New Bioactive Products and Biomaterials from Hyaluronan

by Vittorio Crescenzi, Andrea Francescangeli, Davide Renier, Davide Bellini and Andrea Pastorello

A rather detailed description of the original synthetic routes leading to new hyaluronan (HA) biocompatible derivatives is afforded. One type of derivative is a novel product consisting in water soluble HA alkyl amides (HYADD) of different hydrophobicity. The other type of derivative consists in alkyl esters of C6-oxidised HA (new HYAFF). HYADD samples exhibit remarkable hydrolytic stability, rheological properties and *in vivo* antiadhesive characteristics. New HYAFF samples processability makes these new polymeric esters very versatile starting products from which a variety of materials can be obtained which potential in the biomedical area.

Hyaluronan (HA), chemically identified some seventy years ago [1], is a glycosaminoglycan with important biological performances and versatile structure which still stimulate the formulation of a variety of interesting products/materials with potential in the biomedical area.

In fact, taking advantage of HA chemical reactivity many HA-based functional derivatives and materials have been prepared, a few of which have already demonstrated their usefulness in a number of end uses [2-4].

We wish to report here on the synthesis, preliminary physico-chemical characterization, and potential/actual applications of novel HA derivatives recently obtained in our laboratories, namely:

a) a series of amides (HYADD) prepared by means of partial amidation of D-glucuronic acid residues along HA chains with alkyl amines of different chain length [5];
b) a series of new esters (HYAFF) [6] prepared *via* the regioselective, C(6) oxidation of *N*-acetylglucosamine residues [7, 8] and the subsequent partial-to-total esterification of the regioselectively oxidized species [9].

We found that type a) derivatives have interesting rheological properties in aqueous media and appear as new, stable viscosupplementation agents superior to other water soluble products with, in addition, good antiadhesion performances [5].

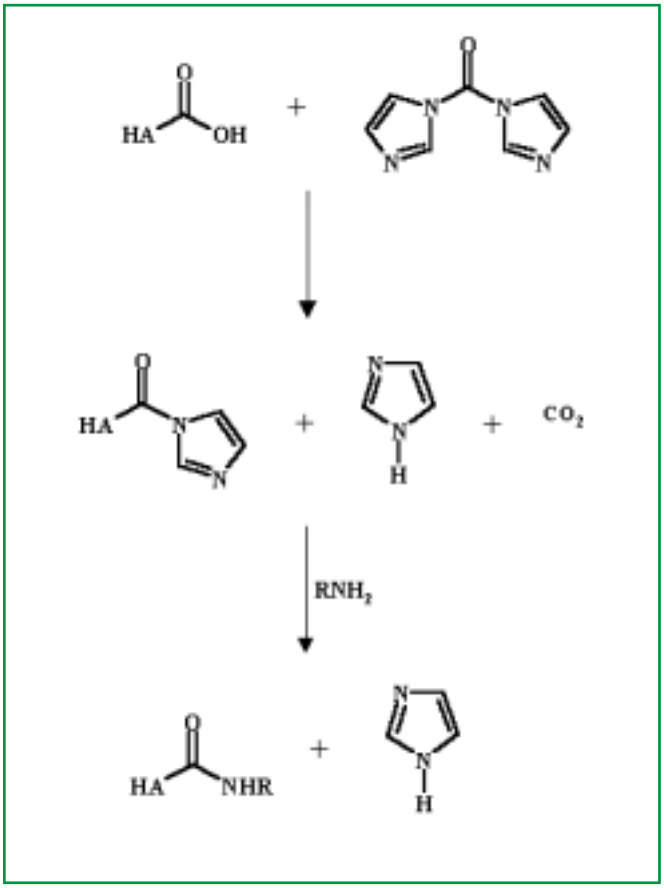

Figure 1 - Scheme of the HA amidation reaction

Esterification of regioselectively oxidized HA leads to new biomaterials, b), with bulk physico-chemical properties - modulated by the degree of esterification and the nature of the substituent groups - different from those of traditional HYAFF products derived from HA [6, 9] and with potential in cells scaffolding and/or nerve regeneration applications.

In this paper a detailed description is given of the preparation of HA derivatives of type a) and b) in as much as contradictory reports may be found in the patent and scientific literature on relevant chemical procedures. A preliminary samples characterization follows. Details on the physico-chemical properties of the ensuing products will be reported in a future publication.

The HA samples employed (FAB products) had average molecular weights in the range 200-700 kDa. All other reagents and solvents were of analytical grade and have been used without further purification.

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Synthesis of HA amides

Samples of the tetrabutylammonium salt of HA (TBA-HA) were obtained passing an aqueous solution of the sodium salt of HA (average molecular weight = 200 kDa) on an ion-exchange resin in the TBA form. After freeze-drying, solid TBA-HA was dissolved in anhydrous dimethylsulphoxide (DMSO). In the solution, added of catalytic amounts of methanesulphonic acid and of 1,1'-carbonyldiimidazole (CDI), appropriate aliquots of a given primary alkylarylamine or alkylamine were finally introduced under stirring at room temperature. The mixtures were left to react for a few hours. The amidation reaction may be summarized as in Figure 1.
The ensuing HA amides (HA-CO-NHR, in Figure 1) were precipitated by addition of excess ethanol, redissolved in water, extensively dialyzed against double distilled water and finally recovered by freeze-drying. Using CDI as carboxyl groups activator, no ester formation (*via* intrachain or interchain reactions) has been detected. As expected, the maximum degree of amidation of HA (% of amidation) for which the derivative is still water soluble decreases with increasing alkyl chain length of the amine employed.
The following are the approximate values of the maximum % of amidation in each case considered: benzyl amide 45%; octyl amide 30-35%; dodecyl amide 10-15%; hexadecylamide 8-10%.

Synthesis of C(6) oxidized HA and esters therefrom

C(6) fully oxidized samples of HA (HYOXX) have been prepared using HA aqueous solutions (average molecular weight = 700 kDa, 1% w/V). These were added of equimolar amounts of NaBr (moles NaBr = moles of primary alcoholic functions), cooled to 0-1 °C, and further added of about 2.5% moles (with respect to the moles of primary alcoholic functions) of 2,2,6,6-tetramethylpyperidine-1-oxyl) radical (TEMPO).
An hypochlorite solution (6-14% active chlorine) was then added dropwise under vigorous stirring while the pH of the mixture was continuously monitored and kept close to 9.2-9.4 (by addition of aqueous NaOH). Completion of the oxidation reaction is revealed by a constant, plateau pH value.
The solution, neutralized with aqueous HCl and added of a slight excess of solid $NaBH_4$, was kept under stirring for a few hours at 0 °C.
After neutralization, the solution was dyalised at room temperature against frequent changes of distilled water until all salts were eliminated and the polymer (HYOXX) finally recovered by freeze-drying.
The oxidation degree of the ensuing HYOXX samples, controlled by means of potentiometric titrations with standard aqueous NaOH of solutions containing known amounts of HYOXX in the H^+-form (prepared by ion-exchange), resulted close to 95% on the average.
The oxidation process described above is outlined in Figure 2.
Esters of HYOXX (HYAFF) have been prepared following procedures very similar to those already reported for HA [7]. In a typical reaction, the tetrabutylammonium salt of HYOXX (TBA-HYOXX: obtained by passage of an oxidised HA solution over an ion-exchange resin in the TBA+ form and subsequently recovered by freeze-drying) was dissolved in an aprotic solvent, especially DMSO or *N*-methylpyrrolidone, The TBA-HYOXX concentration was varied between 1.0 and 2.5%

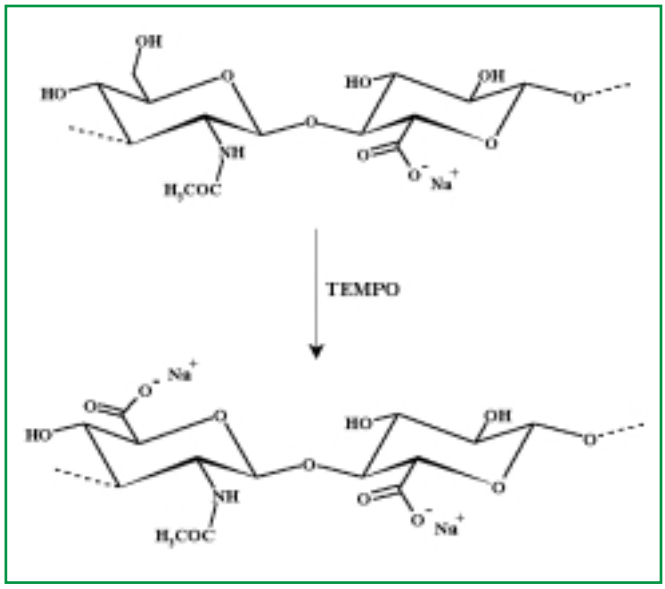

Figure 2 - Scheme of the HA oxidation reaction

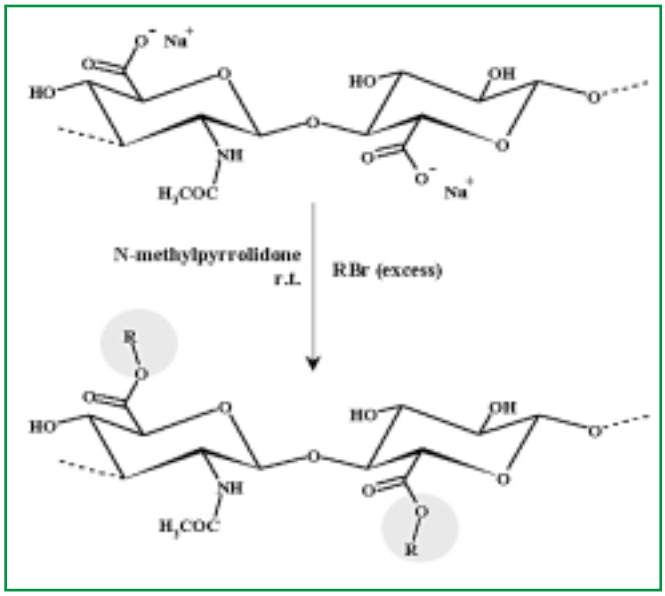

Figure 3 - Synthesis of the new HYOXX-esters

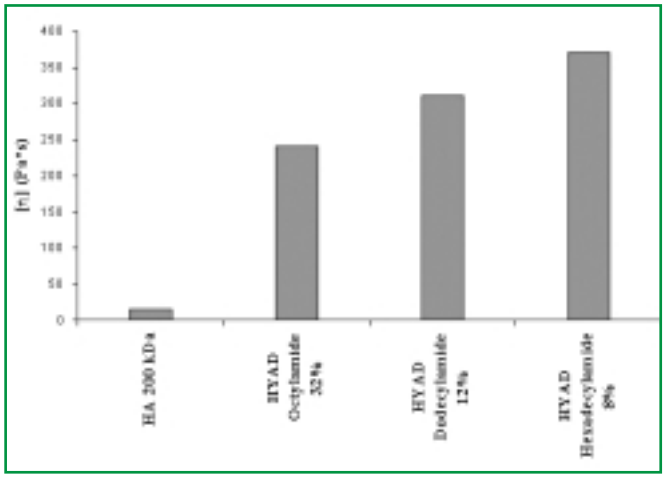

Figure 4 - Zero-shear viscosity of HA and HYADD solutions (c=2.5% w/V; T=25 °C)

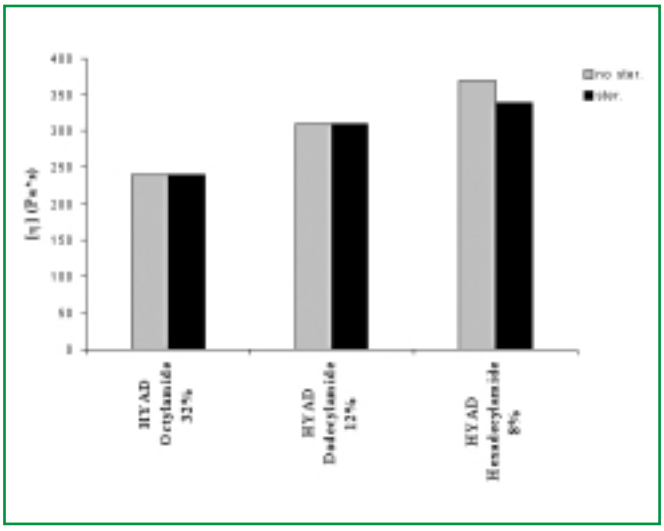

Figure 5 - Sterilization effect on the zero-shear viscosity of HYADD solutions (c=2.5% w/V; T=25 °C)

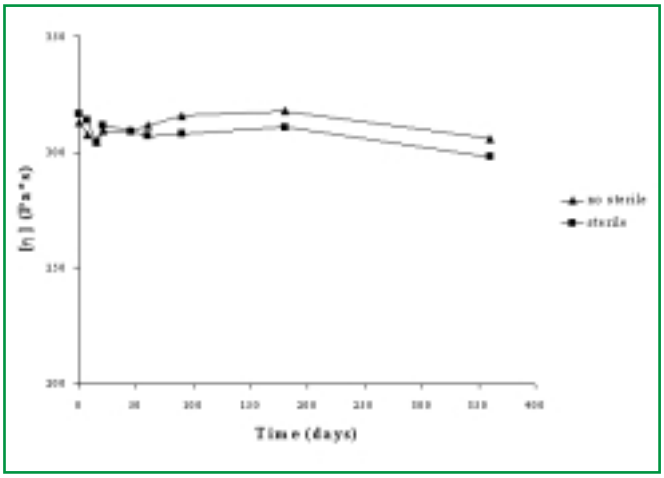

Figure 6 - Sterilization effect over one year on the zero-shear viscosity of HA hexadecylamide (degree of amidation=8%; c=2.5% w/V; T=37 °C)

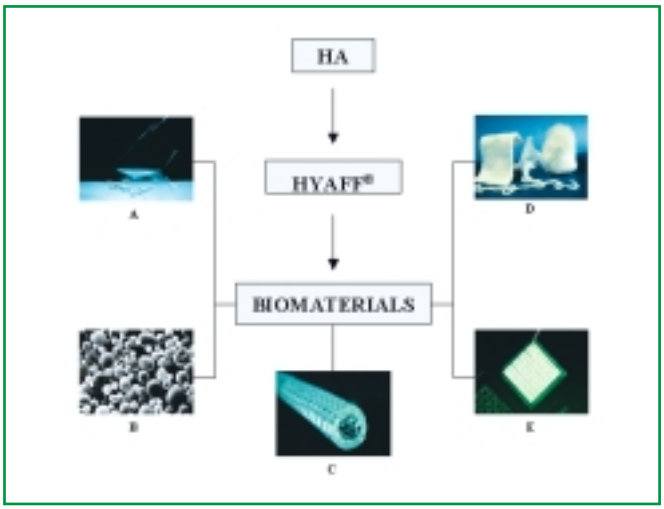

Figure 7 - A list of HYAFF-derived biomaterials; A: wet extrusion (coagulation in ethanol) membrane; B: microspheres (10 mm); C: tube (nerve guide); D: non-woven (scaffolds for cells; wound healing); E: laser perforated membrane (tympanic repair)

w/V depending on sample molecular weight. The mixture, kept at 4-6 °C under stirring in a nitrogen atmosphere, was then added of a controlled amount of the desired alkyl bromide. Subsequently, it was kept under stirring at room temperature for a few hours and then at 40 °C for 3 days. The alkyl bromide had always to be added in excess with respect to the desired HYOXX esterification degree which, in some cases, could be varied at will from 50 to 175% (200% esterification corresponds to a HYOXX fully esterified sample). However, esterification degrees higher than 175% could not be obtained probably because of steric hindrance among alkyl side-groups crowded along HYOXX chains. The esterification reaction is briefly summarized in Figure 3.
The esterified products were recovered by pouring the reaction mixtures into an excess (3:1 in volume) of ethanol or of acetone which were previously added of a saturated NaCl solution (0.1% in volume). The polymers were repeatedly washed with a water:ethanol 20:80 mixture to eliminate residual salts, then with ethanol, and finally dried under vacuum.
Attention is focused in this paper exclusively on a water insoluble HYOXX benzyl ester sample with degree of esterification (d.e.) equal to 150% in which the unesterified carboxylic acid residues (about 45%) are in the sodium salt form.

HYADD solution properties and applications (preliminary data)

HA amides considered include those listed in Figure 4 where the room temperature zero-shear viscosity of a 2.5% w/V solution in water of each sample is reported.
It clearly appears that the viscosity of the amides in aqueous solution is higher of a factor of at least ten in comparison to that of the starting HA sample. Indeed, the viscosupplementation properties appear remarkable in the case of the hexadecylamide derivative.
In addition, of particular interest are the results on the stability of samples considered in aqueous media reported in Figure 5 and 6: the first shows that steam sterilization has a very modest effect and the latter demonstrates that the viscosity of the hexadecylamide of HA (degree of amidation 8%) remains unmodified even after 12 months in water at 37 °C.
Finally, the results of experiments carried out *in vivo* on the antiadhesive properties of the HA amides - relatively concentrated, gel-like mixtures with water applied after surgical operation in a rabbit peritoneum - have so far been particularly satisfactory as demonstrated by the few data collected in Table.

Esters HYOXX properties and applications (preliminary data)

The high HYAFF processability makes these biopolymer-based esters very versatile starting products from which a variety of biomaterials can be obtained. These can be in the form of either membranes, or tubes, or microspheres etc., as pictorially illustrated in Figure 7.
The morphological structures of HA benzyl ester (d.e. equal to 100%) and of HYOXX benzyl ester (d.e. equal to 150%) are very different (Figure 8); the first appears homogeneous and totally impermeable to water while the latter shows a porous structure resembling that of cellulose nitrate/acetate Millipore filters. As a matter of fact, appropriately cast mem-

Table - *In vivo* anti-adhesive properties of viscous solutions of HA-amides

Derivatives	*Concentration (mg/ml)*	*Score surface*	*Adverse events*
HYADD2 (octylamide)	50	0	None
HYADD3 (dodecylamide)	35	0	None
HYADD4 (hexadecylamide)	20	0	None
Control (untreated)	-	4	-

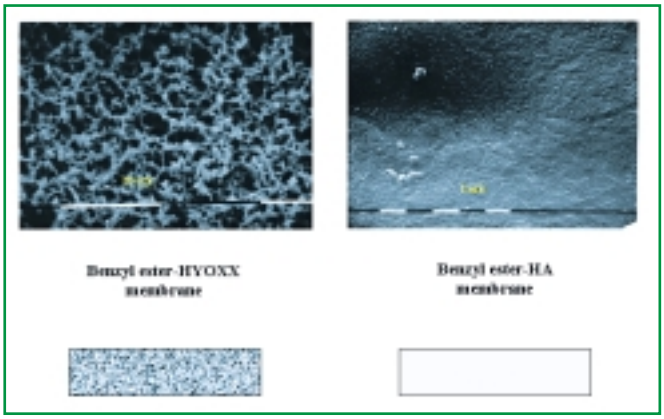

Figure 8 - Morphological structure of HYOXX benzyl ester (d.e.=150%) (right) and HA benzyl ester (d.e.=100%) (left)

branes of HYOXX benzyl ester do equally well perform in filtering aqueous solutions to sterility.
More important, applications of the latter materials - including the type D forms (non-woven) of Figure 7 - in tissue engineering are actually being actively pursued.

References

[1] K. Meyer, J. Palmer, *J. Biol Chem.,* 1934, **107,** 629.
[2] T.C. Laurent, The Chemistry, Biology and Medical Applications of Hyaluronan and its Derivatives, Portland Press, London, 1998.
[3] V. Crescenzi, A. Francescangeli, D. Renier, D. Bellini, *Biopolymers*, in press.
[4] V. Crescenzi, A. Francescangeli, D. Renier, D. Bellini, *Biomaterials*, submitted.
[5] D. Bellini, A. Topai, PCT Pat. Appl. Publ., No WO 00/01733.
[6] A. Rastrelli *et al., Clin. Imp. Mat.,* 1990, **9,** 199.
[7] B. Jiang *et al., Carbohydr. Research,* 2000, **327,** 455.
[8] V. Crescenzi *et al., Macromolecules,* 2001, **38,** 6373.
[9] D. Bellini, V. Crescenzi, A. Francescangeli, Patent application filed.

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