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Hyaluronic acid-based models of the brain microenvironment: Challenges and advances

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While hyaluronic acid (HA) extracellular matrix (ECM) models continue to provide valuable insights into brain physiology and disease, much room for improvement remains in terms of capturing the cellular and structural complexity of the brain microenvironment. Here we review next-generation HA models that are aimed at better capturing brain microenvironmental complexity. We discuss functionalization and crosslinking strategies designed to improve HA stability and biocompatibility. We also cover efforts to incorporate ECM proteins and stromal elements into HA hydrogels, including astrocytes, endothelial cells, and macrophages. We conclude with a brief discussion of nascent advancements and applications of these models, ranging from the reconstruction of multicellular stromal structures to the development of high-throughput screening platforms. This new suite of matrix technologies and the resulting applications should contribute greatly to mechanistic and therapeutic discovery in brain physiology and disease.

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Introduction

The Institute for Health Metrics and Evaluation estimates that neurological conditions, including diseases of the central nervous system (CNS), have a prevalence of 43 % globally [1]. Despite the devastating clinical, societal, and economic consequences of these disorders, we continue to face major gaps in our understanding of how CNS disorders and diseases develop and progress. A major contributing factor fueling this gap is the relative absence of *in vitro* models that incorporate the complexities of tissue while preserving the high throughput and accessibility of cell culture. The development of such advanced models could help improve the field's mechanistic understanding of CNS pathology and serve as the basis for platforms to accelerate discovery of therapeutic targets [2].

One CNS disease where an incomplete understanding of mechanism limits therapeutic progress is glioblastoma (GBM), the most common and aggressive form of primary brain cancer, which accounts for just under half of all primary malignant brain tumors [3]. Even with an aggressive therapeutic regimen, which typically includes surgical resection with adjuvant radiation and chemotherapy [4], the median survival time remains at less than 15 months after diagnosis [5], with only 5 % of patients surviving more than five years after diagnosis [6]. This poor survival is largely attributed to the highly invasive tumor front, which allows cells to evade surgical resection, penetrate portions of the brain that are less accessible to chemotherapy, and ultimately drive resistance and recurrence. For example, it is common for patients to experience tumor reduction in response to an initial course of surgery, radiotherapy, and temozolomide [7], only to experience emergence of resistant tumors weeks to months later. Similar effects are regularly observed with anti-angiogenic agents such as bevacizumab [8], which may paradoxically drive invasion through activation of hypoxia-induced signaling [9]. However, the exact mechanisms by which specific subsets of tumor cells penetrate the brain, evade treatment, and establish secondary tumors remain incompletely understood. Progress in understanding and disrupting the invasion process is limited by a relative absence of paradigms for identifying genes and proteins that can be therapeutically targeted to limit invasion, which remains challenging to infer from traditional two-dimensional cell culture models and mouse models. Specifically, traditional cell culture models such as multi-well plates and Boyden chamber assays omit key geometric, mechanical, and biological complexities of tumor tissue. Conversely, mouse models remain largely refractory to longitudinal, high-resolution studies of tumor invasion [10].

Therefore, there remains a need for models to occupy the sizable gap between highly reductionist culture platforms and animal models. Engineered hydrogel

biomaterial models have emerged as an attractive candidate for this purpose, particularly because the brain extracellular matrix (ECM) is itself rich in biopolymeric building blocks such as glycosaminoglycans and proteoglycans [10,11]. More specifically, hyaluronic acid (HA) is a glycosaminoglycan (GAG) that serves as the most abundant component of brain ECM and plays central roles in both organizing other brain ECM components and directly engaging cellular adhesion receptors, such as CD44 [12]. As a result, HA is increasingly explored as an *in vitro* model for modeling and studying neuro-oncologic disease mechanisms [13]. The adoption of engineered HA hydrogels as 3D culture models of brain matrix is fueled by HA's high solubility and versatile chemical functionality, with the repeating N-acetylglucosamine and D-glucuronic acid units offering hydroxyl, carboxylic acid, and amide moieties that can be exploited for chemical conjugation [14]. These conjugation chemistries, which are described in detail below, can be leveraged to crosslink HA into 3D hydrogels and to append short bioactive peptides to facilitate integrin-based adhesion, recruitment/retention of growth factors, and other important functions. These capabilities can be combined to yield 3D HA hydrogels that support long-term culture of tumor cells that invade the surrounding matrix, a capability that can be harnessed to investigate mechanisms of invasion, capture inter-tumor invasive heterogeneity, and identify molecules and/or pathways that could be targeted to limit invasion. Ambitiously, these materials could serve as the basis of personalized "tumors in a dish" that could support patient-specific modeling and therapeutic design [15]. While early HA models featured tumor cells encapsulated within rigidly crosslinked HA hydrogels, recent years have witnessed a burst of effort in both HA fabrication methodologies and incorporation of stromal components key to driving tumor invasion. This review covers both developments.

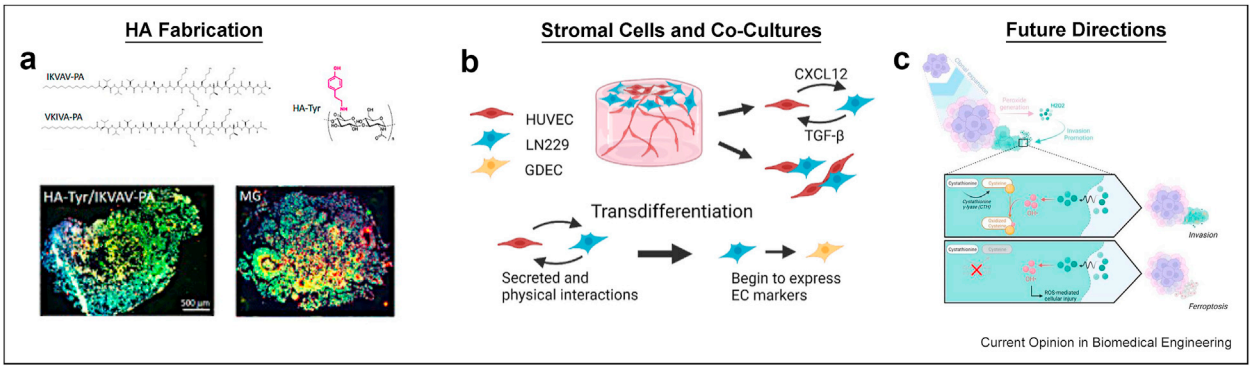
HA fabrication: novel approaches in functionalization, crosslinking, and catalysis

Over the past ten years, different chemistries have been applied to HA systems to develop 3D constructs suitable for cell culture. To construct an HA hydrogel, endogenous HA is chemically modified at one of the available functional groups mentioned earlier. Common conjugation approaches include adding a reactive chemical group, such as a methacrylate, to the hydroxyl group [13] (generating HA-methacrylate, HAMe), or altering the carboxylic acid group into an activated ester via carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) chemistry [16]. Either of these chemistries can then be subject to nucleophilic attack via a Michael addition reaction, often with a dithiol moiety (such as dithiothreitol (DTT)), which crosslinks the modified HA backbone together, creating a 3D construct. Alternatively, the activated ester resulting from the NHS-EDC reaction can be replaced with a dibenzylcyclooctyne (DBCO) moiety, to which an azide-functionalized crosslinker can be attached through strain-promoted alkyne−azide cycloaddition (SPAAC) [17,18]. While these chemistries are relatively efficient and straightforward to use, they are sometimes slow and do not offer precise spatiotemporal control of the crosslinking process. By contrast, photoactivated systems enable much faster crosslinking, with the possibility of spatial and temporal patterning. These systems rely on a photoinitiator, typically a molecule with high potential for generating oxygen radicals. Upon exposure to UV or visible light [18], generation of these oxygen radicals produce nucleophilic attack on the HA backbone, resulting in local crosslinking. Phototoxicity remains an important potential drawback of photocrosslinking approaches, thus limiting their application to cellular systems.

Recent studies have expanded on the above outlined design principles by engineering alternative HA functionalization and crosslinking strategies. For example, *Unal* et al. recently successfully applied photoinitiated DTT crosslinking to a norbornene functionalized HA matrix containing oligodendrocyte progenitor cells [19]. Specifically, photoinitiation was mediated by lithium phenyl-2,4,5trimethylbenzoylphosphonate, resulting in rapid gel formation within 2 min and preserved cell viability, strengthening the case for future application of light catalyzed reactions to cell-containing HA hydrogels [19]. Alternatively, others have combined olefin conjugation with thiolated zwitteronic polymers to develop a cytocompatible hydrogel with minimal nonspecific protein adsorption, which can be valuable for certain modeling and *in vivo* delivery applications [20].

Approaches in crosslinking strategies have varied, including crosslinking catalyzed by horseradish peroxidase and choline oxidase [21], as well as utilizing peptide amphiphiles [22] to crosslink the matrix and recapitulate ECM nanofibrous architecture (Figure 1A). Extensive crosslinked hydrogels are often purely elastic, whereas soft tissues are typically viscoelastic or stress relaxing. Brain tissue has a particularly pronounced stress-relaxing character, which is often ascribed to its high cellularity and water content and thought to play a role in protecting the brain from mechanical injury [23–25].For example, our own recent atomic force microscopy indentation measurements revealed that mouse brain relaxes ∼20% of applied stress [26]. As we recently reviewed [27], many viscoelastic ECM systems have been introduced to capture these effects, and our own laboratory developed a stress-relaxing hydrogel in which polymer backbones were crosslinked with mismatched pendant DNA oligonucleotides. We initially deployed this system in 2D polyacrylamide gels

Figure 1



Novel approaches and future directions for HA-based *in vitro* systems. **(a)** Two-component hydrogels are generated through the combination of laminin-mimetic peptide amphiphiles and tyramine-functionalized HA; cerebral organoid encapsulation in this system reveals comparable morphology and molecular signatures to Matrigel systems. Coculture of epithelial and GBM **(b)** cells enables modeling of paracrine signaling and its contributions to GBM aggression. Through multi-omic *in vitro* screens and validation of hits against GBM patient tumor samples, cystathionine is identified as a key gene for GBM invasion **(c)** due to its role in helping GBM cells clear elevated levels of reactive oxygen species, which are generated during invasion. Reproduced with permission from Refs. [22,29,30]. GBM, glioblastoma; HA, hyaluronic acid.

and demonstrated that adult hippocampal neural stem cells (NSCs) preferentially differentiate into astrocytes as stress relaxation increases [28]. We then extended this crosslinking strategy to 2D and 3D HA hydrogels [17]. In a related study, we constructed stress-relaxing 3D HA matrices by lightly crosslinking methacrylated high molecular weight HA, enabling us to capture the stress-relaxing properties of brain ECM and recapitulate the rapid cellular invasion that is reminiscent of the invasive GBM front [26]. Hence, application of novel crosslinking and functionalization chemistries not only strengthens HA hydrogel stability and biocompatibility but also presents opportunities to more closely match endogenous HA, improving the physiological relevance of *in vitro* models.

Toward true ECM recapitulation: multi-component incorporation and microenvironment remodeling

While HA hydrogels have been validated in their ability to recapitulate biomechanical and biochemical facets of the brain microenvironment [29], they typically omit non−HA brain ECM components known to regulate brain biology in important ways. These HA-centric matrices limit the degree to which the mechanisms of matrix remodeling and turnover can be incorporated and studied. For example, in GBM, tumor cells both degrade the surrounding matrix through matrix metalloproteinase secretion [31] and stiffen the tissue through the secretion of ECM components, such as HA, tenascin, fibronectin, and collagens [32]. Thus, developing chemistries that both incorporate additional ECM components and allow for ECM secretion/degradation represents an important next conceptual step for improving the biomimicry and predictive power of HA matrices.

Approaches to incorporate ECM components into HA hydrogel models generally fall into one of two categories: interpenetrating/semi-interpenetrating polymer networks (IPNs, sIPNs) and direct covalent functionalization. IPNs are two-component polymer networks in which each component is self-crosslinked to form an interlocked network; sIPNs are two-component networks in which one polymer is self-crosslinked and the other polymer is physically entrapped within the primary network [33]. sIPNs have proven valuable for orthogonally controlling the mechanical and biochemical properties of the network through each polymer component. A recent study described a strategy in which the primary network was formed through covalent crosslinking of HA or chondroitin sulfate, another GAG abundant in the brain ECM, via EDC-NHS chemistry, with collagen forming the secondary network [34]. Using this model, the authors of the study were able to discern how the presence of these different ECM components affected the expression of nestin, a key neural stem cell marker. Others have expanded on this approach (combining collagen and HA) but with the addition of a poly(ethylene glycol) diacrylate scaffold to control for matrix stiffness independent of HA concentration [35].

Covalent functionalization serves as an alternative, more permanent way to incorporate additional ECM components into HA-based hydrogels. Multiple researchers have focused on the incorporation of laminin motifs onto HA hydrogels (via SPAAC or Michael addition) to study

the growth and differentiation of NSCs [36]. Alternatively, in one study, researchers grafted dopamine moieties onto HA-chondroitin sulfate to sequester cell-secreted laminin [37], thereby enabling the analysis of how cell-secreted ECM supports neuronal growth and network formation.

While ECM incorporation into HA hydrogels enables perturbation of cellular-ECM biochemical signaling, another critical component of the cell−ECM relationship is ECM remodeling, i.e. the cellular digestion and secretion of ECM components. A primary challenge in understanding ECM remodeling is distinguishing secreted matrix content from matrix already present in the hydrogel scaffold. One solution has been to tag secreted matrices using an azide-containing analog [38], which is incorporated into the newly synthesized matrix and can subsequently be tagged and visualized using a DBCO conjugated fluorophore. This method has been applied to understand how mesenchymal stem cell protein secretion changes among agarose, alginate, PEG, and HA hydrogels [38]. Others have used this matrix-tagging strategy to visualize matrix degradation during some dynamic process. For example, secretion of both HA (in a GelMa system) [39] and collagen VI (within an HAMe system) [40] have been visualized and correlated with increased GBM invasion.

Stromal cells: modulators of disease

While previously underappreciated, stromal cells have received a growing amount of attention in the CNS disease space as important modulators of disease progression. As a result, incorporation of stromal cells into HA models is important to fully recapitulate the microenvironment and to capture and/or study cellular interactions that contribute to disease progression.

Astrocytes are the most abundant cell type within the brain's stromal component (and the brain as a whole) and are involved in various homeostatic and disease-related processes, including: maintaining the blood−brain barrier (BBB) [41], becoming activated by GBM cells to promote GBM invasion [42], and secreting antioxidants to combat oxidative stress, a common aspect of neurodegeneration [41]. In addition, astrocytes can also be directly influenced by the changing ECM to contribute to disease processes. A recent study of astrocytes in an IPN HA hydrogel found that decreased HA concentration resulted in an increase in inflammatory markers and decrease in cellular process lengths [35]. These findings further support a need to study stromal cells in HA hydrogels to probe the effects of the matrix both on stromal cells alone and on the interaction between stromal cells and normal/diseased cells.

Hence, recent studies have sought to incorporate astrocytes into hydrogel platforms to better understand astrocytes' innate biology and interactions with diseased cells. Recently, a modular, 3D printed, HA hydrogel system was developed and applied to probe the effects of cell adhesion peptides on astrocyte−glioma interactions [41]. Others have perturbed GBM−astrocyte interactions in Matrigel vs. thiolated HA to further understand the influences of ECM biochemical signaling on stromal−GBM interactions [43]. Another approach has been to merge the principles from both of these studies by incorporating collagen into an HA hydrogel system and characterizing the effects of astrocytes on GBM cell migration and growth [44].

Incorporation of brain-resident and exogenous immune cells into *in vitro* models is also gaining momentum given the role of these cells in inflammation and disease. Microglia (the resident macrophages of the brain) and tumor-associated macrophages (TAMs) are increasingly featured within disease models with an eye toward better understanding immunological contributions to pathophysiology. For example, GBM cells were shown to activate microglia in a gelatin methacrylate (GelMa) coculture system [45], and secreted factors from TAMs (in an HA hydrogel) [46] or microglia (in GelMa) [45] can either promote or inhibit GBM invasion. These efforts can be expanded further into 3-component coculture models; for example, neurons, astrocytes, and microglia have been cocultured in 3D hydrogels and subjected to screening to identify formulations that support optimal neuronal and glial network formation [47]. Much like astrocytes, TAMs have been shown to directly engage HA; for example, disrupting GBM cell HA synthesis or blocking HA-CD44 binding altered TAM polarization [48].

Finally, there are ongoing efforts to incorporate endothelial cells (ECs) into *in vitro* models due to the essential role these cells play in regulating vascular permeability (e.g. in the BBB) and driving angiogenesis, the formation of new blood vessels associated with later stage tumors. ECs also compose the perivascular niche (PVN), which provides complex signaling to tumor cells as well as tracks upon which tumor cells can quickly migrate [49]. Hence, incorporation of ECs into hydrogel systems as well as recapitulation of the perivascular niche is important for elucidating mechanisms of later stage brain tumors. Recently, capillary structures were recapitulated within GelMa hydrogels (with and without methacrylated HA), allowing for the perturbation of how triculture of GBM cells, ECs, and fibroblasts influences GBM cell vessel cooption and regression [50]. *Hatlen* et al. built upon this work by featuring ECs, astrocytes, GBM, and stromal cells within a single hydrogel system [30] to characterize the effects of transforming growth factor beta (TGF-β) and CXCL12 paracrine signaling (Figure 1B). Others have taken a top-down approach, utilizing additive

manufacturing (3D printing) tools to recapitulate macroscopic portions of the PVN. These advanced fabrication tools were leveraged to create microtissues with spatially varying matrix mechanical properties and precisely positioned ECs and GBM cells in a manner that permitted next-generation sequencing and characterization of drug resistance [51].

Limitations of HA hydrogel chemistries and approaches

Despite these advancements in next-generation HA model design, much room for improvement remains. Most notably, the incorporation of ECM components (including HA) is still largely done through some degree of covalent modification chemistries that are not found in the brain. There is ample evidence that these chemistries do not affect cell viability, and chemically modified HA hydrogels have been used in advanced drug delivery systems *in vivo* [52] without noticeable disruption of cellular behavior, suggesting that cell sensation of chemically modified HA may be minimal. Nevertheless, the degree to which HA covalent modification influences ligand presentation, signaling, and other downstream biological events remains controversial [53]. There is therefore a strong need to better define effects of covalent backbone modification on HA-dependent biology as well as an expanded suite of conjugation approaches that better mimic *in vivo* presentation. For example, we and others have conjugated heparin fragments to the HA backbone, which noncovalently sequester growth factors and cytokines analogous to their presentation *in vivo* [54].

Further, while the addition of stromal components increases physiological relevance of these models, doing so also introduces important practical challenges, such as identifying coculture conditions that support all cell types. Such efforts may be accelerated by new machine learning-based tools to speed optimization of media formulations [55]. Moreover, while coculture models allow investigation of how different cell types influence one another in disease states, the isolation of these effects becomes increasingly difficult as model complexity (i.e. the number of cell types) increases. To address this gap, one promising approach has been to couple traditional genomics and single-cell analyses with techniques that provide spatial information in real time, such as multiplexed fluorescence, DNA, RNA, and isotope labeling [56]. Integration of these data with multiomics datasets presents a potential gateway into understanding thousands of cancer variants and biomarkers within complex, multicellular models. Future work should continue in this direction, focusing on new approaches to systematically dissect and understand causal interactions among multiple cell types.

Future directions

HA hydrogel systems continue to show great promise for modeling CNS physiology and disease as well as for serving as a foundation for screening and precision medicine technologies. We have reviewed recent advancements in HA hydrogels for CNS applications, particularly in GBM, with a focus on hydrogel crosslinking/functionalization and inclusion of stromal cells. In the future, it will be fruitful to continue integrating these approaches to more closely mimic the brain microenvironment, such as capturing brain-mimetic ECM component profiles and multiple stromal cells within a common platform. Additional areas for future development include the reconstruction of stroma at larger length scales with tissue-like geometries, such as blood vessels and white matter tracts [51,57], which is particularly important in GBM given these tumors' well-known propensity to invade along such structures. Integration of brain organoids into HA hydrogel platforms [22] could also advance progress toward these goals and facilitate longer-term culture in which a diversity of cell types could organically arise and self-assemble. Ideally, these paradigms could be designed to be compatible with microscopic dissection and analysis [29], including single-cell analysis, which can capture heterogeneities that are often lost in averaged analyses of homogenized tissue. In this spirit, development of high-throughput and high-content screening technologies or platforms [58] will prove useful in the identification of biomarkers as well as disease responses to therapeutics (Figure 1C). Finally, our understanding of matrix degradation mechanisms will be further strengthened by novel techniques to label and visualize degraded or secreted matrices [38], particularly when the secreted matrix and surrounding hydrogel are composed of the same material.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data were used for the research described in the article.

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** of outstanding interest

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