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Controlled drug delivery to the lung: Influence of hyaluronic acid solution conformation on its adsorption to hydrophobic drug particles

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Abstract

This work reports investigations into the interaction and adsorption of the hydrophilic polymer hyaluronic acid (HA) onto the surface of the hydrophobic corticosteroid drug fluticasone propionate (FP). The eventual aim is to formulate a bioadhesive pulmonary drug delivery system with prolonged action that avoids rapid clearance from the lungs by the mucociliary escalator.

Adsorption isotherms detailing the adsorption of HA from aqueous HA solution concentrations ranging from 0.14 to 0.0008% (w/v) to a fixed FP particle concentration of 0.1% (w/v) were investigated. The method of preparing FP particles with HA molecules adsorbed on their surfaces (FP/HA particles) involved suspension of the FP either in hydrated HA solution or in water followed by addition of solid HA, centrifugation of the solids to form a pellet, washing the pellet several times with water until no HA was found in the supernatant and then freeze drying the suspension obtained by dispersing the final pellet. The freeze dried powder was then analysed for adsorbed HA using a Stains-all assay. The influence of order of addition of HA to FP, time for the adsorption process, and temperature of preparation on the adsorption isotherms was investigated.

The non-equilibrium adsorption isotherms produced generally followed the same trend, in that as the HA solution concentration increased, the amount of HA adsorbed increased to a maximum at a solution concentration of ~0.1% (w/v) and then decreased. The maxima in the adsorption isotherms were close to the change from secondary to tertiary conformation in the HA solutions. Below the maxima, adsorption occurred via interaction of FP with the hydrophobic patches along the HA chains in the secondary structures. Above the maxima, secondary HA molecules aggregate in solution to form tertiary network structures. Adsorption from tertiary structure was reduced because strong interactions between the HA molecules limited the availability of hydrophobic patches for adsorption of HA onto FP. The influence of preparation variables on adsorption was also related to the availability of hydrophobic patches for adsorption.
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1. Introduction

Drug delivery to the lung for the local treatment of pulmonary disease with its two to four times daily dosing is far from ideal. The highly efficient clearance mechanisms that have evolved in the human respiratory tract give little time for drug action after dosing. Ideally, once daily therapy with prolonged drug action would allow more effective treatment of conditions such as asthma. An improved delivery system would allow for drug action during periods of the day where current therapies achieve none and the symptoms are most prevalent (Barnes, 1984). Recent research suggests that achieving sustained drug action in the lung is highly desirable (Surendrakumar et al., 2003; Cook et al., 2005).

Corticosteroid anti-inflammatory agents are widely used to prevent acute exacerbations of pulmonary disease. They reduce airway inflammation, oedema and microvascular leakage, secretion of mucus in the airways and also the hyperreactivity of bronchial smooth muscle. The mechanisms by which they act, however, is complex and still not fully understood. They are known to act on a subcellular level and on a variety of cells in the lung including mast cells, lymphocytes, neutrophils, macrophages, eosinophils and airway epithelial cells (Guyre and Munck, 1989; Schleimer, 1989). They are cleared rapidly from the site of deposition by the mucociliary escalator so that to achieve clinical efficacy regular two to four times daily dosing is

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required. Fluticasone propionate (FP) is a hydrophobic corticosteroid that has advantages over the earlier corticosteroids in that systemic side effects (such as skin thinning and osteoporosis) are reduced. The usual adult dose of FP is 100–250 μg twice daily increased to 1 mg in severe disease. A modified delivery system that provides constant levels of drug at the prime site of action for a prolonged time would enable greater control of the disease.

This work reports attempts to prepare a bioadhesive delivery system that avoids the mucociliary escalator by the adsorption of hyaluronic acid onto the surface of FP particles. The polysaccharide hyaluronan (hyaluronic acid, HA) was first isolated by Meyer and Palmer (1934) and has since been extensively investigated (Scott et al., 1991; Fraser et al., 1997; Esposito et al., 2005; Yamamoto et al., 2004). It plays many roles in mammals including regulation of water balance via osmotic pressure and flow resistance, interaction with plasma proteins by sieve and exclusion effects, lubrication through its rheological properties and stabilisation of structures by electrostatic and other interactions. HA was chosen for this work for a number of reasons. Firstly, because HA and the enzymes which metabolise it (mainly hyaluronidase) are endogenous to the pulmonary environment (Bollet et al., 1963). It has been isolated from the lungs of mammals (sheep, guinea pig, rat (Fraser et al., 1997), bovine (Westerman, 1972) and human lung parenchyma and pleura (Hallgren et al., 1985)). The quantity of HA in human lung secretions was found to be ∼66 ng/mL with values ranging from 34 to 423 ng/mL (Dentener et al., 2005).

Secondly, asthma is a disease characterised by immune hypersensitivity and the presence of inflammatory cells. HA has been shown to play a role in the function of various inflammatory mediators including the agglutination of alveolar macrophages (Shannon and Love, 1980) the function of lymphocytes (Darzynkiewicz and Balazs, 1971), monocytes, macrophages (Shannon and Love, 1980) and neutrophils (Forrester and Balazs, 1980). Although HA turnover in the lung has not been investigated, we anticipate that the use of small quantities of HA in a pulmonary delivery system is unlikely to lead to undesirable accumulation.

Finally, for a successful formulation, the delivery system must be bioadhesive, yet avoid clearance by the mucociliary escalator. We consider that high molecular weight HA may have these properties. It is bioadhesive (Barbault-Foucher et al., 2002) and it has been shown that high molecular weight HA present in the lung anchors proteins and enzymes preventing their removal by the mucociliary escalator, although HA of a lower molecular weight had the opposite effect in that it increased ciliary beat frequency to increase particle clearance in the lung (Forteza et al., 2001). High molecular weight HA was thus investigated in this work, in an attempt to anchor the FP long enough in the lung for a prolonged action.

Although HA is a hydrophilic molecule and FP particles are hydrophobic ($\log P = 4.6$), we hypothesised that interaction might occur under certain circumstances via hydrophobic patches within the HA chains. Other investigators have reported that HA can interact with the hydrophobic regions of molecules such as lecithin (Ghosh et al., 1994; Pasquali-Ronchetti et al., 1997) and the hydrophobic surface, graphite (Spagnoli et al., 2005) via such hydrophobic patches. In the current work, adsorption isotherms derived to characterise adsorption from different solution concentrations of HA under different experimental conditions are presented. The influence of adsorbed HA on the suspending properties of FP particles in water, and on the patterns obtained microscopically after freeze drying unwashed suspensions in situ is also reported.

2. Experimental

2.1. Materials

Micronised fluticasone propionate, FP, was donated by GlaxoSmithKline Research Laboratories, Ware, UK. Particle size analysis in 0.1% (w/v) Tween 80 using the Mastersizer 2000 (Malvern Instruments, UK) gave a volume based mean particle diameter of 1.88 μm, with a 10–90% range of 0.70–4.8 μm. Two samples of prokaryotic hyaluronic acid, HA (approximate molecular weight 1.49×10^6) produced by fermentation were donated by Vitrolife (Edinburgh, UK). Both samples of HA were from the same batch but either recrystallised from water (sample 1) or propan-2-ol (sample 2), respectively. The water content of the two HA samples determined by TGA (not shown) were 20.85% (w/w) and 7.54% (w/w), respectively. Reagents acetic acid (glacial GPR reagent 99.5% min), Stains-all (approximately 95%), and L-ascorbic acid (99.7%) were obtained from Sigma–Aldrich (Dorset, UK), 1,4 Dioxan (99.5% stabilised with ∼25 ppm of 2,6-di-*tert*-butyl-4-methylphenol) from BDH (Leicester, UK).

2.2. Assay for HA in the presence of FP

Initial experiments were performed to screen the suitability of several common assays for HA in the presence of FP particles. The assays investigated included Alcian Blue (Whiteman, 1973), uronic acid determination using microtiter plate assay (Hoogen et al., 1998) and fluorimetric determination with 1,4-aminothiophenol (Zhu and Nothnagel, 1991). All were unsuitable due to either interference by FP, especially at higher concentrations of HA, or incomplete dissociation of HA from the FP particles (Alcian Blue). Finally a spectrophotometric method based on the dye Stains-all (Benchetrit et al., 1977; Homer et al., 1993) was chosen due to its relative simplicity, lack of interference with FP and complete dissociation of the HA/dye complex from the FP particles which was confirmed microscopically.

Stains-all solution was prepared by dissolving the dye (final concentration 0.1 mM) in 50% (v/v) water and 50% (v/v) 1,4 Dioxan containing 1 mM acetic acid and 0.5 mM ascorbic acid. The 1,4 Dioxan was stabilised with approximately 25 ppm 2,6-di-*tert*-butyl-4-methylphenol. Dye solutions once prepared were stored in the dark at 25 °C and used within 14 days of preparation. A calibration line was prepared using HA solutions at concentrations ranging from 8×10^{-5} to 0.01% (w/v) by placing a 0.2 mL aliquot of HA solution in a 3 mL stoppered cuvette of 1 cm light path and adding 1.8 mL of dye solution and 1 mL of distilled water. The cuvette was inverted five times to mix

the components and the spectral absorbance was determined at 650 nm using a UV spectrophotometer (Unicam UV1). Above 0.01% (w/v) HA solution concentration a precipitate formed. Any HA solution assayed at a higher concentration was diluted appropriately prior to assay.

2.3. Adsorption isotherms

Adsorption of HA from aqueous solution onto FP particles suspended in water was undertaken at 4 °C at a fixed FP concentration of 0.1% (w/w) and HA solution concentrations varying between 0.08 and 0.2% (w/v). Initial experiments were performed using HA (sample 1) at four HA solution concentrations (0.14, 0.11, 0.07, 0.04% (w/v)) to investigate the effect of experimental variables on adsorption including: (i) method of addition of FP and HA and (ii) the time required for the adsorption processes to occur. Experiments were also performed at (iii) 25 °C (instead of 4 °C) and (iv) using a different sample of HA (i.e. sample 2, 7.54% water). Allowances were made for the water content of HA in all calculations. More extensive adsorption isotherms were then prepared at a fuller range of HA solution concentrations (seven concentrations between 0.14 and 0.008% (w/v)) using sample 1 HA and the best experimental conditions determined above. Concentrations of HA above ∼0.2% (w/v) were too viscous to recover FP particles completely after the centrifugation step of preparation (below).

Experiments were performed to determine the time required for HA to hydrate completely at 4 °C. Samples of HA (10 mL) at concentrations of 0.008 and 0.14% (w/v) were prepared in water, and 1 mL aliquots were removed from the top, middle and bottom of each sample at 12 h intervals ($n = 3$). Separate, undisturbed samples were used at each time interval. Complete hydration was considered to be when the three values were within 5% of each other, and this occurred between 60 and 72 h.

Two methods were used to investigate the influence of addition of HA and FP. In method 1, HA was added to 10 mL of water and allowed to hydrate for 72 h at 4 °C. Ten milligrams of FP was then added to the hydrated HA solution and dispersed using a pestle and mortar, followed by sonication. For method 2, a crude suspension of FP in water was prepared by adding l0 mL of distilled water in aliquots to 10 mg of FP using a pestle and mortar. The dispersion was sonicated, and then the dry un-hydrated HA was added to the sonicated dispersion.

The suspensions of FP prepared by each method were left for 168 h after sonication at 4 °C for adsorption to take place whilst stirring continuously (magnetic stirrer 75 rpm). To monitor the progress of adsorption with time, samples (3 × 1 mL) were taken at 24, 72 and 168 h after sonication and centrifuged at 13,000 rpm for 60 min. The resultant pellet was washed and centrifuged several times. The supernatants were collected, allowed to stand for 72 h to ensure solution uniformity, and then assayed for HA. When the assay indicated that the supernatant did not contain HA, the final FP/HA pellet was re-suspended and the suspension freeze dried for 26 h in an Advantage freeze drier (Virtis, UK). For a control, the micronised FP was processed by exactly the same methods but without the addition of HA, i.e. the FP was suspended in water, sonicated, then freeze dried.

To analyse for adsorbed HA, the freeze dried samples of FP control or FP/HA were re-hydrated by adding 5–10 mg of material to 0.2 mL of distilled water and incubating at 4 °C for 12 h. 1.8 mL of dye solution and 1 mL of distilled water was added to 0.2 mL of the re-hydrated dispersion in a 3 mL stoppered cuvette of 1 cm light path, the cuvette was inverted five times to mix the components, and the spectral absorbance measured at 650 nm. A mass balance calculation was used to ensure that all the HA (adsorbed and from the supernatant samples) was accounted for. If this correlation was out by more than 1% the whole experiment was repeated. Adsorption isotherms were then plotted of the amount of HA adsorbed onto FP particles expressed as a % (w/w) of HA adsorbed onto 1 g of FP/HA particles versus the HA solution concentration from which adsorption occurred. To investigate the influence of temperature the procedure was repeated at 25 °C, and the effect of using sample 2 HA for adsorption was also investigated.

2.4. Characterisation of FP/HA particles

2.4.1. Suspension formation

Suspensions (∼10 mg/mL) from the processed FP control and freeze dried FP/HA prepared from different solution concentrations of HA by method 1 (0.07% (w/v)) and method 2 (0.04, 0.07, 0.14% (w/v)) were obtained by vortexing the solid material in water for 30 s. Suspensions were also prepared from material taken at various stages of preparation, namely before the washing and centrifugation process (i.e. containing excess HA) and after the final washing but before freeze drying (i.e. no excess HA). All suspensions were monitored visually for sedimentation over a 1 week time period.

2.4.2. Microscopical evaluations

Suspensions prepared before and after washing were freeze dried on a microscope slide ($n = 3$) and examined in brightfield and between crossed polars using a Polyvar compound microscope (Reichart-Jung, UK).

2.4.3. Theoretical calculation of HA adsorption

The % (w/w) of HA required to cover 1 g of FP/HA particles as a monolayer adsorbed flat on the surface was calculated theoretically, using the following information:

FP particles	
The density of FP (GSK personal communication)	1.36 g/mL
Mean particle diameter (Mastersizer data in Tween 80)[a]	1.884 μm
Number of FP particles per gram	2.1×10^{11} particles
HA molecules	
Length of HA repeat unit (Winter et al., 1975)	1.96×10^{-9} m
Width of HA unit (confirmed using molecular models)	0.196×10^{-9} m
Theoretical molecular weight of repeat unit	376
Average molecular weight of HA chain	1.49×10^{6} Da

[a] The particle size distribution of FP, a drug used for pulmonary delivery, is narrow. Calculations were also performed at the limits of the 10–90% particle size range, i.e. particle diameters of 0.695 and 4.750 μm, respectively. Theoretical values were then compared to the experimental values to estimate the quantities of HA required to cover the surface of 1 g of FP/HA particles as a monolayer.

3. Results

3.1. Adsorption isotherms

Initial adsorption experiments were performed at a few concentrations to investigate the effect of preparation variables on adsorption and to evaluate the most reproducible method of preparation. Fig. 1 shows the influence of using pre-hydrated HA (method 1) or allowing HA to hydrate during adsorption (method 2) on adsorption isotherms prepared at 4 °C and left for 72 h (a) or 168 h (b) after sonication for adsorption to occur. The 24 h adsorption data (not shown) showed a large variability in adsorption isotherms (standard deviations > 100%). Fig. 2 shows adsorption isotherms at a greater range of concentrations after 168 h using method 2. The isotherm shows increased adsorption up to a HA solution concentration of about 0.1% (w/v), then reduction in adsorption. The influence of (a) changing the sample of prokaryotic HA from sample 1 (21.85% water) to sample 2 (7.54% water) on adsorption values and (b) the effect of temperature (4 and 25 °C) on adsorption values with sample 1, are shown in Table 1. The data indicate firstly that adsorption data at 25 °C is similar for samples 1 and 2, when the water content is allowed for in the initial calculations and secondly, although there are differences in the concentrations adsorbed at 4 and 25 °C for sample 1, the adsorption maxima are at the same concentration (0.11% (w/v)). In all the experiments, the majority of the starting quantity of HA was recovered from the washings

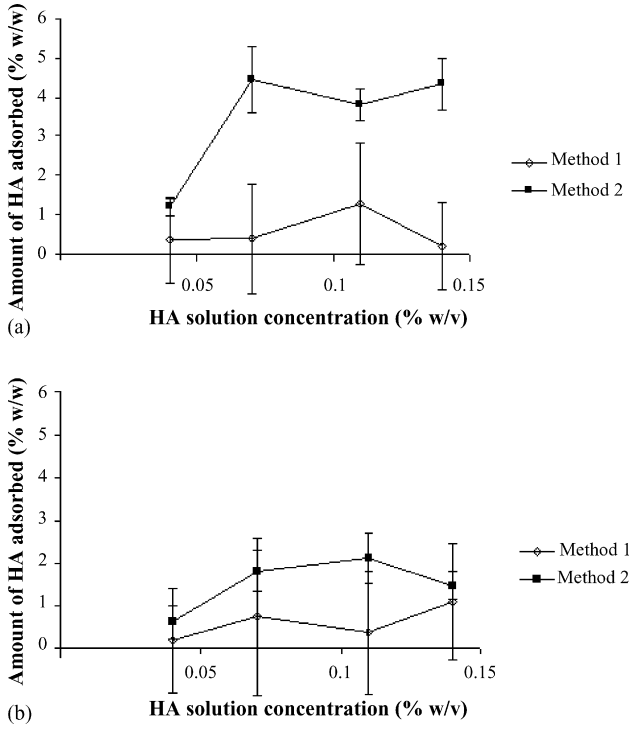

Fig. 1. Preliminary adsorption isotherms of the amount of HA adsorbed onto FP particles (expressed as % (w/w) of HA adsorbed onto 1 g of FP/HA particles) vs. the HA solution concentration from which adsorption occurred. The influence of method 1 (using pre-hydrated HA) or method 2 (simultaneous HA hydration and adsorption) on adsorption isotherms prepared at 4 °C and left for 72 h (a) or 168 h (b) for adsorption to occur.

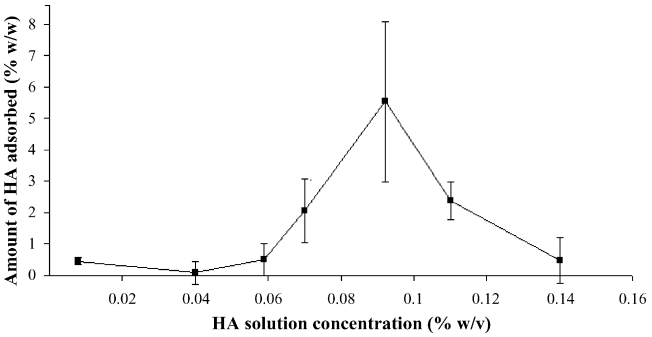

Fig. 2. Detailed adsorption isotherm of the amount of HA adsorbed onto FP particles (expressed as % (w/w) of HA adsorbed onto 1 g of FP/HA particles) vs. the HA solution concentration from which adsorption occurred prepared at 4 °C by method 2 and left for 168 h for adsorption to occur.

Table 1
Adsorption data for HA samples with different % moisture content (in parenthesis) and different temperatures of preparation

HA solution concentration (% (w/v))	Amount of HA adsorbed onto FP particles (% (w/w) FP/HA)		
	Sample 1 (21.85%) at 25 °C	Sample 2 (7.54%) at 25 °C	Sample 1 (21.85%) at 4 °C
0.14	0.55 ± 0.02	0.10 ± 0.05	0.45 ± 0.31
0.11	1.66 ± 0.42	1.35 ± 0.12	2.37 ± 0.25
0.07	0.41 ± 0.07	1.07 ± 0.27	2.05 ± 1.40
0.04	1.63 ± 0.29	0.60 ± 0.01	0.06 ± 0.01

prior to freeze drying, indicating that only a small quantity of the starting material was adsorbed and the HA concentration of the final supernatant prior to freeze drying was zero.

3.2. Evidence for interaction between FP and HA (FP/HA)

3.2.1. Suspension formation

Fig. 3 illustrates the appearance of suspensions prepared by re-suspending freeze dried FP/HA particles prepared by method 2 in water. The particles prepared from a HA solution concentration of 0.07% (w/v), i.e. close to the maximum in the adsorption isotherm, gave a smooth suspension that showed little sedimentation after 1 week storage (Fig. 3c). In contrast, suspensions prepared from HA solution concentrations below 0.04% (w/v) and above 0.14% (w/v) the adsorption maximum (Fig. 3b and

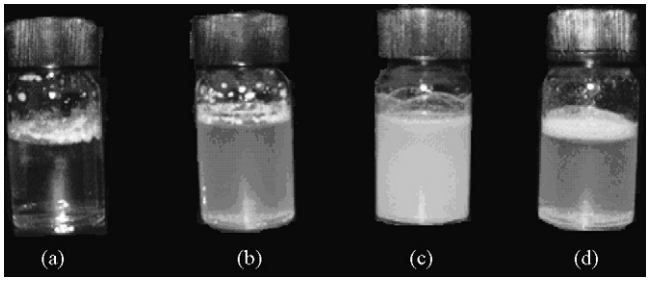

Fig. 3. Photographs of suspensions (~10 mg/mL) prepared from the processed FP control (a) and freeze dried FP/HA prepared by method 2 from HA solution concentrations of 0.04% (w/v) (b), 0.07% (w/v) (c), and 0.14% (w/v) (d).

d, respectively) formed cruder suspensions with many particles either at the surface or settled at the bottom of the vial. The FP control did not form a suspension as the hydrophobic particles floated on the surface. FP/HA particles prepared by method 1 (0.07% (w/v)) (not shown) formed a poor suspension. Similar results were obtained with suspensions prepared from material taken after the washing process was complete but prior to freeze drying, indicating that the freeze drying process did not influence the suspending properties of the material.

3.2.2. Microscopy

Fig. 4 shows photomicrographs of unwashed suspensions of FP/HA particles prepared using method 2 from HA solution concentrations of 0.04% (w/v) (a and b), 0.07% (w/v) (c and d)

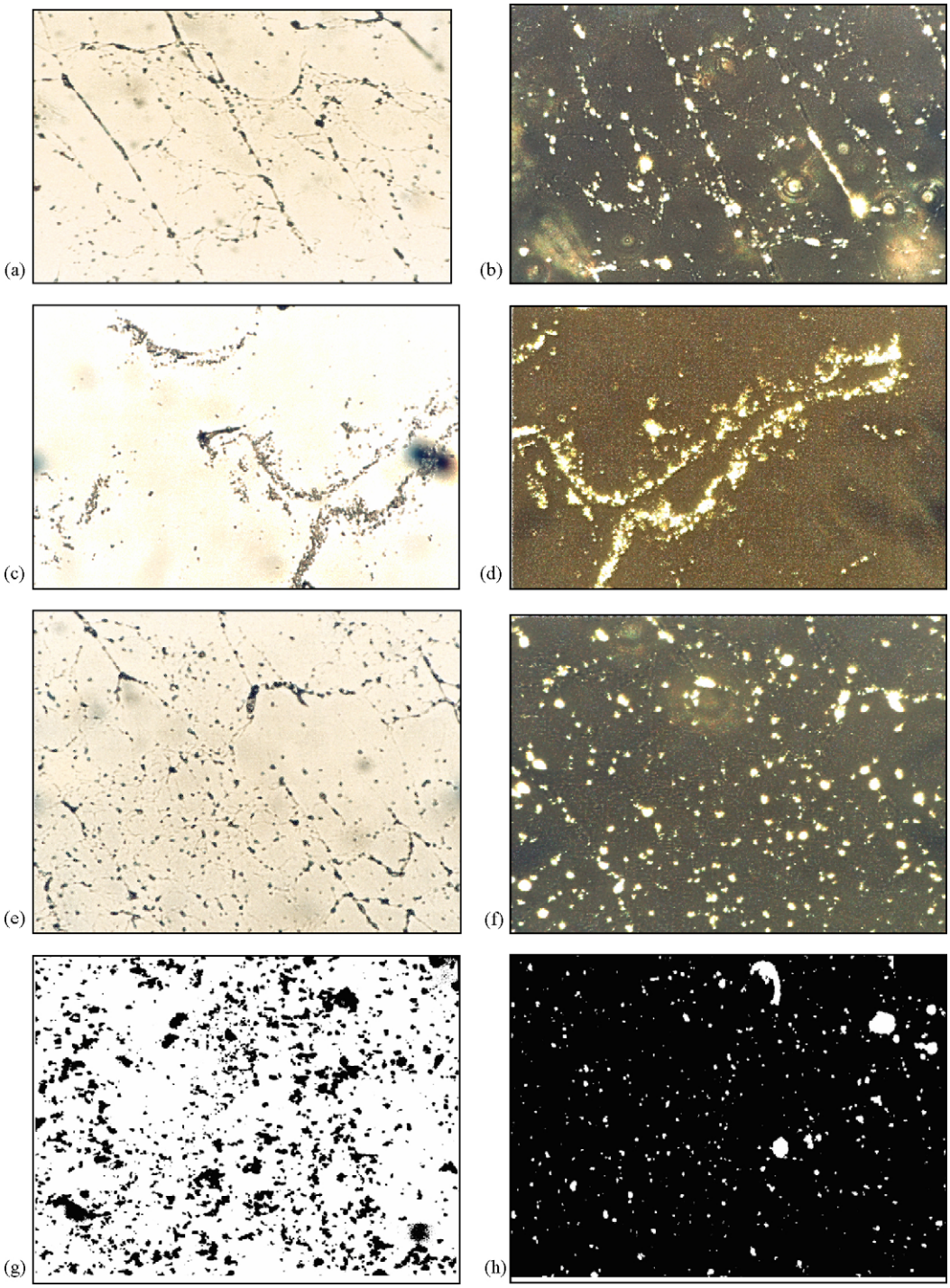

Fig. 4. Brightfield and polarizing microscopic images of FP/HA suspensions adsorbed from different solution concentrations of HA, and freeze dried in situ on the microscope slide prior to washing and centrifugation, i.e. in the presence of excess HA (a–f) and after washing and centrifugation, i.e. no excess HA (g and h). Solution concentrations: (a and b) 0.045% (w/v), (c and d) 0.07% (w/v), (e and f) 0.11% (w/v), and (g and h) 0.07% (w/v). Scale: 1 cm = 50 μm.

and 0.14% (w/v) (e and f) after freeze drying in situ. Fig. 4a, b and e shows some string-like ordering of the particles whereas Fig. 4c and d shows the majority of the particles lying in a large loop or necklace. Slides examined from suspensions formed after washing (i.e. with no HA excess) showed random particles and floccules of FP/HA particles (Fig. 4g and h).

3.2.3. Calculation of HA adsorption

The theoretical calculations using the mean particle diameter indicate that if the HA molecules are lying flat on the surface of FP particles as a monolayer, then ~0.3% (w/w) of the FP/HA particles will be composed of HA. When the diameter limits of the 10–90% particle size range are used, the values range from 0.1 to 1.5% (w/w).

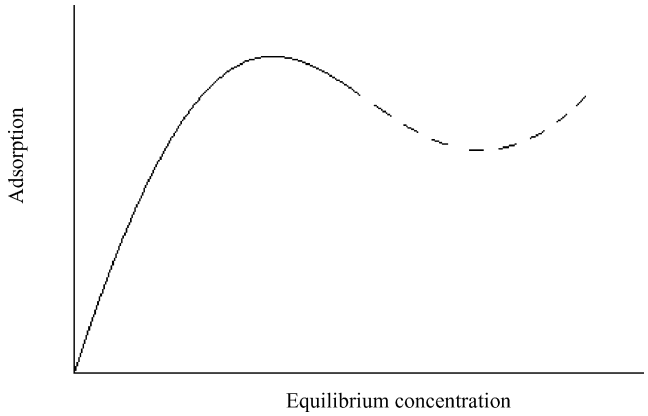

Fig. 5. An atypical adsorption isotherm (Giles et al.,1960).

4. Discussion

HA is a network forming polymer which takes up preferred conformations forming primary, secondary and tertiary structures in water at different polymer concentrations (Scott et al., 1991). The primary structure of HA in solution has not been isolated, but is probably a simple sequence of sugars at very low concentrations of HA acting independently in solution. At higher concentrations the secondary structure forms. In this structure HA molecules aggregate in pairs in solution to form ordered two-fold helical structures adopting extended random coil conformations (Scott et al., 1984). HA in the two-fold helix has extensive hydrophobic patches of about eight C–H units sequenced along its chain on the interior faces of the helix which stabilise the structure, whilst the exterior is stabilised by H bonds (Scott, 1989). At higher concentrations, secondary structure aggregates become entangled to form an extended three-dimensional network, the tertiary structure (Scott and Heatley, 1999). The concentrations of HA for conversion to tertiary network structure for HA of similar molecular weights to that used in this work has been reported to be ~0.1 mg/mL (Ghosh et al., 1994; Mikelsaar and Scott, 1994).

Samples were stored in a dessicator containing silica gel immediately after receipt from the manufacturer, and at all times thereafter. TGA data (not shown) obtained immediately after removal from the dessicator indicated that sample 1 HA contained $21.85 \pm 2.8\%$ water and sample 2, $7.54 \pm 1.2\%$ water ($n = 3$). Hence the moisture content of prokaryotic HA is appreciable and varies within the same batch of HA depending on the method of crystallisation when 10 mg samples are taken. To prevent fluctuations in HA water content when preparing samples for adsorption, HA was weighed and the required amount of water added immediately after removal from the dessicator. The appearance of HA was dependent on water content and varied from a hard rock-like appearance at low moisture content to light fluffy strands at the higher moisture content and such differences may influence hydration processes. We also found that tissue extracted HA generally contains in the region of 40% water (unpublished data). To avoid errors in reporting the concentration of HA in dilute solutions it is therefore essential to either dry the HA thoroughly or to account for the moisture content when calculating the actual weight % of HA in solution (as was done in this work). The extensive HA literature is sometimes confusing in that it is not always clear whether the HA sample has been dried, or whether the water content has been allowed for in aqueous concentration calculations.

The adsorption isotherms indicate that the amount of HA adsorbed to a 0.1% (w/v) FP dispersion from different concentrations of HA solution increased to a maximum as the concentration of HA increased and then decreased (Figs. 1 and 2). The concentration of the HA solution to give the maximum adsorption varied between 0.07 and 0.11% (w/v) depending on method of preparation, the temperature during adsorption, and HA moisture content. When higher solution concentrations were used, adsorption decreased with increasing HA concentration. It should be emphasised that our adsorption isotherms are not equilibrium adsorption isotherms in the normally accepted sense. Although the initial part of the adsorption isotherm is similar to a type III isotherm for a pure substance at equilibrium (Martin et al., 1993) the reduction in adsorption at higher HA concentrations indicates non-equilibrium conditions. Giles et al. (1960) reported a theoretical isotherm shape (c.f. Fig. 5) similar to those found throughout this work. Other researchers (Parfitt and Rochester, 1983) have related similar non-equilibrium isotherms to molecular associations in solution, for example the concentration-dependent association of surfactants and certain dyes which contain highly surface-active impurities. In our work, aggregation of HA molecules in the secondary conformation to form tertiary network structures may be responsible for the non-equilibrium isotherms. The maxima in the adsorption isotherms are close to literature values (0.1% (w/v)) for this change.

At equilibrium, the meshwork of secondary structure HA solution is held together by a large number of fairly weak interactions that can be broken apart by mechanical stresses such as stirring, pouring and centrifugation thereby exposing the hydrophobic patches along the HA chains. Our data imply that the HA anchors onto the hydrophobic FP surface via hydrophobic interactions with these patches. Similar mechanisms are believed to be responsible for the interaction of HA with other hydrophobic molecules and surfaces. For example, it has been shown that phospholipids also form complexes with HA in the secondary structure. The phospholipid opens out the HA

molecule by preventing intra molecular associations that occur in HA solution and the hydrocarbon tails of phospholipids to bind to HA hydrophobic patches (Ghosh et al., 1994). More recently the different conformations of HA has been shown to interact in different ways with the hydrophobic surface graphite (Spagnoli et al., 2005). In our system, as the concentration of secondary structure HA in solution increases, there are more chains in solution available for adsorption, and adsorption increases with increase in concentration as with normal type III adsorption isotherms.

The maximum in each adsorption isotherm correspond to a conversion of HA solution conformation from secondary to tertiary network structures. Reductions in adsorption at higher solution concentrations of HA implies that adsorption of HA onto FP from a tertiary network structure is not as effective as from secondary extended structures. The strong HA–HA interactions favoured in the tertiary network structures at high concentrations appear to limit the availability of hydrophobic patches for interaction with FP and adsorption reduces. This may explain the observations of Spagnoli et al. (2005), who noted that there was much greater adsorption to graphite at lower solution concentrations of HA compared to higher solution concentrations.

The availability of hydrophobic patches for adsorption may explain the influence of preparation variables on the adsorption isotherms. Method 1 (using previously hydrated HA) always gave reduced adsorption and larger error bars than method 2, involving the addition of non-hydrated HA to FP suspension. This is supported by visual observations. The ability of the particles to suspend in water was related to the quantity of HA adsorbed. FP particles at maximum adsorption (0.07% (w/v)), re-hydrated after the final freeze drying stage, formed a smooth suspension after preparation by method 2, indicating that the surface of the drug particle had been made more hydrophilic by the presence of adsorbed HA. In contrast, the FP/HA particles (0.07% (w/v)) generally formed a crude suspension after preparation by method 1 with many particles either at the surface or settled at the bottom of the vial, implying little adsorption of HA onto the surface of the particles. A possible explanation for reduced adsorption with method 1 is that fully hydrated HA is less likely to be adsorbed onto the surface of FP because its solution structure, stabilised by the hydrophobic patches, has already formed, whereas when hydration and adsorption occur simultaneously, as in method 2, FP particles can compete with the HA molecules for the hydrophobic patches so that some of the HA will be adsorbed onto FP rather than onto another HA molecule to form secondary structure.

The amount of HA adsorbed was found experimentally to vary up to a maximum of 4.83% (w/w). Although it is not possible to determine whether single or multilayers of molecules are adsorbed from the atypical adsorption isotherms obtained in this work, or even whether a full covering of each FP particle with HA is obtained, theoretical calculations give useful information about the adsorption. The calculations, where the extreme case of adsorbed HA molecules lying flat on the surface of the FP particles was assumed, gave an adsorption value for the quantity of HA to be between 0.1 and 1.5% (w/w) of FP/HA, with 0.3% (w/w) adsorbed at the mean particle size. The experimental values were higher than this figure, but in the correct range to speculate that the HA molecules are adsorbed via hydrophobic patches essentially flat on the FP surface separated by loops.

There were only minor differences in the position of the maximum and the percentage of HA adsorbed at this maximum when the experimental conditions such as the temperature of adsorption and the water content of the HA samples were varied (Table 1). This implies that factors including the higher viscosity of the HA solution in which the FP particles are dispersed and the increased density of water at 4 °C compared to adsorption at 25 °C have only a minor effect on the solution conformation of HA from which adsorption occurred. Differences in adsorption between the samples of HA containing different amounts of water may be due to minor differences in their hydration processes.

Further evidence for the interaction of HA with FP particles was obtained by a microscopical examination of unwashed suspensions freeze dried in situ on microscope slides. The suspensions, containing excess HA, showed distinct patterns microscopically depending on the solution concentration from which adsorption occurred, i.e. the amount of HA adsorbed onto the FP surface (c.f. adsorption isotherms). The relatively hydrophilic FP/HA particles produced from HA solution concentration 0.07% interacted strongly with the excess HA such that the particles appear to be linked as a necklace or loop (Fig. 5b). In contrast, when adsorption occurred from HA solution concentrations either considerably below (0.04%) or above (0.14%) the maximum in the adsorption isotherm, ordered strings of the less hydrophilic FP/HA particles are observed (Fig. 5a and c). These microscopical images, extending over several hundred microns are remarkably similar to images obtained mainly on a nanometer scale for molecular combing of DNA or HA (Spagnoli et al., 2005). The combing process generally relies on the action of a receding air water interface created during evaporation to uniformly straighten and align the molecules on solid surfaces such as mica, glass or graphite. In our work, the forces during the freeze drying process appear to cause the excess HA to link the FP/HA particles together on the glass slide. Samples dried after the washing process was complete and all the excess HA had been removed showed random particles and aggregates of FP/HA rather than highly ordered structures (Fig. 5d). Although the photomicrographs confirm interaction between FP and HA, further work is required to fully explain these images.

5. Conclusions

This work has shown firstly, that hydrophilic HA molecules can adsorb under specific conditions onto the surface of hydrophobic FP particles. Secondly, the extent of adsorption is influenced by the conformation of the HA molecules in the solution from which adsorption occurred. Significant adsorption occurred at HA solution concentrations in the secondary structure below 0.1% (w/v) due to hydrophobic interactions between the surface of FP drug particles and hydrophobic

patches along the HA chains. At higher concentrations, adsorption reduced because the tertiary network structures form in which the hydrophobic patches are used for strong HA–HA interactions and hence are not available for interaction with FP surfaces.

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