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Synthesis and properties of melt-processable hyaluronan esters

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A series of melt-processable hyaluronan (HA) esters were synthesized for potential biomedical applications (e.g., hot molding with thermoplastic ultra high molecular weight polyethylene for total joint replacements or molding tissue engineering scaffold). A silylated complex of HA with cetyltrimethylammonium cations (silyl HA-CTA) was used as the starting material. The reactions were performed with acid chlorides as the acylation agents in xylenes or no solvent other than the acid chloride. The disappearance of all characteristic FT-IR vibration bands associated with the $-OSi(CH_3)_3$ groups and the appearance of the strong ester carbonyl peak at 1753 cm^{-1} demonstrated the success of esterification. Thermoplasticity was achieved when the length of aliphatic chains in the HA esters was equal to or greater than 10 carbon atoms. It was found that the longer the ester chain, the lower the melting point; thus, to meet various needs different melting temperatures can be obtained by adjusting the acid chloride chain length.

1. Introduction

As a natural polysaccharide present in all vertebrate tissues and body fluids [1], hyaluronan (HA) has a unique set of physical and biological properties, including viscoelasticity [1], hydrophilicity [2–4], lubricity [4, 5] and cell-activity regulation [6]. Since Balazs prepared the first non-inflammatory fraction of sodium hyaluronate (NIF-NaHA) and used it for arthritic joint therapy of racehorses [7], the clinical uses of HA and HA-based materials continue to expand. These include: tissue separation and protection as a viscoelastic gel or solution in ophthalmic, abdominal and orthopedic surgery; postoperative adhesion prevention [8–10]; drug delivery [11]; scaffolds in tissue engineering [12]; and dressings in wound healing [13].

However, the extreme hydrophilicity and quick turnover [14] in the body environment limit the application of HA for permanent implants, and its use in conjunction with durable, hydrophobic biomaterials, such as polyethylene or polypropylene. Furthermore, HA and the currently available derivatives of HA degrade before melting. Thus, they cannot be hot molded with thermoplastic biomaterials or into unique, custom shapes. The objective of this study was to esterify HA to obtain melt-processable HA derivatives and improve HA's hydrophobicity and compatibility with other more hydrophobic materials.

Two classes of esterified HA have been patented, or have pending patent applications. Fidia Advanced Biopolymer in Italy developed one under the trademark Hyaff. Hyaff is a class of hyaluronan esters with the free carboxyl groups of HA esterified using different types of alcohols [15]. These HA esters can be used in medicine, surgery, cosmetics and other biomedical fields, but they are not thermoplastic (i.e. no melting endotherms) [16]. Yui *et al*. [17] modified the hydroxyl groups of HA with an acid halide carrying photoreactive groups, such as cinnamoyl chloride, in DMF solution in the presence of pyridine. The product was dissolved in DMF, and was subjected to ultraviolet radiation to crosslink HA. The HA derivatives are useful for pharmaceuticals, foodstuffs, cosmetics, etc., but the crosslinked polymer cannot melt.

It is well known that a variety of melt-processable cellulose esters have been synthesized to confer thermoplastic properties on cellulose. Generally the acid anhydride with a suitable catalyst or the acid chloride in the presence of a tertiary base was employed to esterify plain cellulose [18], but Stein and Klemm [19] reported a very different method. They used silyl cellulose as the starting materials to prepare cellulose esters of high aliphatic acids. This quick, non-solvent reaction was also applied to the esterification of HA in the present study.

A series of novel, melt-processable HA esters with varying aliphatic chain lengths were created from silyl HA-CTA (silylated HA complex with cetyltrimethyl ammonium salt, a hyaluronan derivative). The HA

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esters were then saponified to regenerate the HA. The synthesis and characterization of these novel HA esters are reported herein.

2. Experimental

2.1. Materials

Silyl HA-CTA was obtained through the methods described elsewhere [20–22]. The synthesis and characterization of the silyl-HA is reported elsewhere. Briefly, the silylation process is performed by first complexing the HA with cetyltrimethyl-ammonium bromide. XPS results demonstrate that the cetyltrimethyl-ammonium (CTA) bromide-HA complexing is stoichiometric (i.e., the ratio between cetyl groups and original carboxyl groups was 1:1) and the resulting degree of silylation at the –OH groups of the HA-CTA [21, 22] was equal to 4 (i.e., complete silylation).

The original sodium hyaluronate (HyluMed®, medical grade, MW: 1.36×10^6 daltons) was purchased from Genzyme (Cambridge, MA). Acid chlorides, including hexanoyl (caproyl), octanoyl (capryloyl), decanoyl (caprinoyl), lauroyl, palmitoyl, and stearoyl chlorides (see Table I), were purchased from Aldrich (Milwaukee, WI) and used as received. Xylenes, hexane and acetone were purchased from Fisher (Pittsburgh, PA), and were respectively dried by refluxing over Na, CaH_2 and anhydrous $CaSO_4$ and distilled just before use. Dimethylsulfoxide (DMSO), tetrahydrofuran (THF), Pyridine and potassium hydroxide (certified A.C.S) were also obtained from Fisher, but used as received. Ethanol (ACS/USP grade) was provided by Pharmco (Brookfield, CT), and used as received.

2.2. Synthesis of HA esters

Silyl HA-CTA, 200 mg, was added to 5–10 equivalents of acid chlorides under N_2 atmosphere. The mixture was heated for 0.5–1 h at 80°C. The reaction product was a clear, light yellow or brown solution, but once cooled, the solution became a turbid, viscous paste or, in the case of HA stearate, a solid. Hexane was used to precipitate the HA caproate, caprylate, caprinate and laurate from the product mixtures. Acetone was used to wash the excess of palmitoyl and stearoyl chlorides from the HA esters. After precipitating or washing several times, the resulting HA esters were dried in a 50°C vacuum oven until constant weight was obtained.

When performing synthesis with solvents, xylenes were used to form a homogeneous solution and promote reaction. After esterification, xylenes were removed under vacuum, while excess acid chlorides were removed with the same method used in the non-solvent synthesis.

2.3. Saponification of HA esters

For those esters soluble in acetone, such as HA caproate, caprylate, caprinate and laurate, 200 mg of HA ester were dissolved in 20 ml of acetone-ethanol mixture solvent (v/v 1:1), forming a clear solution. Aqueous 1 M KOH solution (5 ml) was slowly added to the solution through a pipette while swirling. The saponified HA ester gradually precipitated from solution. After addition of 5 ml water, the solution stood at room temperature for another hour to completely saponify the ester residue. The precipitate was filtered and the KOH was removed by dissolving the precipitate in water and re-precipitating with ethanol several times. The final white particle product, regenerated HA, was vacuumed dried in a 50°C oven until a constant weight was obtained.

For those HA esters insoluble in acetone, such as HA palmitate and stearate, 200 mg of ester were dissolved in 20 ml of a pyridine-ethanol mixture solvent (v/v 1:1). The rest of the saponification procedure was the same as above.

2.4. Fourier transform infrared spectroscopy (FT-IR)

A Nicolet Magna-IR 760 Spectrometer (E.S.P.) (Nicolet Instrument Corporations, WI) was used to record FT-IR spectra. Transmission absorption spectra were collected over a range of 600–4000 cm^{-1} at a resolution of 4 cm^{-1} with 128 scans.

HA caproate, caprylate, caprinate and laurate were dissolved in acetone and coated on NaCl disks for FT-IR analysis. HA palmitate and stearate were dissolved in xylenes and cast onto NaCl disks. Regenerated HA from ester saponification was mixed with KBr and pressed into pellets.

TABLE I Properties of HA and its aliphatic esters

Material	Acid chlorides	Formula of acid chlorides	Melting point (°C)	Starting point of degradation (°C)
HA			No[a]	212
HA-CTA			No	175
Silyl HA-CTA			No	161
HA Caproate	Hexanoyl	$CH_3(CH_2)_4COCl$	191.7	170
HA Caprylate	Octanoyl	$CH_3(CH_2)_6COCl$	188.9	184
HA Caprinate	Decanoyl	$CH_3(CH_2)_8COCl$	156.7	187
HA Laurate	Lauroyl	$CH_3(CH_2)_{10}COCl$	97.8	191
HA Palmitate	Palmitoyl	$CH_3(CH_2)_{14}COCl$	96.4	200
HA Stearate	Stearoyl	$CH_3(CH_2)_{16}COCl$	88.3	194

[a]No melting temperature: polymer degrades before melting.

2.5. X-ray photoelectron spectroscopy (XPS)

XPS analyses were performed on a PHI 5800 spectrometer (Physical Electronics, Inc., MN). The instrument was equipped with a monochromatic Al K_α (1486.6 eV) X-ray source. A low energy (5 eV) electron gun was used for charge neutralization on the non-conducting samples. Measurements were taken with an electron takeoff angle of 45° from the surface normal (sampling depth ~ 50 Å). High-resolution spectra (C1s, N1s) were obtained at a pass energy of 25 eV. Component peak analysis of high-resolution spectra was performed using XPSPeak 4.1 software. HA laurate was dissolved in xylenes and cast into a film on a glass slide for XPS analysis.

2.6. Differential scanning calorimetry (DSC) and thermal gravimetric analysis (TGA)

The thermal properties of all the HA esters were determined using Seiko DSC SCC 2200 differential scanning calorimeter and Seiko TG SCC 5200 thermal gravimetric analysis at a heating rate of 10°C/min in air.

2.7. Hot stage microscope analysis

HA ester film samples were prepared by evaporating solutions of HA ester in organic solvents (e.g., acetone for HA caproate, xylenes for HA laurate), which were placed between a microscope slide and a coverslip. The slide was placed on a hot stage mounted on a Nikon polarizing light microscope. The polymer was heated at a rate of 20°C/min and photomicrographs were taken with a Nikon FX-35DX camera.

2.8. Solubility studies

The solubility of the different HA esters in various organic solvents (DMSO, THF, acetone, xylenes, hexane) was visually examined. Approximately 50 mg of the given HA ester were mixed with 10 ml of solvents and stirred for 30 min. The HA ester was categorized as "soluble" in the given solvent if all HA ester particles were gone and a clear solution was formed. The HA ester was categorized as "swollen" in the given solvent if HA ester particles remained, but were visibly greater in volume. If no changes occurred to the particles, the HA ester was categorized as "insoluble" in the given solvent.

3. Results and discussion

3.1. Synthesis of HA esters

Compared with native HA, silyl HA-CTA used as a starting material is more reactive, making it useful as the intermediate of many further modifications of HA, including esterification. The trimethylsilyl groups of silyl HA-CTA are more easily replaced than the active hydrogen in HA hydroxyl groups. Esterification with silyl HA-CTA as a starting material (Fig. 1) was carried out without addition of any catalysts, and the reaction

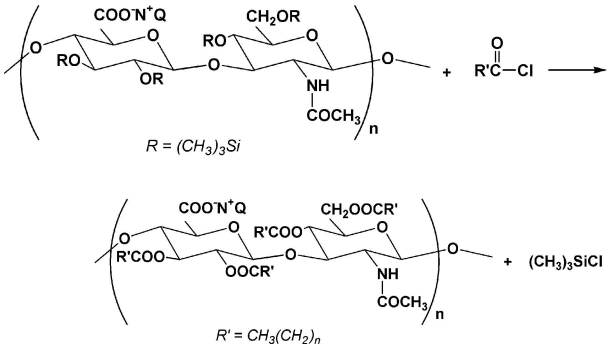

Figure 1 Synthesis of hyaluronan esters.

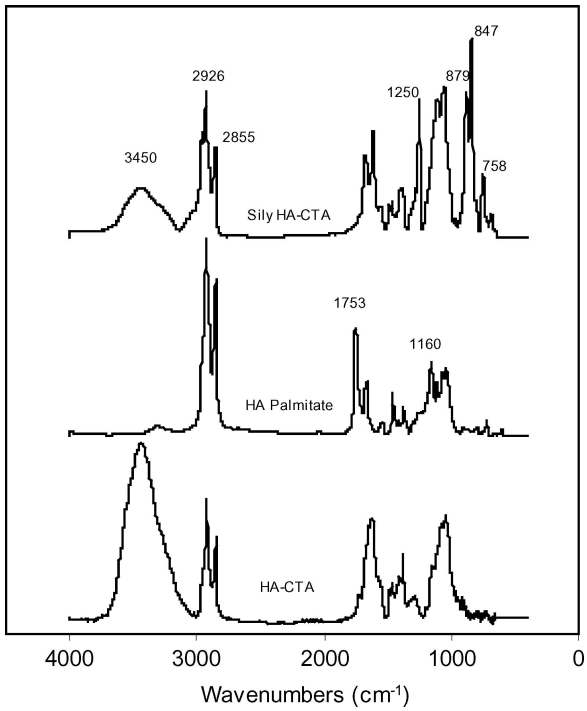

Figure 2 FT-IR spectra of silyl HA-CTA, HA palmitate and HA-CTA.

took place at a very high rate (within 0.5–1 h). The only by-product was trimethylchlorosilane (TMCS), which has a low boiling point (57°C), and can be evaporated at the reaction temperature (80°C).

The fact that the acylation takes place at the oxygen of the trimethylsilyloxy group —O—Si$(CH_3)_3$ in the silyl HA-CTA can be demonstrated by the complete removal of trimethylsilyl groups from HA esters. The FT-IR spectrum of HA palmitate is shown in Fig. 2 in comparison with HA-CTA (complex between HA and with cetyltrimethyl ammonium salt) and silyl HA-CTA. The spectra of all HA esters are similar, so the spectrum of HA palmitate is used as a representative. All the characteristic absorption bands related to trimethylsilyl groups, including bands at 1250, 879, 847 and 758 cm^{-1}, disappear in the HA ester spectra. A strong, new peak at 1753 cm^{-1} is observed for the HA ester, attributable to the carbonyl groups in saturated esters [21], indicating the introduction of a large amount of acyl groups with acylation. A strong peak near 1160 cm^{-1} associated with ester C—C—O asymmetric stretching also shows in the HA ester spectrum. The intensity of bands at 2926 and 2855 cm^{-1} also

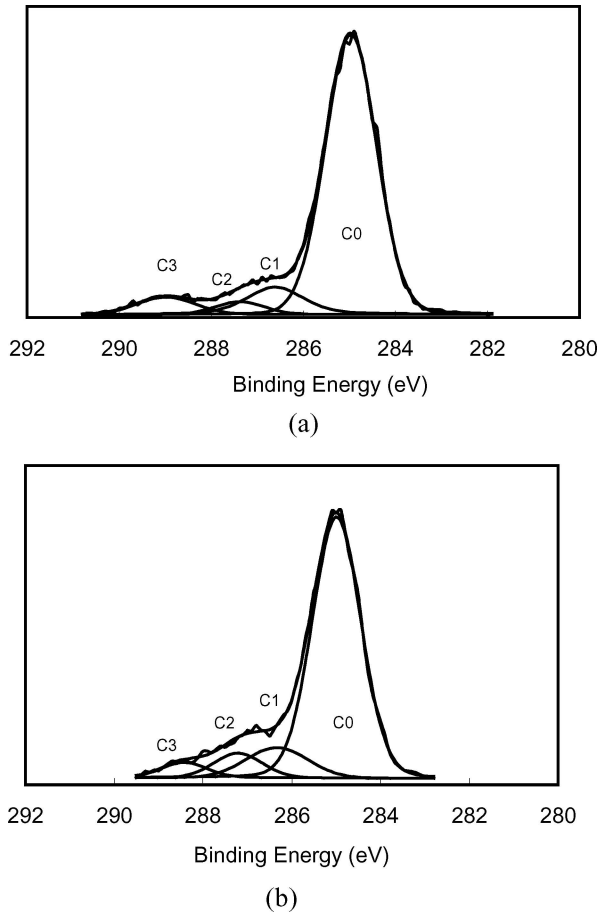

Figure 3 High-resolution XPS C1s spectra of (a) HA laurate; (b) silyl HA-CTA.

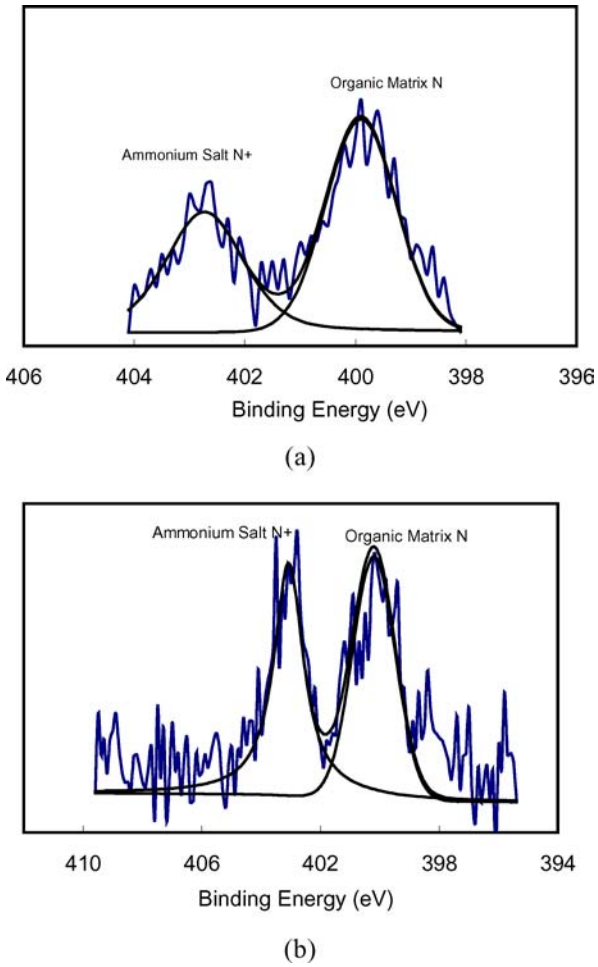

Figure 4 High-resolution XPS N1s spectra of (a) HA laurate; (b) silyl HA-CTA.

significantly increases compared to the silyl HA-CTA spectrum. This is due to the introduced long aliphatic chains of HA esters. Thus, this rapid esterfication reaction between the silylated hydroxyl groups and the acid chlorides results in complete esterification rather quickly. This is in direct contrast to traditional esterification reactions between ─OH groups and acid chlorides, which proceed slowly and show conversions dependent on the aliphatic chain length of the acid chloride.

The C1s XPS high-resolution spectrum of HA laurate is shown in Fig. 3. Like silyl HA-CTA, the C1s spectrum of HA ester can also be best fitted with four peak components based on the number of carbon-oxygen/nitrogen bonds: C0 (CHx, C─C; 285.0 eV, reference), C1 (C─O, C─N; 826.5 eV), C2 (O─C─O, C═O; 287.8 eV) and C3 (─O─C═O, ─HN─C═O; 289.2 eV) [22]. In comparison with silyl HA-CTA, the C3 component of the HA ester increases significantly, and its intensity almost doubles that of the C2 peak. In silyl HA-CTA, the C3 peak area is inferior to C2. The increase of C3 peak percentage and intensity relative to C2 in HA laurate can be explained with the introduction of large amounts of ester groups during esterification.

The N1s XPS high-resolution spectra of HA laurate and silyl HA-CTA are shown in Fig. 4. The N1s signal of the ester consists of two components: ammonium salt N^+ (402.8 eV) and amide N (399.8 eV) [23]. The intensity of ammonium salt N^+ signal is smaller than that of amide N signal in the ester sample, but they are almost equal to that observed in silyl HA-CTA. This may suggest that the acid chloride also attacked –CTA groups during esterification, but this attack was not dominant, because most ammonium salt N^+ groups still remain.

3.2. Regeneration of HA from HA esters

The FTIR spectrum of HA regenerated from saponification of HA caprylate is shown in Fig. 5. All the peaks characteristic of absorptions for –CTA and capryloyl esters are gone. There is no difference between the

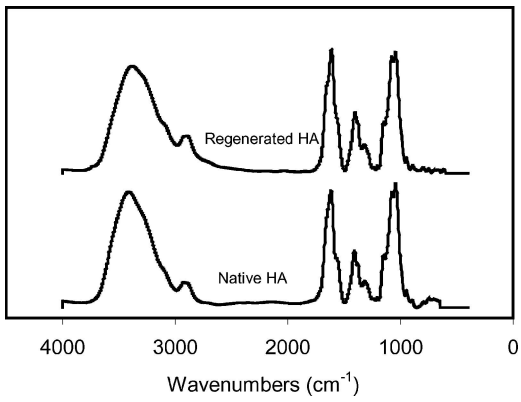

Figure 5 FT-IR spectra of regenerated HA from saponification and native HA.

spectra of regenerated HA and original HA, indicating the complete removal of –CTA and ester groups through saponification.

Thermal degradation/depolymerization of the HA under the HA ester synthesis conditions should be considered. The silylation studies [21, 22] included an investigation of the molecular weight change of HA in aqueous solution, using viscosity to measure molecular weight. It was found that the molecular weight of HA decreased by less than 11% after 48 h at 75°C. In the non-aqueous environments used for the silylation and esterification, the depolymerization is expected to be much slower. Thus, the 0.5–1 h, 80°C esterifcation reaction should not cause significant depolymerization. This is verified by the FTIR analysis: HA regenerated from the HA ester has the same spectrum as native HA. It is further verified by the following TG data, which indicates the onset of degradation for both the HA and HA esters is well above 80°C. Future work will use GPC and NMR to further confirm this lack of depolymerization.

Ester bonds are susceptible to hydrolytic cleavage, and under alkaline conditions, an irreversible saponification of ester groups can occur [18]. Therefore, the ester groups can be removed from HA esters with saponification when they are not desirable. Furthermore, alkaline metal cations (e.g. Na^+ and K^+) with sufficiently high concentration can displace the –CTA groups in the ester [24], resulting in a regenerated HA, as demonstrated in Fig. 5. It was reported that saponification of cellulose esters was normally performed in an alcoholic alkali solution [25, 26]. In order to increase wetting and thus reaction of the hydrophobic ester, a water-miscible and ester-soluble organic solvent was often added. In ASTM D 817-96, acetone or pyridine was used with methanol to dissolve cellulose esters with high content of propionyl or butyryl substitutions. A mixture of acetone and ethanol was used for saponification of these esters soluble in acetone, including HA esters from caproate to laurate. HA palmitate and stearate are insoluble in acetone, but dissolve in pyridine, so a pyridine-ethanol mixture was used for them.

3.3. Properties of HA esters

From the TGA data in Fig. 6, it can be seen that HA begins to degrade around 212°C, but no melting point

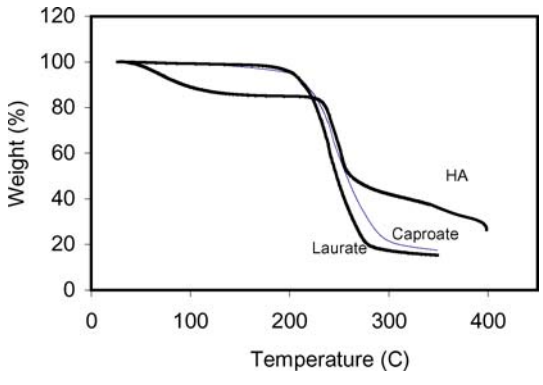

Figure 6 TGA of HA, HA caproate and laurate.

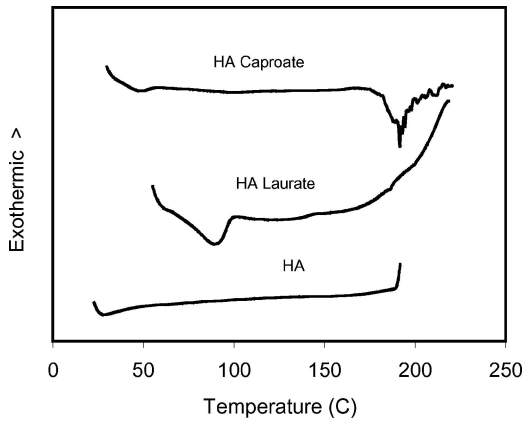

Figure 7 DSC of HA, HA caproate and laurate.

can be found below that temperature in the DSC data (Fig. 7). Each disaccharide unit of HA contains four hydroxyl groups, one amide and one carboxyl group. Due to the strong intra- and inter-molecular hydrogen bonds, HA is highly crystalline and insoluble in organic solvents, and cannot be transformed into the molten state before its decomposition.

Introduction of aliphatic acyl groups to HA was effective in disrupting the strong HA intermolecular bonding, reducing the crystallinity and producing appreciable thermoplasticization, similar to that observed in esterified cellulose [26]. This is confirmed by the melting endothermic peaks in the DSC thermograms of HA laurate and caproate (Fig. 7). For HA laurate, a broad endotherm is observed ranging from 76°C to 105°C with a peak at 97.8°C, while HA caproate shows a melting peak starting from 175°C with the peak at 191.7°C. The TGA results (Fig. 6) show that HA laurate begins degrading at about 191°C, while HA caproate begins degrading at approximately 170°C.

The hot-stage microscope photographs of HA laurate are shown in Fig. 8. Melting liquid droplets became obvious in HA laurate at approximately 100°C, and with increasing temperature, the droplets became bigger and bigger until they joined to form a flowable melt. The HA laurate exhibited complete, simultaneous melting of all the material under the hot stage microscope with reformation of a film (without visual evidence of degradation) upon cooling. On the hot stage the HA caproate did not appear to melt until 190°C, but at this temperature the liquid also showed a brownish appearance typical of degradation. Therefore, HA laurate is melt-processable, but HA caproate melts with decomposition due to insufficient disruption of hydrogen bonds and crystal structure. The melting onsets determined using the hot-stage microscope coincided with the peaks of the endotherms in the corresponding DSC thermograms, but not with the endotherm onsets. This is probably due to the higher heating rate used in the hot-stage and the difficulty of detecting the first-formed liquid drops in the micrographs.

The melting temperature (from DSC) and the starting points of degradation (from TGA) for various HA esters are summarized in Table I. The intermolecular interaction between the polymer chains decreases with increasing length of ester side chains, as indicated

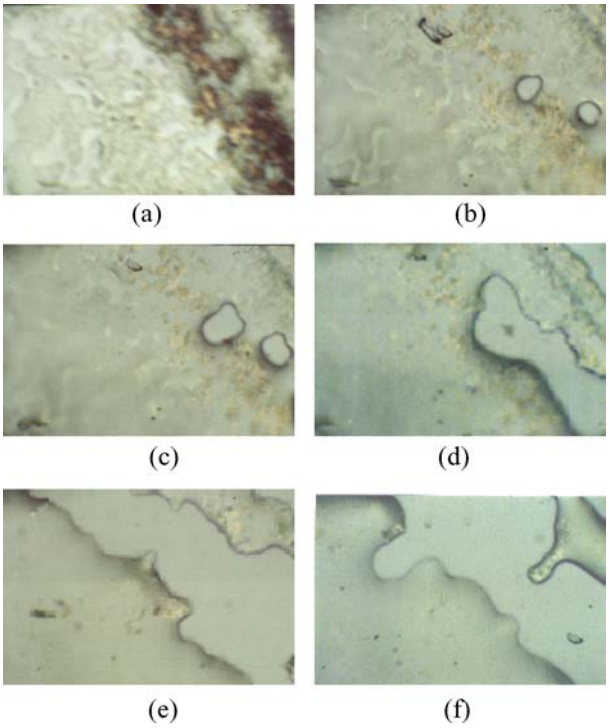

Figure 8 Optical micrographs of HA laurate at (a) room temperature; (b) 100°C; (c) 120°C; (d) 140°C; (e) 160°C; (f) 180°C.

by the change in melting points. The higher aliphatic acid chloride was more effective in conferring thermoplasticity to HA. Starting from HA laurate, higher aliphatic esters of HA melt far before the degradation begins, and thus can be melt processed. The HA caprinate is a borderline thermoplastic polymer with a relatively narrow window between melting and the onset of degradation. On the other hand, caproyl and capryloyl are not sufficiently large enough to disturb the molecular order of HA, so HA caproate and caprylate did not achieve thermal fluidity before the onset of degradation.

Degradation points of HA esters depend on the balance between two factors: a decrease in crystallinity (molecular order) and an increase in the length of acyl groups. From HA caproate to palmitate, the effect of acyl group chain length seems to dominate, so the degradation temperature increases with the carbon number of the ester chain. However, with HA stearate, the effect of molecular order disruption seems to dominate, so the degradation temperature of HA stearate is lower than that of HA palmitate.

The solubility of HA esters were also investigated and the results are summarized in Table II. HA esters from caproate to laurate are soluble in acetone and THF, while palmitate and stearate are soluble in hexane, indicating that the hydrophobicity of HA esters increases with the side chain length. DMSO is not a good solvent for any of the HA esters, while xylenes are a good solvent for all of these esters.

From the above evidence, HA ester melting points and solubility in organic solvents can be controlled by modulating the chain length of acyl groups. The resultant thermoplastic HA esters can be hot molded into films, sheets, and any desired shapes. They can be used alone or mixed with some other biomedical grade thermoplastics, such as ultra high molecular weight polyethylene. Crosslinkers, such as blocked polyisocyanate, can also be used during molding to obtain a permanent three-dimensional HA ester network. Finally, the acyl groups can be easily removed through saponification in order to return HA to its native state after molding.

TABLE II Solubility of HA esters in organic solvents

HA esters	DMSO	THF	Acetone	Xylenes	Hexane
HA Caproate	swollen	+	+	+	−
HA Caprylate	swollen	+	+	+	−
HA Caprinate	swollen	+	+	+	−
HA Laurate	−	+	+	+	−
HA Palmitate	−	−	−	+	+
HA Stearate	−	−	−	+	+

"+" soluble; "−" insoluble.

4. Conclusions

A series of HA esters were successfully created through the acylation reaction of silyl HA-CTA with acid chlorides. The disappearance of all characteristic infrared absorption bands assigned to the $-OSi(CH_3)_3$ groups and the appearance of the strong ester carbonyl peak confirmed the completion of esterification. In this esterification, some –CTA groups attached to carboxyl groups might also be attacked by acid chlorides. This was reflected by the decrease in intensity of the N^+ peak component in high-resolution N1s XPS spectrum.

Esterification with high aliphatic acid chlorides was demonstrated to be an effective method for imparting thermoplasticity to HA. HA caprinate, laurate, palmitate and stearate had melting peaks far below their degradation temperatures, providing a large window for safe hot processing of the polymers. Caproyl and capryloyl are not long enough to disrupt the strong HA intermolecular interactions and molecular arrangement, so their corresponding HA esters were not melt-processable.

The ester groups could be completely removed through saponification. The FT-IR analysis showed that the HA regenerated from HA esters had the same structure as native HA, indicating esterification and saponification did not damage the backbone of HA. Future work will examine whether the molecular weight of the HA is altered by the esterification process.

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