = 18$. Where error bars are invisible they are within size of data points. *Significance $p < 0.05$ compared to control.

When HA–trypsin was exposed to HAase for up to 18 h prior to enzyme activity assessment, there was a significant increase in the observed enzymatic activity of the HA–trypsin conjugate. While K_m remained similar to HA–trypsin, both V_{max} and K_{cat} were increased to mirror the values of free trypsin (0.0214 mM/min and 2.79 s^{-1}, respectively).

3.4. HA–trypsin conjugate stability in the presence of elastase

When trypsin was exposed to physiological concentration of human leukocyte elastase (0.45 U/mL) it was rapidly degraded by proteolytic degradation (49% degradation after 24 h) (Fig. 4d). However, the HA–trypsin conjugate was much more stable, with only 14% degradation after 24 h.

3.5. Cell proliferation of HEp2 cells incubated with HA fragments, EGF and HA–EGF

When cells were exposed to EGF or HA–EGF, native EGF caused the greatest increase in growth which peaked after 5 days (Fig. 6). When HA–EGF was added to the cell culture medium, HEp2

cell proliferation was the same as for control cells. Even when HAase-activated conjugate was added to the culture medium, cell proliferation was not significantly different to untreated cells. However, when culture medium was supplemented with HA fragments with or without EGF, cell proliferation was significantly greater than control cells and growth peaked at days 4 and 5, respectively. The greatest stimulation of cell proliferation was seen when cells were treated with a mixture of free HA fragments + EGF.

4. Discussion

Polymer-masked unmasked protein therapy was first described using dextrin as a model polymer (Duncan et al., 2008). Here, two model conjugates have been synthesized to assess the feasibility of PUMPT employing HA for protein masking in the presence of endogenous or bacterial HAase as the activating enzyme. HA is one of the predominant components of the extracellular matrix and is found widely throughout connective, epithelial, and neural tissues. Unlike dextrin, however, HA exhibits some inherent biological activity, including cell proliferation and migration and tumor progression (Gaffney et al., 2010). HAase is also widespread throughout the body and is found both intra- and extracellularly (Kreil, 1995).

Since enzymatic degradation of the polymer is required for PUMPT, it was first necessary to characterize the degradation of HA by HAase. While the most abundant type of HAase in the body is endogenously produced, the presence of exogenous HAase is relevant to a number of clinical conditions where bacterial pathogens (e.g. *Staphylococcus aureus*, *Streptococcus pyogenes*) colonize host tissues (e.g. chronic infections, skin wounds, periodontal disease and burns), releasing HAase as a virulence factor (Hynes and Walton, 2000). In these experiments, to test the potential degradation of the HA within the wound environment, we utilized both mammalian and bacterial HAases. While all species of HAase were capable of breaking down HA, bacterial HAase degradation did not follow the same kinetics as the mammalian HAases – probably due to its distinct cleavage mechanism. While mammalian HAases randomly cleave HA chains to produce tetrasaccharides as the final product, bacterial HAases are capable of translocating along the HA chain releasing disaccharides (Ludowieg et al., 1961; Stern et al., 2007) (Fig. 1a). Therefore, it was not surprising that the initial degradation of HA by bacterial HAase was not as effective as that observed with mammalian HAases. Moreover, these results, however, highlight the potential clinical impact of bacterial contamination and its role in controlling conjugate unmasking at any site involving colonization/infection by HAase-secreting bacteria.

4.1. HA–trypsin conjugates

The HA–trypsin conjugates synthesized here contained, on average, 2.45% (w/w) trypsin, corresponding to ~10.9 HA chains per protein molecule. Although free trypsin was easily removed by FPLC (to <5% unbound trypsin), unreacted HA was not so easy to eliminate. In these studies, we have not determined how much free HA is present in the purified conjugate, however, since both free and trypsin-bound HA elute in the void volume of the FPLC it is expected that unbound HA will be present. Here, we attempted to limit the amount of free HA by controlling the ratios of the reactants. In previous studies, free dextrin was detected in dextrin–trypsin conjugate samples by two-dimensional diffusion-ordered NMR spectroscopy (DOSY) (Duncan et al., 2008). It is important to note that, while dextrin is an inert polymer, HA stimulates a wide range of molecular weight-dependent biological effects, including fibroblast adhesion and proliferation, angiogenesis and inflammation (David-Raoudi et al., 2008; Huang et al., 2009). Fortunately, the molecular weight at which HA stimulates inflammation and angiogenesis – both vital steps in the wound repair process – is within the same range as the HA used here for conjugation and its degradation products (Gao et al., 2010). Nevertheless, the purification and characterization steps require careful optimization to ensure complete removal of free polymer to minimize conjugate heterogeneity prior to clinical testing.

The potential value of HA conjugation in resisting degradation at sites of inflammation is highlighted by the enhanced stability of HA–trypsin conjugates in the presence of elastase. In these experiments, trypsin degradation was reduced by ~70% compared to free trypsin. This is likely to have important therapeutic implications; sites of inflammation in human disease being characterized by increased proteolytic activity. Moreover, such improved stability may allow less frequent administration of the conjugate and reduced dose.

The ability of physiological concentrations of mammalian HAase (bovine) to reinstate biological activity of trypsin was assessed after an 18 h incubation of HAase and conjugate. Previously, conjugation of trypsin to dextrin, monomethoxy-PEG (mPEG) or semi-telechelic poly[N-(2-hydroxypropyl)methacrylamide] (ST-HPMA) has resulted in reduced enzyme activity in all cases (15–19, 17–96 and 13.8%, respectively) (Treetharnmathurot et al., 2008, 2009). Surprisingly, the enzymatic activity of the HA–trypsin conjugate was not significantly different to that of free trypsin. Masking of a protein's activity has been previously demonstrated; for example, when HA was bound to α-interferon, the conjugate retained 71% of the antiviral activity of the unbound protein (Parent and Larsen, 2006). In this study however, 'unmasking' of the HA–trypsin conjugates by HAase increased the enzymatic activity by almost 1.5-fold that of free trypsin. Although this increased activity was not predicted, enhanced enzyme activity following polymer–protein conjugation is not unprecedented for HA conjugates. HA–superoxide dismutase (SOD) conjugates have been synthesized and investigated as a novel anti-inflammatory agent. Here, the conjugate retained 70% of the activity of free SOD in vitro, but when it was assessed in vivo, they exhibited much higher anti-inflammatory activities than either HA or SOD alone (Sakurai et al., 1997). HA-anti-Flt1 peptide conjugates have also been investigated for the treatment of corneal neovascularization (Oh et al., 2009). In this case, the HA-anti-Flt1 peptide significantly reduced the binding of Flt1-Fc to VEGF compared to the same amount of free peptide (28 and 35%, respectively).

In contrast to these studies, Gilbert and Duncan (2006) demonstrated PUMPT using HA–trypsin conjugates (3.7%, (w/w) trypsin). Here, trypsin activity was effectively masked (6.4%) following conjugation to HA. However, unmasking by HAase was only able to reinstate 23.3% of native trypsin's activity. It should be noted that these conjugates used a larger molecular weight of HA compared to these studies (135,000 and 87,000 g/mol, respectively), which suggests that longer chains of HA are required to mask the trypsin's activity by steric hindrance.

4.2. HA–EGF conjugates

HA–EGF conjugates were purified by FPLC to yield conjugates with <1% (w/w) free EGF. As expected, HA conjugation reduced the proliferative effects of EGF in vitro. Interestingly, however, the addition of HAase to the cell culture medium, failed to restore the biological activity of the EGF. These results contrasted markedly with previous experiments, in which α-amylase-activated dextrin–EGF greatly enhanced proliferation of HEp2 cells (Hardwicke et al., 2008). EGF has just two potential lysine residues for conjugation – K28 and K48 (Lu et al., 2001). Since K28 is likely to be buried within the tertiary structure of EGF, HA is most likely to bind to K48. However, this residue is situated in close proximity to EGF's receptor binding domain. It is therefore, proposed

that binding of HA inhibits receptor binding, preventing the proliferative activity of EGF. Even after HA degradation, oligosaccharides are likely to remain bound to the surface since HAase cleaves along the main HA chain rather than the bond between polymer and peptide.

In theory, the presence of serum in the cell culture medium may degrade HA and HA–EGF conjugates, leading to difficulties in creating controlled conditions. Although heat-inactivated serum was used here to minimize the degradation of HA by enzymes in the serum, HAase is a lysosomal enzyme that would also be expected to be produced by the cells during the experiment (Aronson and Davidson, 1967). Indeed, due to their cancerous origin, HEp2 cells have been reported to secrete significant levels of HAase in vitro (47.3 ± 6.7 mU/mg in 16 h) (Franzmann et al., 2003). Nevertheless, this does not justify the lack of biological activation in the untreated HA–EGF conjugates. It is, however, important to note that addition of HA and EGF (individually or combined) at the same concentrations was able to stimulate significant increases in the maximal proliferative response, such that maximum proliferation occurred 72 h earlier than was observed in the untreated cells.

In these studies we have further demonstrated the potential of HA in PUMPT. Importantly, however, we have shown that this effect is not universal and is dependent upon the proteins and peptides to which it is conjugated. HA has many important roles in wound healing, including anti-oxidant, pro-inflammatory and immune-inducing properties (all of which support exogenous application in human wound healing). HA was confirmed here (as it has been in a number of other systems (Ellis and Schor, 1996; Brecht et al., 1986)) to represent an important "trigger" of proliferation. While the application of PUMPT using HA does not appear to be universal, it must be remembered that the other potential benefits of polymer conjugates (e.g. targeted delivery and resistance to degradation in vivo) have not been studied in these systems and these may be important unrecognised benefits of using HA in polymer conjugates. Indeed, the enhanced stability of HA conjugates was clear when HA–trypsin demonstrated enhanced stability to proteolytic degradation by elastase.

5. Conclusions

This study evaluated HA conjugates as novel bioresponsive polymer conjugates and explored their suitability for PUMPT. HA conjugates were reproducibly synthesized and incubation with clinically-relevant concentrations of HAase was capable of degrading HA to release free trypsin. However, enzyme activity was not consistently masked, nor could it be readily reinstated in all instances. These data suggest that the effectiveness of the application of PUMPT is variable between both polymer and protein/peptide conjugate. Despite this, the conjugate's enhanced resistance to proteolytic degradation, coupled with the proven biological activity of HA, indicates that further physicochemical and biological evaluation of HA conjugates for enhanced tissue repair is warranted.

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