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Hyaluronan in cancer - From the naked mole rat to nanoparticle therapy

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Hyaluronan, a glycosaminoglycan, abundant in the tumour microenvironment, is a key player in many processes associated with cancer. Recently the cancer resistance of the naked mole rat has been attributed to the presence of an ultra-high molecular weight form of this molecule. The physical properties of this multifunctional biopolymer have been extensively studied in the context of synovial joints. However, relatively little has been reported with regard to the soft matter properties of hyaluronan in relation to cancer. In this review we examine the role of hyaluronan in cancer, paying particular attention to its mechanical interactions with malignant cells and its soft matter properties. In addition we discuss the use of hyaluronan based gels to study cancer invasion as well as nanoparticle based strategies for disease treatment.

Introduction

Cancer in humans is very common with a third of us developing some form of it during our lifetime. However a number of animal species have been described to have either a very low incidence or resistance to cancer.[1] This can usually be attributed to extra tumour suppressive cell signalling pathways, however there is one exception. The naked mole rat is a remarkable creature in that it has an exceptionally long life span for its size and is resistant to cancer.[2] Thus when a breakthrough paper was published attributing the naked moles cancer resistant to the production of an ultra-high molecular weight glycosaminoglycan[3], the inevitable wild speculation, regarding injecting humans with this as a preventative measure against future development of cancer was brandished.

It turns out that this molecule, albeit in a lower molecular weight form, is a ubiquitous extracellular matrix (ECM) molecule in humans, and has been attributed to play important roles in many aspects of cancer pathology.[4–10] These roles can be roughly split into two categories, although there is obvious crossover. The first is biological in nature, involving the binding of hyaluronan with cell membrane based receptors to initiate signalling and subsequent gene expression. The second is mechanical, relating to purely physical interactions regulating cell adhesion, mobility and invasion.

The cancer cell microenvironment comprises non-cancerous cells, ECM biomolecules and vasculature. Cues within the microenvironment can regulate the various processes involved in cancer progression.[11] Within the microenvironment the ECM, a "glue like" substance, has an intricate relationship with the cells in which it encloses.[5,12–14] Abnormality of the ECM within the microenvironment is a characteristic of cancer, and abnormal levels of hyaluronan within the ECM are indicative of a poor prognosis for the patient.[15–20]. A symbiotic relationship exists whereby tumour cells have the ability to remodel the ECM around them, and the matrix has the ability to affect the behaviour of the tumour cells.[21–24] Soft matter properties of the matrix are an important factor in the mechanotransduction between these two environments.[25] A second hyaluronan rich interface exists called the pericellular matrix. This micron thick cell coating can also play an important role in the cell mobility necessary for metastatic spread .[26]

A somewhat confusing paradox exists in that the presence of hyaluronan has been attributed to cancer resistance in the naked mole rat, whereas the presence of hyaluronan in human malignancies is seen as an indicator of poor prognosis. However in the human case the hyaluronan has been cleaved into fragments of varying molecular weights, which themselves may have disruptive effects on cell/microenvironment linkages.[27,28] Given this strong link between hyaluronan abundance/abnormality in cancer, an opportunity exists to target hyaluronan in the microenvironment.[29,30] Such an extra line of attack is particularly attractive in cancers that have been hard to treat with conventional therapies, for example pancreatic and colon cancer.[31,32]

In this review we will concentrate on the mechanical role of hyaluronan in cancer in so far as it can be deconvoluted from its function in cell signalling. This will lead to a discussion on the hyaluronan biomaterials that can mimic the tumour microenvironment and how hyaluronan-cell interactions can be exploited for nanoparticle based therapy.

Structure and synthesis of hyaluronan

Hyaluronan, also known as hyaluronic acid (HA), is a linear

glycosaminoglycan. It is comprised of a repeating disaccharide unit, which is composed of N-acetyl-glucosamine and glucoronic acid. Depending on function and location, its molecular weight (in humans) can vary between 0.5 to 2 MDa.[33–35] This compares with naked mole rat hyaluronan (NMR-HA) which varies from 6 to 12 MDa.[3] The molecular weight of this biopolymer determines its physicochemical properties and these determine its function.[36] Accurate determination of the molecular weight of HA from tissue can be achieved using gel electrophoresis and is possible for both low and high molecular weight polymer.[37,38] In contrast to most other glycosaminoglycans which are produced in the Golgi apparatus and attached to other proteins, HA is synthesised in the inner cell membrane and extrudes out of the cell surface as further polymer units are added,[7,39](Fig.1). In eukaryotic cells, there are 3 main enzymes identified which synthesise HA into different sizes: Hyaluronan synthase (HAS) 1, 2 and 3.[40] These different isoforms of HA have been attributed to specific roles in normal biological processes. HAS3 is the most active enzyme leading to the production of large amounts of low molecular weight HA (0.5-1 MDa) required for normal cellular processes (growth and tissue repair). It is in itself a key component of the ECM with roles including regulation of tissue homeostasis, resistance to compressive tissue forces, and lubrication of articular joint surfaces. HAS2 is particularly active during embryonic development producing a high molecular weight HA (2 MDa), important for facilitating co-ordination of numerous essential cellular processes during early development. HAS1 is the least active enzyme producing HA of similar weight to HAS2 and its roles in health and disease remain relatively poorly understood.[41]

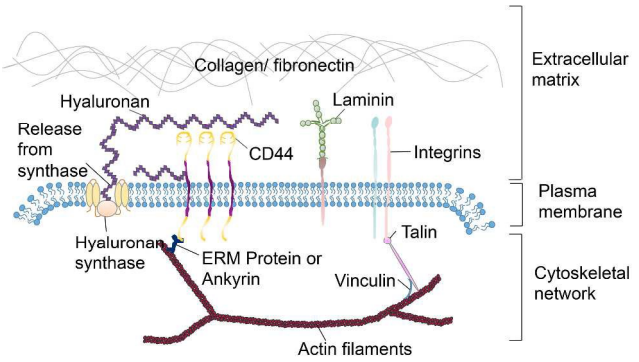

Fig. 1 Hyaluronan is synthesised via a transmembrane located enzyme, hyaluronan synthase and is encompassed amongst the extracellular matrix. Its main receptor is CD44 and this linkage between the ECM and the cell is fundamental to many aspects of cancer pathology. The balance between hyaluronan production and destruction may be key to understanding this role.

Interaction with cell membrane receptors

Beyond the oncological event that transforms a cell into malignancy, the processes involved in cancer progression have both biological and mechanical origins. HA interacts with cells though transmembrane receptors, the main such receptor being CD44[42,43], (Fig.1). It is these linkages and others like these, that regulate the adhesion and mobility of cells.[44–46] There are of course other ECM-cell interactions[47–49] for example those involving the protein fibronectin and the cell surface receptor integrin.[50] The complex interactions between CD44 and HA have received intensive investigation including the role for membrane bound enzymes, which can cleave the CD44/HA complex and therefore facilitate cancer cell invasion.[51] Fig. 2 shows how a cancerous cell in a human can break through an HA rich matrix and invade into a blood vessel via remodelling of its ECM. Enzymes degrading the HA and enzymes cleaving the CD44 receptor create a path for cell movement.

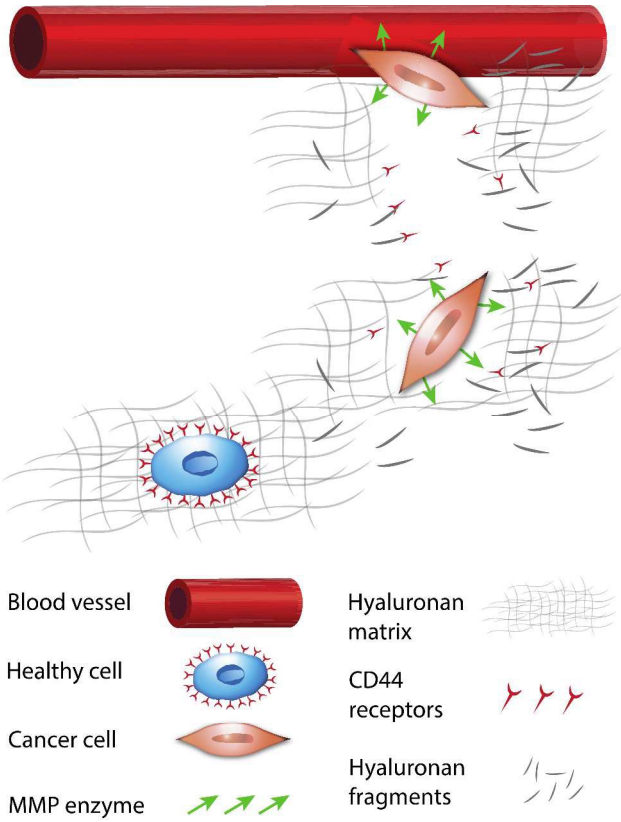

Fig. 2 Remodelling of the hyaluronan rich matrix by cancer cells leading to invasion. In healthy cells CD44 receptors bind to hyaluronan in the matrix. Cancerous cells over express the membrane bound enzymes MMPs which cleave CD44 receptors. They also over produce the enzyme hyaluronidase which digests hyaluronan into smaller fragments. In this way the cancer cells can "break" through the matrix and invade into the circulatory system.

Soft Matter Properties of HA

Given the importance of intermolecular and mechanical interactions both within the ECM and with the cell membrane, understanding the soft matter properties of HA is critical to determining its biological function. These can be examined at both the gross and microscopic levels. A particular characteristic of HA containing solutions are their high viscosity[52] and their ability to form cross-linked networks.[53] This coupled with HA's ability to form gel like structures has been linked to increased density and fluid pressure in the tumour microenvironment, a consequence of which is increased resistance to chemotherapy.[54,55]

HA has an established biological role in the dissemination of

cancer cells to distant sites with the mechanics of circulating tumour cells adhering to the blood vessel wall critical to the initial steps of metastasis. An elegant method for elucidating these mechanical interactions involves microfluidic experiments which can reveal the behaviour of cancer cells under flow conditions, either encountering a cultured layer of endothelial cells or immobilized biomolecules.[56] Such model systems can represent the binding of cancer cells to endothelial cells in blood vessels, a critical step in metastasis.[57] When encountering HA coated surfaces various types of cancer cells exhibit characteristic rolling and adhesion (Fig. 3). If CD44 receptors are blocked such rolling and adhesion is prevented, thus proving the importance of this interaction in phenomena related to metastasis.[58–60] The nature of this ligand receptor linkage is force dependent, meaning it has the ability to regulate cell rolling via force induced conformational changes in the CD44.[61]

Much can be learnt about the mechanics and assembly of HA when examined at the single molecule level. Atomic force microscopy (AFM) reveals that HA can adopt a number of different conformations when adsorbed onto surfaces. In tapping mode, AFM reveals branched structures for high molecular weight HA with both intramolecular and intermolecular interactions.[62,63] Force spectroscopy, the unfolding of single biomolecules with an AFM tip, has shown HA to exhibit hydrogen bonding networks in aqueous solutions[64] with the molecules undergoing a non-random to random coil transition upon heating to 46 °C. Single molecule stretching using an optical trap has allowed the measurement of persistence length, a measure of molecular stiffness, to be established.[65] This was found to be 4.5 ± 1.2 nm, indicative of a single molecule measurement. However given the tendency of HA to form branched and network structures a more useful quantity would that be the persistence length of higher order fibrous structures. Force spectroscopy has also quantified the tensile strength of the CD44-HA bond.[66] These single molecule measurements revealed a rupture force of between 70 and 80 pN depending on contact duration. For comparison these were significantly higher than the rupture strength of the CD44 - fibrinogen bond. Extending this methodology further by attaching HA molecules to an AFM cantilever, it has been possible to examine the forces of interaction between HA and live glioma cells.[67] As well as binding with CD44 and other membrane based receptors, HA can interact directly with lipids.[68] Evidence for these non-specific interactions have been observed in the interaction of HA with liposomes, giving rise to cylinder and sheet like super structures .[69] It has also been proposed that hydrophyllic regions of HA could attract the polar head groups of phospholipids[70], a phenomenon which could explain the association of HA with cell membranes and by extension tumour cell membranes.

Using soft matter approaches to assemble model systems can help deconvolute physical interactions from biological. This has been achieved by reconstituting CD44 receptors into lipid bilayers, allowing HA adsorption behaviour to be studied using techniques such as the quartz crystal microbalance and ellipsometry.[71]

Mechanical stress generated in a confining matrix has been shown to control tumour spheroid shape and morphology.[72] A logical inference from this observation is that the material constituents of the ECM can influence the growth behaviour of a tumour. It is one thing measuring the mechanics/material properties of ECM components but novel approaches are required to study the material properties of HA in the pericellular matrix (PCM), the micron scale coating that surrounds many cells and has been implicated in many of the physical processes associated with disease progression. One such technique is the use of particle tracking microrheology (to obtain a mechanical map of PCM) combined with fluorescence microscopy, (to identify the biomolecular constituents).[73]

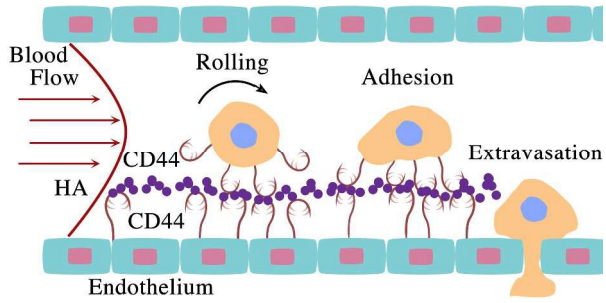

Fig. 3 Hyaluronan mediated rolling and adhesion of flowing tumour cells. CD44 receptors on both the tumour cells and blood vessel epithelial cells bind to hyaluronan. Weak attachment promotes rolling behaviour. Strong attachment can lead to full adhesion followed by extravasation though the blood vessel wall, a critical step in the establishment of a metastasis.

Elevated levels of hyaluronan

A paradox exists between the high levels of HA found in the naked mole rat, the presence of which is attributed to the prevention of cancer, and the high levels of HA associated with cancerous tissues in humans, an indicator of poor prognosis. In order to understand this fundamental issue one must consider what causes an accumulation of HA in human tumours. In healthy tissue there is a balance between HA synthesis and its degradation. For the naked mole rat HA is of the ultra-high molecular weight variety, whereas in human cancers, the molecular weight is much lower. In some cancers (squamous cell carcinoma and melanoma), HA levels overall are low and linked to metastatic potential[74,75], whereas in other cancers such as breast[76] and lung adenocarcinomas[77], HA accumulation is high, but clinical outcomes are still poor.[78,79] The levels of HA are assessed using histochemical staining. For example a HA positive tumour having 71-100% of cells stained positive for pericellular HA would be classified as high, and a tumour categorised as having reduced HA having 0-70% HA positive pericellular staining. The precise concentration of HA in various tumours has not been determined and methods for quantification of high versus low HA tumour content vary among the literature. Several theories have developed explaining the role of HA in a poor prognosis in cancer. The first is that some cancer cell types overexpress enzymes such as MMP's that breakdown the ECM and the HA-CD44 bond to facilitate invasion and metastasis.[80] The second is that there are particular cancer cells that can overexpress HA in response to chemotherapy, thereby producing a protective effect for the tumour.[81] A further concept is that the

presence of large amounts of HA can prevent chemotherapeutic agents accessing the cancer cells.[82] In order to illustrate the first theory, Fig. 4 presents a magnetic resonance image (MRI) of a large malignant tumour in the thigh of a patient subsequently diagnosed with a sarcoma. Sarcomas are rare cancers arising in the connective tissues and usually appear in the limbs. Alongside the MRI is the pathology slide from the tumour. The lighter grey colour of the tumour in the MRI is due to the large numbers of rapidly dividing cancer cells and the relatively high glycosaminoglycan content, mainly HA, in the ECM.[83] The cells in the pathology image are stained for a matrix metalloproteinase enzyme which cleaves the HA receptor CD44. In this tumour there is intense MMP staining because the cells are constantly destroying their microenvironment using these enzymes to cleave key ECM components such as collagen and HA. This HA release via initial cleavage of the CD44/HA complex followed by further HA breakdown into smaller fragments will further promote the metastasis of cancer cells via the facilitation of cancer cell migration.[39] Moreover it has been demonstrated that the HA fragments themselves can begin a positive feedback loop whereby their very presence increases production of CD44 cleaving enzymes.[84,85]

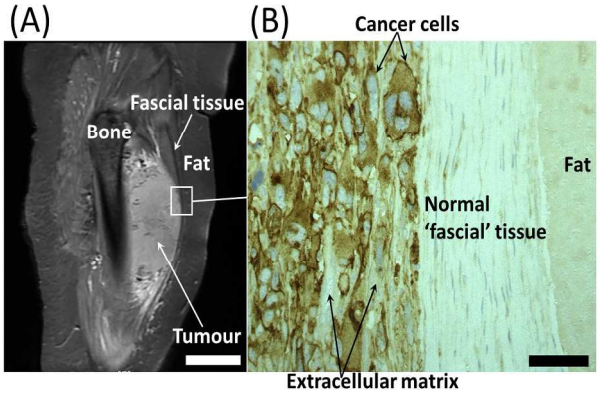

Fig. 4 (A). Magnetic Resonance Image of a soft tissue tumour in the thigh. The lighter grey appearance of the tumour is due to the high cellular content and different ECM components of the tumour compared to the bone, normal fascial tissue and fat. Scale bar=20mm. (B) Histological section of the interface between the tumour and normal tissues. The intense brown staining in the cancer cells is due to high expression of the invasive enzyme membrane type-1 matrix metalloproteinase. This enzyme cleaves the hyaluronan receptor CD44. Scale bar=50μm.

Abundance of HA in tumours has been found to have serious implications in the delivery of therapy. Such HA related resistance is usually cancer and drug dependent. Examples include resistance to Adriamycin in head and neck cancer,[86] and resistance to Carboplatin in ovarian cancer,[81] In terms of more recent treatment strategies which include utilisation of antibodies to perturb cancer function, the accumulation of HA in solid tumours can act as a barrier to monoclonal antibody therapy.[87] In order to mitigate these effects, it has been shown that it is possible to minimise drug resistance in peripheral nerve sheath tumours by disrupting the CD44-HA bond.[88] This disruption was achieved by delivering small oligosaccharides of HA which could compete for the receptor sites with the larger HA polymers. [89]

Hyaluronan based nanoparticle therapy

The use of HA based nanoparticle systems in cancer therapy has several advantages over existing formulations including selective attachment (via the CD44 receptors of the target cells), the fact that many carcinomas over-express CD44, and the ease of chemical functionalisation.[90]

With its intricate role in cancer biology, it is of no surprise that HA and the CD44/HA interaction are being targeted as a strategy for both disruption and drug delivery. One such approach has been to load lipid based nanoparticles with HA oligosaccharides with the aim of breaching HA barrier found in breast cancers. The HA oligosaccharides, in essence HA fragments, compete with the native HA for cell surface CD44 receptors. Moreover these oligosaccharide containing particles have the potential to overcome chemoresistance.[91] A related approach has been to graft HA lipid vesicles for the delivery of gene silencing RNA designed to interfere with key cancer cell signalling pathways. The over-expression of CD44 receptors on cancer cells results in HA accumulation which in turn facilitates the internalisation of the HA coated vesicles into the cancer cells. Once internalised, the interfering RNA renders the cancer cell susceptible to chemotherapy.[92] HA coating of lipid vesicles containing chemotherapeutic agents is relatively straight forward if cationic lipids are used. This is because HA has a negative charge and an ionic exchange mechanism can be exploited. Such a strategy was applied to target CD44 positive colon cancer cells.[93] As an alternative to liposome based strategies HA decorated polymer nanoparticles have been engineered to target CD44 receptors on malignant cell surfaces with the particles being internalised via receptor mediated endocytosis.[94] Such polymer vesicles make use of the so called enhanced permeability and retention effect to reach the innards of solid tumours.

One approach to treating skin cancer involved targeting the HA itself. This was achieved via a nanoformulation of the HA degrading enzyme hyaluronidase. Its modus operandi was to degrade tumour associated HA reducing the gel like properties of the ECM and thus allowing subsequent administration of chemotherapy drugs to reach the tumour.[95] Given the many adverse effects that accumulation of HA has on tumour progression, depleting the tumour of this antagonistic biopolymer presents opportunities for therapy.[89]

HA based hydrogels for studying invasion

Given the role of HA in the ECM, its role in invasion, the process by which cancerous cells break through the extracellular matrix, is directly related to its over-expression. A particularly revealing method to study this phenomenon *in vitro* has involved biologists adopting soft matter approaches to mimic the tumour microenvironment.[96] Using gels of defined compositions to mimic the tumour ECM, entrapped cells are observed and their trajectories through the gel analysed (Fig. 5).[97–99] A prime example are gels used to mimic ECM in the brain where HA is known to play a major role in glioma cell invasion. Rao et al formulated composite HA-collagen gels to examine the behaviour of patient tumour derived glioblastoma cells [100]. They showed that by adding HA they could increase the modulus of pure collagen hydrogels from 300 Pa to greater than 1000 Pa. These gel mechanical properties compare favourably to brain tissue reported to have a modulus of between 200 and 1000 kPa.[101]

Pedron et al demonstrated it was possible to regulate glioma cell phenotype using brain mimetic HA hyrogels.[97,102] This approach has been generalised to numerous different cancer cell lines allowing a comparative study of invasion and suggest that such gels represent some of the essential features of the ECM.[103] HA in isolation does not form robust gels, however under low pH and over an extremely narrow pH range it can form viscoelastic putty like gels. It is also possible to form weak cryotropic gels by using a freeze and thaw technique[104]. To form more robust gels one of two strategies are used. The first is chemical modification of HA so that it can form the covalent cross links necessary for gel formation. Methods to create such crosslinkable HA involves targeting either carboxylic acid or hydroxyl groups.[105] A second strategy is to combine HA with a component that does readily form robust gels. Collagen is often used for this purpose and has the added value that it is also an extracellular matrix component.[106] The gelation process of collagen with HA has been closely followed using confocal reflectance microscopy and correlated with viscoelastic properties via rheology. This combined approach demonstrated that the HA altered the mechanical properties in a temperature dependant manner and could be explained in terms of the HA distribution either on ore between the collagen fibres[106]. Yang et. al. demonstrated that by incorporating HA into acid solubilised collagen they could modify the viscoelastic properties as characterised by the storage and loss modulus. HA had the effect of increasing the loss modulus of the composite with the HA viscoelasticity dominating the mechanical properties of the gels.[107] Commercially available gels derived from tumour secretions are also useful models and have been utilised to demonstrate that the HA-CD44 interaction facilitates invasion of colon carcinoma cells.[108] These gels also allow the relationship between matrix mechanical properties and cell behaviour to be investigated. For example Shen et al were able to show that the stiffer the HA hydrogel, the less cancer cell invasion was observed.[109] This was consistent with the general findings that cells can respond to the stiffness of their ECM.[110] In order to further exploit the soft matter properties of model systems, researchers have attempted to recreate the tumour microenvironment using a combination of hydrogels, HA and growth factors.[111] They were able to grow tumouroids from prostate cancer cells and tune the mechanical properties of the gels to simulate the microenvironment. The gels produced had an average modulus of 234 ± 30 Pa and importantly could be degraded by hyaluronidase, the HA digesting enzyme. Non-HA based gels, for example agarose also have their uses in studying HA based phenomena. Their mechanical properties can easily be tuned and thus can be used to systematically examine the effect of mechanical stress on tumour related behaviour. Using this principle Koike et al were able to exert a controlled mechanical environment on neoplastic cells grown under confinement, with the size and rate of growth of the spheroids depending on the stiffness of the gels. The HA produced by the growing spheroids was identified by hyaluronan binding protein allowing a linkage between the role of HA and spheroid growth to be proposed[112].

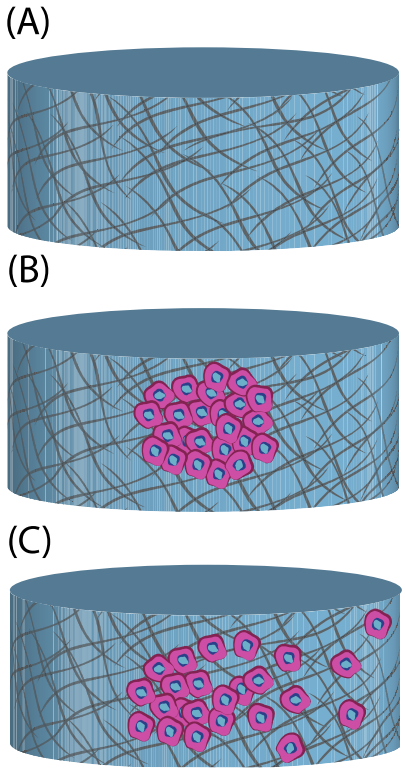

Fig. 5 Hyaluronan gels used for cell invasion studies. (A) The gel is composed of hyaluronan that has either been chemically modified to cross link, or mixed with another gel forming polymer such as collagen. (B) Cells of interest are cultured inside the gels, they are observed over time. (C) Depending on the type of cancer the cells will digest the gel components and spread out, simulating invasion of the cancer through the ECM.

Perspective

Understanding the soft matter properties of hyaluronan in the context of its role in cancer remains a challenge due to both the complexity of biological systems and the disease itself. However simplified model systems such as gels and microfluidic assays can reveal the physics of HA mediated process, many of which are involved in various stages of metastasis. Moreover gels can be tuned to have similar mechanical properties to the extracellular matrix, allowing the effect of mechanics on cell invasion and model tumour growth to be investigated. As these gel based systems become more complex by incorporating additional components of the ECM, the studies will become more accurate in replicating *in vivo* behaviour. Nanoparticle base formulations exploiting HA-receptor interactions and targeting HA itself offer the potential to modify the tumour microenvironment. This could be advantageous both for disease treatment and reducing tumour resistance to chemotherapy.

It is obvious that a large gap in knowledge exists in terms of quantifying and physical characterisation of HA from the tumour microenvironment. To date quantification has been performed in a histological sense, rather than a physically accurate method involving polymer physics. This presents an opportunity for soft matter scientists to work with clinicians in order to extract and characterise HA from the tumour microenvironment.

The mechanisms by which ultra-high molecular weight HA confers its anti-cancer properties in the naked mole rat may involve several different routes, both signal based and physical in origin.[113] However from a soft matter perspective a tantalising question remains, what is the significance of the molecular weight of NMR-HA and how could this be exploited to treat human cancer?

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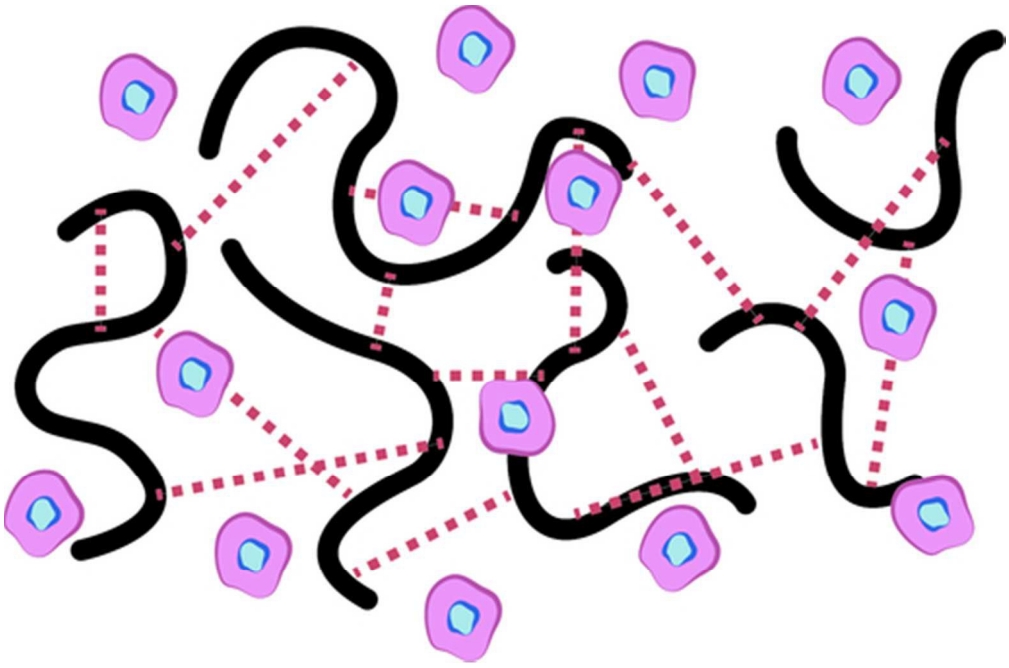

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