Gadolinium Diethylenetriaminepentaacetic Acid Hyaluronan Conjugates: Preparation, Properties and Applications

*Sébastien Gouin, M. Victoria Valencia Grayeb, Françoise M. Winnik**

Department of Chemistry and Faculty of Pharmacy, Université de Montréal, CP 6128 Succursale Centre Ville Montréal QC H3C 3J7 Canada

Summary: We have prepared new hyaluronan (HA) gadolinium diethylenetriaminepentaacetic acid (DTPA) conjugates that have potential as tumor specific contrast agents for magnetic resonance imaging. Conjugates were synthesized, starting with a high molecular weight HA or with HA oligomers, by an efficient 2-step procedure involving first, reaction of ethylenediamine with HA carboxylic acid groups and, second, covalent linkage of DTPA to aminated HA. The final polymers were compared in terms of molar masses and DTPA content. Tapping mode atomic force microscopy has been used to examine the morphology of the polymers in aqueous solution.

Introduction

Hyaluronic acid (HA) is a naturally occuring linear high molecular weight polymer consisting of alternating N-acetyl-β-D-glucosamine and β-D-glucuronic acid residues linked (1→ 3) and (1→ 4), respectively (Figure 1). It plays an important structural and mechanical role in various tissues, participates in the control of tissue hydration and water transport, and affects numerous biological processes, such as inflammation, tumor metastasis and development.[1] Hyaluronan interacts with cells via specific receptor proteins, the hyaladherins, and it has been shown that several extracellular matrix HA-binding receptors are overexpressed in tumor cells or in the inflammation process,[2, 3] resulting in abnormally high levels of HA near tumor or inflammation sites. This high affinity of HA for cancerous cells can be exploited in the design of targetted contrast agents in order to improve the diagnostic value of images produced by medical imaging techniques, including X-ray, magnetic resonance imaging (MRI), or ultrasound.[4] In MRI, the contrast agents of choice are organic chelates of high spin paramagnetic metals, such as Fe and Gd. Water molecules bound to the metal relax orders of magnitude faster than free water, resulting in a dramatic change in T_1 relaxation time that can be observed by MRI.[5] A contrast agent used routinely in MRI is the chelate of Gd^{3+} and diethylenetriaminepentaacetic acid (DTPA). It has great utility for imaging the brain,

 CCC 1022-1360/00/$ 17.50+.50/0

central nervous system, and other organs, but it is rather ineffective in other parts of the body, as it is essentially a first pass agent.

There is a significant need for agents that target specific organs, regions of the body, such as the gastroinstestinal tract, and sites of lesion, such as tumors. To address this issue, a variety of approaches have been investigated, including the conjugation of paramagnetic chelates to macromolecules, such as serum albumin or immunoglobulins,[6] polylysine,[7] dextrans,[8] as well as synthetic linear polymers[9] and dendrimers.[10] The macromolecular conjugates increase the blood pool lifetime of the contrast agents and show higher relaxivity as a consequence of increased rotational correlation times of macromolecules, compared to free Gd chelates. However most polymeric contrast agents tend to have broad biodistribution and fail to target specific organs or tissues, unless they carry, not only a contrast agent, but also a moiety, such as a folic acid residue,[11] recognized by specific cellular receptors. Since HA receptor sites are overexpressed on certain tumor cells, it is expected that HA-conjugated contrast agents will exhibit high tumor selectivity even without the incorporation of specific targeting groups. To assess the validity of this hypothesis, we undertook the synthesis of hyaluronan-Gd/DTPA conjugates by linking the chelator via a short diamine spacer group to the carboxylic acid groups of glucuronic acid residues. Several methodologies will be described in this report and their effectiveness will be compared with particular attention to the efficiency of the conjugation reaction and the extent of polymer degradation.

Preparation of Gd/DTPA hyaluronan conjugates

The synthesis of Gd/DTPA conjugates was performed in two steps (Figure 1), starting with hyaluronans of different molecular weights (Table 1), a high molecular weight polymer of high binding affinity for HA receptors,[12] and an oligomeric sample obtained by controlled enzymatic degradation of the original HA sample.[13] The size of the HA oligomer was such that it consisted of more than eight disaccharide units, the smallest motif necessary for recognition by HA receptors. In the first step of the synthesis, carboxylic acid groups of the glucuronic acid residues were allowed to react with ethylenediamine (EDA) by means of a coupling agent, either 2-ethoxy-1-(ethoxycarbonyl)-1,2-dihydroquinoline (EEDQ), a compound soluble in organic media, or the water soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). The pH selection of the reaction medium was critical to the success of the coupling with either EDC or EEDQ. The acidity of the medium controls the delicate balance between the rates of decomposition of the coupling

agent, formation of the active intermediate, and formation of the amide bond.[14] Optimal pH conditions were 3.5 and 4.7, respectively, for EEDQ and EDC-mediated reactions. In our hands, higher coupling efficiencies were obtained when EDC was used as coupling agent (Table 1).

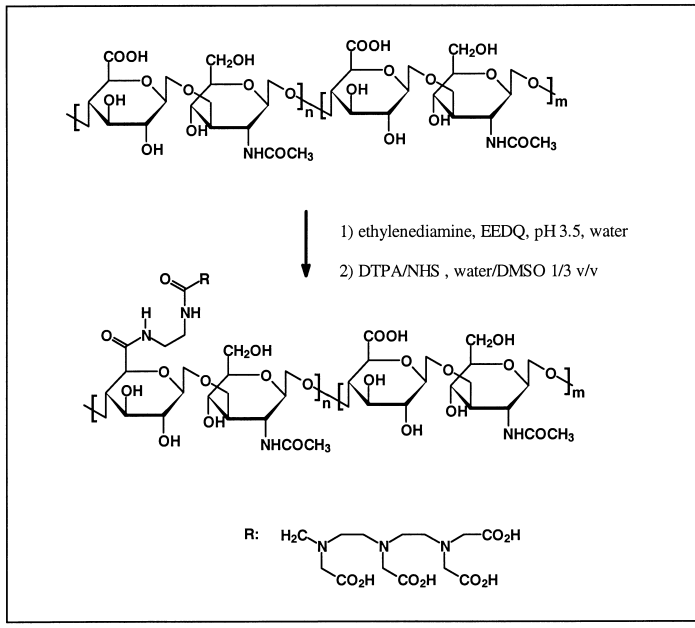

Fig. 1. Synthesis of HA-DTPA (bottom structure) from hyaluronic acid (top structure); EEDQ: 2-ethoxy-1-(ethoxycarbonyl)-1,2-dihydroquinoline; NHS: N-hydroxysuccinimide.

The conversion of HA-EDA to the desired DTPA conjugate was achieved by treatment with N-hydroxysuccinimide-activated DTPA of an HA-EDA water/DMSO solution maintained at pH 10 throughout the reaction. Under these conditions, remarkably high conversion rates were achieved, especially in the case of HA oligomers. The formation of the HA-DTPA/Gd complexes occurred quantitatively, relative to the level of DTPA incorporation, upon addition of an aqueous $GdCl_3$ solution to HA-DTPA. It was important to ensure (1) that all the DTPA chelates linked to the polymer had been converted to the Gd^{3+} complexes and (2) that the polymer was devoid of free Gd^{3+}, a highly toxic ion. This was achieved by quantitative titration of $GdCl_3$ in a solution of HA-DTPA, for which the level of DTPA incorporation had been measured by isothermal titration calorimetry as well as by a colorimetric assay.[15] The Gd^{3+} content of the resulting polymers ranged from 10.2 to 15.9 weight % , in direct proportion to the level of DTPA incorporation.

Table 1: Composition and molecular weights of the polymers prepared

Polymer (coupling agent)	Composition		Molecular weight		
	EDA[a] mmol/g	DTPA[b] mmol/g	Mn[c]	Mw[c]	End-group Analysis[d]
HA-EDA-1 (EEDQ)	0.73	----	19,900	32,900	55,000
HA-EDA-2 (EDC)	1.28	----	10,000	12,000	54,000
HA-DTPA-1	----	0.74	42,700	82,000	69,000
HA-DTPA-2	----	0.78	91,700	122,000	72,000
Oligomers					
HA-EDA-3 (EEDQ)	0.89	----	7,100	9,800	4,200
HA-EDA-4 (EDC)	1.38	----	< 3,000	< 3,000	4,800
HA-DTPA-3	----	0.81	11,900	22,100	4,300
HA-DTPA-4	----	1.20	9,500	13,300	5,000
HA	----	----	553,000	900,000	485,000
HA-oligomer	----	----	9,100	10,000	6,100

[a] determined by a colorimetric assay.[16] [b] determined by isothermal titration calorimetry and colorimetry.[15]
[c] determined by GPC, ultrashydrogel colums, 0.1 M $NaNO_3$ as eluent, calibrated against pullulan standards.
[d] Determined by a colorimetric assay.[17]

Characterization of the hyaluronan derivatives

The average molar masses of the polymers were measured after each step of the synthesis by GPC analysis and by end group analysis based on the conversion of the polysaccharide reducing end into a UV active group.[17, 18] The molecular weights obtained by end-group analysis are lower for the HA-EDA and HA-DTPA samples, compared to those of the corresponding starting polymer, an indication that some depolymerization occurred during the chemical modification of HA, in particular in the case of the high molecular weight samples. We note significant discrepancy between the molar mass values obtained by end group analysis and GPC, in particular in the case of all HA-EDA samples (Table 1). The dramatic decrease in GPC molecular weights of HA-EDA samples, compared to starting HA, reflects a decrease in hydrodynamic radius of the polymer upon conversion of polymeric carboxylates to primary amines. It also confirms that some depolymerization of HA took place during the amidation of HA. It is known that native HA adopts an extended conformation in solution attributed to specific intra chain hydrogen bonds between the carboxylates of glucuronic acid and the amide groups of N-acetylglucosamine.[19] In HA-EDA, amidation of the carboxylic groups precludes the formation of extensive, long range, intrapolymeric hydrogen bond, hence increases the

chain flexibility and, consequently, decreases the hydrodynamic volume of the polymer. GPC analysis of the HA-DTPA samples, performed under identical conditions, yielded molar masses significantly higher than those of the corresponding HA-EDA precursors. This increase reflects the enhanced chain extension of HA-DTPA, caused by electrostatic repulsion between the highly charged DTPA substituents.

Tapping mode atomic force microscopy (TMAFM) imaging of native hyaluronan, HA-EDA, HA-DTPA, and HA-DTPA-Gd was performed on a Nanoscope 3A Digital Instrument using a silicon cantilever probe (length: 125 nm). Data were analyzed with the Nanoscope software (version). A solution of the polymer (5 μg/mL or 200 μg/mL) in a pH 7.4 phosphate buffer was deposited on freshly-cleaved mica and air-dried. The specimen was rinsed 3 times with deionized water, air dried, and observed immediately under ambient conditions. Imaging of a solution of HA-DTPA-Gd at a concentration of 5 μg/mL yielded a network of rather linear ribbons, clustered in bunches or isolated from each other (Figure 2A). The length of the ribbons ranges from ~200 to 450 nm, their average witdth is ~ 40 nm and their height is approximately 6.5 nm. This morphology is different from that of native HA, which upon imaging under identical conditions, forms thin curved strands ~ 200 nm long, less than 3 nm thick, and ~ 1.5 nm high (Figure 2A, inset), in agreement with previous TMAFM imaging of native HA.[20] Imaging of HA-DTPA (Figure 2B) yielded a fibrous network (Figure 2b, inset) of intertwined strands along which one can discern small clusters with diameters of 28 nm and height of 5.2 nm.

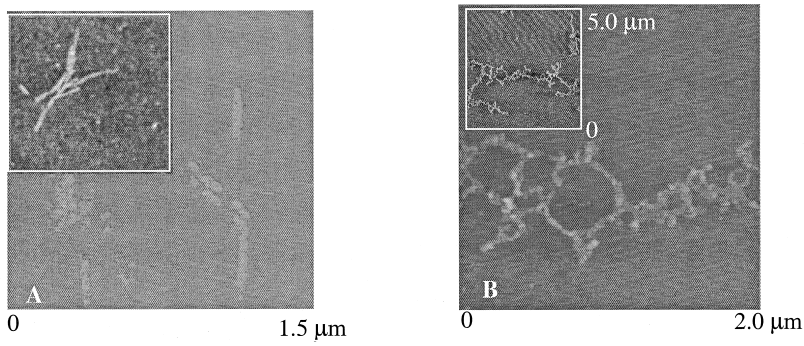

Fig. 2. TMAFM images of HA-DTPA-Gd (A) and native HA (A, inset) and of HA-DTPA (B) imaged with two different magnifications; grey scale height: 50 nm.

The differences in appearance of TMAFM images obtained from solutions of HA-DTPA and HA-DTPA-Gd is quite striking, but to what extent do they reflect the conformation of the macromolecules in solution? As noted previously, HA-DTPA is a highly charged polyanion due to the uncomplexed DTPA moiety, which carry four

carboxylate groups each. These groups form tight complexes with Gd^{3+} alleviating the electrostatic repulsion between saccharide units with resulting decrease in the stiffness of the polymer chain.

Conclusion

We have synthesized novel hyaluronan DTPA conjugates via an efficient procedure yielding samples of high level of modification. Initial in vitro imaging of an invasive (malignant) breast cancer cell line (MDA-231), and a non-invasive (benign) human breast cancer cell line (MCF-7) with three different preparations of HA-DTPA-Gd showed a strikingly higher signal intensity increase for the MDA-231cells, compared to MCF-7 cells.[21] This result confirms that, while the chemical modification of HA affects its conformation in aqueous solution, it does not preclude the uptake of hyaluronan conjugates by HA-specific receptors permitting differential imaging of the malignant *vs* benign cells.

Acknowledgments

This work was supported by a University/Industry collaborative grant of the Natural Sciences and Engineering Research Council of Canada and Hyal Pharmaceutical Corporation (Mississauga, ON Canada).

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