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## Introduction

Hyaluronic acid (HA) is a linear polysaccharide consisting of alternating 1,4-linked units of 1,3-linked glucuronic acid and *N*-acetylglucosamine (Figure 1), and is one of several glycosaminoglycan components of the extracellular matrix (ECM) of connective tissue [1]. Its remarkable viscoelastic properties account for its importance in joint lubrication [2] and its complete lack of immunogenicity makes it an ideal building block for biomaterials needed for tissue engineering [3,4] and drug delivery systems [5,6]. Sodium hyaluronate, the predominant form at physiological pH, and HA are collectively referred to as hyaluronan; in this paper, the chemical modifications always begin with the carboxylic acid form, and we use the abbreviation 'HA'. The three-dimensional structures adopted by HA and HA oligosaccharides in solution [7,8] show extensive intramolecular hydrogen bonding that restricts the conformational flexibility of the polymer chains and induces distinctive secondary (helical) and tertiary (coiled coil) interactions.

In this chapter, we will first summarize background information on (1) key HA–protein interactions important in cell biology, (2) the role of HA in cellular signalling, (3) the biomedical applications of HA, (4) biodegradable polymer scaffolds and (5) chemical modification of HA, leading to the discovery of the hydrazide modification method. Second, we present an overview of our current work on (1) new cross-linkers, (2) optimization of HA hydrogel formation, (3) hydrogel degradation by hyaluronidase (HAse), (4) use of chemically modified hydrogels for tissue engineering, (5) synthesis and applications of functionalized HA probes in cell biology and (6) structural studies of HA–HA binding domain interactions.



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HA binding proteins, are essential in tissue remodelling processes [1
HA–protein interactions are important in cell adhesion, growth and migrat
[9,12,13], stimulation of cell motility and promotion of focal adhesion turno
[14], in cartilage [15], in inflammation and wound healing [16] and in can
[17,18]. Many of the molecular details of the interactions of HA with HA bind
domains (HABDs) of hyaladherins have been elucidated. Indeed, a solut
structure of the link module, a hyaluronan-binding domain involved in EC
stability and cell migration, has recently been published [19]. However, HA is
simply 'extracellular glue'; the critical importance of HA receptors in
regulation of intracellular signalling to the cytoskeleton has been recen
reviewed [20], and key elements are described below.

## Role of HA and HA receptors in metastasis

Extracellular HA acts as a signalling molecule by activating regulatory kin
pathways important for cell cycle progression and movement [21,14,20]. T
types of cellular HA receptors have confirmed roles in signalling: (i) CD4
family of glycoproteins originally associated with lymphocyte activation, and
RHAMM (receptor for hyaluronan mediated motility), originally identified fr
transformed fibroblasts. CD44 isoforms found on tumour cells bind HA w
higher affinity and promote cell migration [22]. Turley's group found t
RHAMM overexpression is itself transforming and regulates events downstre
from H-ras [23,14]. Moreover, RHAMM plays a role in the cell cycle, induc
mitotic arrest by suppression of cdc2 and cyclin b1 production [24]. In cancer,
also has effects on angiogenesis and on an increased HA production in and aro
tumours [25].

While the predicted secondary (and tertiary) structures of RHAMM
CD44 are different, both share the HA binding motif common to all known
binding proteins: $BX_7B$, where B is His, Arg or Lys [26]. The importance of th
two domains for HA binding activity has been determined by deletion muta
and synthetic peptide competition experiments. HA–CD44 complexes med
lymphocyte–endothelial cell adhesion [27]. A critical $Arg^{41}$ in CD44
identified by mutagenesis [28]. A series of Lys substitutions in RHA
confirmed their importance in the mediation of metastatic transformation indu
by H-ras [14]. The role of CD44 and RHAMM in determining whe
transformed cells become aggressive and metastatic has been extensi
investigated [29,30,22,31–37]. The importance of HA–receptor interaction

[40].

## Biomedical applications of HA derivatives

Both naturally occurring and chemically modified HA have found use in a broad range of biomedical applications, including ophthalmic surgery [41] and the treatment of arthritis [5,42,43], in particular via the technique known as viscosupplementation [44,45]. A wide variety of uses are described elsewhere in this volume, but several examples are described here to place the present chemical modification work in context. The chemical derivatization of HA by our laboratories and others provides an opportunity to develop materials for new applications that exploit some of the viscoelastic properties of this high-molecular-mass polymers. For example, controlled release of pharmacologically active compounds such as prednisolone [46,47] for use in eye surgery, moisturizing agents, swellable gels, adhesion management aids, cell encapsulation matrices, hydrophilic coatings of plastics [48], mammary implants [49] and certain aspects of wound treatment [41] are some of the applications envisioned for these new materials. Native sodium HA was shown to serve as a template for nerve regeneration [50]. HA is suitable for all of these applications since it is bioerodable and compatible with systemic functions. Insoluble HA derivatives that retain significant bioerodability [51,52] have been used as membranes for the culture of keratinocytes for transfer to human wounds [53]. Other chapters in this volume will provide an expanded repertoire of the specific uses of HA in human medicine. New HA hydrogels can in principle be fashioned into prosthetic implants capable of drug delivery [54], into porous microspheres for culturing cells [55,56] and into gels for bridging gaps in bone or cartilage during cell regrowth [57–59].

## Biodegradable polymers

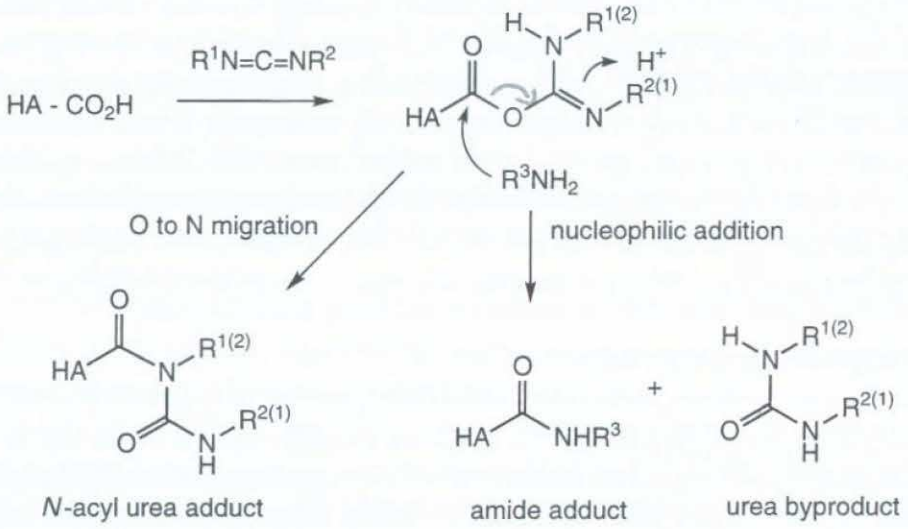
Biodegradable polymer scaffolds based on polyglycolic acid, polylactic acid and related co-polymers have been investigated recently as substrates for tissue engineering [3], and important advances in neocartilage regeneration [60] and joint resurfacing have been described. A high-molecular-mass viscous HA material has also been used alone or in gel-like formulations with decalcified bone matrix [57,58]. Despite promising results, in these cases, the realization of a shape-retaining implant was not achieved. As a result, the need for new biomaterials [4] for tissue engineering and for drug delivery [6] continues to drive the development of new technologies.

peptide or drug attachment, in addition to producing a biocompat
bioerodable hydrogel, should provide access to a unique new set of biomateria

## Chemical modification of HA

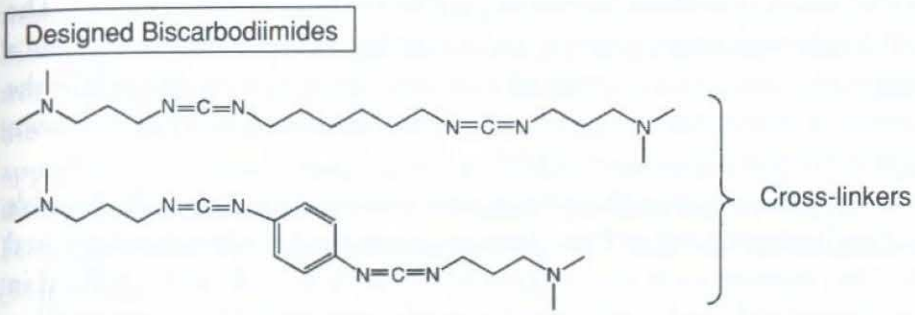
As detailed below, HA can be chemically modified at hydroxy groups of glucuronic acid or *N*-acetylglucosamine units, the carboxylic acid of glucuronate units, or the reducing end of the polysaccharide chain. Since chemical reactions described herein require the protonated carboxylic acid and conducted between pH 2.0 and 5.0, we will refer throughout to hyaluronic or simply HA. The resulting materials used in a biological context woul correctly called chemically modified hyaluronan derivatives, referring mixture of protonated and sodium and/or potassium salts present in physiolo systems. In this chapter, we will focus on controlled modifications of carboxylic acid under mild aqueous conditions, using water-soluble carbodiir chemistry.

**Figure 2**




**Attempted chemical modification of HA with carbodiimides ($R^1N=C=N$ and primary amines ($R^3NH_2$) leads to formation of *N*-acyl ureas rearrangement of the intermediate *O*-acyl ureas**

*The amide and urea by-product are only minor products.*




**Designed monofunctional (top) and bisfunctional (bottom) carbodiimides**

*The compounds were synthesized and used to prepare N-acyl urea derivatives and N-acyl urea cross-linked derivatives of HA.*

Carbodiimide modification of glycosaminoglycans has three decades of history; unfortunately, for most materials the chemical structures were not well-characterized. We initiated our studies of the modification of HA with ethyl(*N*,*N*-dimethylaminopropyl)carbodiimide (EDCI) and a variety of 'designer' carbodiimides in 1986. The production of glucuronamides requires the activation of the carboxylic group, which can be accomplished using a water-soluble carbodiimide such as EDCI as the condensing agent. The first report of an HA-glycine methyl ester derivative [68] could not be repeated in our laboratories [69]. We found that the *O*-acyl urea activated complex formed between EDCI and high-molecular-mass HA (2 MDa) at pH 4.75 did not give the expected intermolecular coupling with added diamines or other primary amine-containing nucleophiles (Figure 2). Instead, the *O*-acyl ureas preferentially rearranged to *N*-acyl ureas rather than undergoing coupling to added amine-containing reagents [69]. This was readily seen by a characteristic quartet and triplet pattern for the *N*-ethyl urea moiety in the NMR spectrum of these derivatives. Partially degraded HA (60 kDa) also followed this reaction pathway. We nonetheless took advantage of this observation to prepare lipophilic, aromatic and functionally reactive derivatives of HA based on the robust covalent *N*-acyl urea linkage formed with customized carbodiimides (Figure 3, top) [69]. Biscarbodiimides offered the possibility of cross-linking HA to form stable gels, and both aliphatic and aromatic biscarbodi-

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