Towards clinical translation of 'second-generation' regenerative stroke therapies: hydrogels as game changers?

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Abstract

Stroke is an unmet clinical need with a paucity of treatments, at least in part because chronic stroke pathologies are prohibitive to "first-generation" stem cell-based therapies. Hydrogels can remodel the hostile stroke microenvironment to aid endogenous and exogenous regenerative repair processes. However no clinical trials have yet been successfully commissioned for these "second-generation" hydrogel-based therapies for chronic ischemic stroke regeneration. This review recommends a path forward to improve hydrogel technology for future clinical translation for stroke. Specifically, we suggest that a better understanding of human host stroke tissue-hydrogel interactions in addition to the effects of scaling up hydrogel volume to human-sized cavities would help guide translation of these second-generation regenerative stroke therapies.

1. Stroke is an unmet clinical need.

Stroke is one of the leading causes of death and disability globally [1]. In ~85% of cases, stroke is caused by an **ischemic** event due to a blockage in the blood supply in the brain (see Glossary). Strategies to treat ischemic stroke are limited to reperfusion medications (**tissue plasminogen activator**) and surgical interventions (**thrombectomy**) that aim to restore oxygenation and reduce lasting damage. However, only up to 10% of ischemic stroke patients actually receive

these therapies due to constraints such as narrow therapeutic windows for tissue plasminogen activator administration (< 4h) and thrombectomy (mostly < 6h). In many patients the disease progresses into a **chronic phase**, characterised by persistent disability and reduced likelihood of full recovery 6 months after the ischemic event [1].

Stem cell therapies are regenerative medicine approaches to restore neuronal function and improve clinical outcomes. Over 50 human clinical trials have been commissioned to study the viability of such therapies for stroke regeneration, including using mesenchymal, **hematopoietic**, neural and **induced neural cells** [2]. Of these "first-generation" cell-based therapies, many utilise the intravenous route of administration during acute stroke to infiltrate the brain via the permeabilized **blood brain barrier** and release of pro-regenerative factors locally. While this method is considered less invasive than intracranial injection, the recovery of the blood brain barrier in the **sub-acute** (3-9 days) to chronic phases (beyond 30 days) limits the timescale for such systemic applications. Despite their positive safety profiles in early clinical trials [3-5], first-generation acute stroke therapies have failed to demonstrate a marked clinical recovery observed through patient scoring (for review see [6]). Whilst some promise has been shown with chronic stroke therapies, there are growing concerns that the pathology of stroke cannot be overcome solely with cell-based therapy [7]. These concerns have prompted a return to preclinical strategies with a focus on tuning regenerative therapies to the specific pathology of chronic stroke.

Hydrogels are emerging as a platform to improve regenerative payload delivery due to their unique physicochemical properties that are tuneable to the tissue type required for site-specific delivery [8-9]. These **biomaterial** technologies are manufactured from natural or synthetic **polymer** solutions that are induced to undergo solution-gel transition using chemical, physical or thermal triggers to yield three-dimensional water-based hydrogels. Hydrogels are already used extensively in the clinic in the form of contact lenses, cosmetic fillers (e.g. Restylane®,

Radiesse®) and as medical implants during spinal fusion surgery (e.g. INFUSE®) and prostate cancer (e.g. Vantas®) [10] and are at the forefront of regenerative medicine efforts for osteoarthritis (e.g. HYMOVIS®) [11-12] and heart failure (e.g. Algisyl-LVR®, Ventrigel®) [13, 14]. Despite the various advantages of hydrogels, no technologies have yet successfully advanced from the preclinical laboratory into clinical trials for chronic stroke patients. A phase I/II clinical trial was designed for implantation into the stroke cavity after intracerebral **hemorrhage** of alginate microcapsules encapsulating mesenchymal cells transfected to secrete glucagon like peptide-1 (GLP-1 CellBeads®, NCT01298830). However, the trial was terminated with 'the need for improvement of study medication' and 'no further gain in knowledge is expected'. A phase I trial transplanting Collagen Scaffold™ with MSCs after brain injury is recruiting (NCT02767817) and another trial designed to test the safety of extracellular matrix-mimicking scaffold after acute ischemic stroke is not yet recruiting (NCT04083001). The present mini-review therefore will highlight the key lessons learned from first generation regenerative stroke therapies with a focus on pathological considerations when designing therapies to treat chronic stroke. In the search for more advanced "second generation" treatment strategies, five leading hydrogels will be reviewed to determine their versatility for overcoming some of the key pathological features of chronic stroke, with suggestions for a path forward to improve this technology for future clinical translation.

2. Regenerative Intervention Impediments for Stroke

First-generation regenerative stroke therapies can be sub-divided into two classes depending on whether their stage of intervention is at the acute or chronic phase. Whilst this mini-review will focus primarily on therapies for chronic stroke, consideration must be given to **acute phase** interactions as they give rise to the key chronic pathologies. Details of the pathology of the stroke lesion are captured in **Box 1** and how this creates an inhospitable microenvironment for

regenerative repair processes are illustrated in **Figure 1A, Key Figure**. Regenerative technologies for chronic stroke are typically administered directly via intracranial injection into or beside the stroke cavity due to restoration of the blood brain barrier in the sub-acute stroke phase. The stroke cavity is an ideal delivery site because it is adjacent to a key zone of **neuroplasticity** and because it can accommodate the injection volume without compressing surrounding tissue. However, it is prohibitive to first-generation regenerative stroke therapies due to a lack of an **extracellular matrix**; being surrounded by a **glial scar**; and being filled with extracellular fluid, debris and inflammatory mediators (**Figure 1A**). Exogenous bone marrow derived- and **neural stem cells** have shown the most promise for chronic stroke regeneration as their ability to release growth and immunomodulatory factors are expected to reprogram the inhibitory stroke microenvironment with **neurogenic** and **angiogenic** properties. The ACTIsSIMA (**Allogeneic** Cell Therapy for Ischemic Stroke to Improve Motor Abilities; NCT01287936 [4]; NCT02448641 [15]) and PISCES (Pilot Investigation of Stem Cells in Stroke trials (NCT01151124 [16]; NCT02117635 [17] represent the most advanced regenerative therapies for chronic stroke under clinical investigation. Whilst such studies have encountered setbacks, such as distribution and survival of cell deposits, potentially due to a lack of vascular microenvironment [18], both studies have shown significant promise in early trials at effecting patient recovery.

3. Regenerative Hydrogels: A "Second-Generation" Therapeutic Approach?

First-generation cell-based therapies for chronic stroke need to improve in efficacy to boost translation into the clinic. Hydrogels are emerging as a strategy to achieve this, with substantial preclinical work already highlighting the key technological considerations for their use in chronic stroke. Hydrogels for regenerative neurological applications can be manufactured using biomaterials from synthetic materials such as poly(ethylene) glycol, and poly(lactic-co-

glycolic acid) (PLGA) [19], or from naturally occurring materials such as collagen, alginate and silk [20-22]. Synthetic hydrogels are reliable due to their scalability, and in most cases, ease of chemical modification during manufacturing and reduced likelihood of inducing immune reactions when compared to natural materials due to their lack of cell binding motifs for example [23]. Conversely, natural materials suffer from batch-limiting scalability issues [24], yet are typically desirable for regenerative applications as most materials retain features such as anti-inflammatory properties (**Figure 1B**) [25], integrin binding sites [26] and high tissue resorption [27] (albeit this is material dependent, for example alginate and *B. mori* silk lack RGD sequences). Regardless of material choice, all preclinical hydrogel formulations must undergo robust biocompatibility and biodegradability testing to validate their safety for chronic stroke applications. This includes local and systemic safety testing, as well as long term degradation studies to assess their potential to release toxic by-products during breakdown [2]. Such anti-inflammatory properties, biocompatibility and biodegradation in response to human stroke tissue have yet to be tested (**Table 1**). The physicochemical properties of the hydrogel should also be tuned to the specific requirements of the stroke microenvironment (**Figure 1B**). For example, *in situ* forming hydrogels are desirable as they can extend throughout the entire stroke cavity prior to gelation, enabling full re-scaffolding and structural support of irregularly shaped lesions [28]. Good **space conformity** without swelling are essential prerequisites for minimally invasive intracerebral administration to preclude compression of surrounding tissue (**Figure 1B**) [29]. Such a tight host tissue-hydrogel interface allows interaction with the glial scar (**Figure 1B**), helps the delivery of regenerative payloads, and provides good support for host cell infiltration and proliferation [22, 27]. *In vivo* MRI brain imaging of hydrogels using their molecular components (e.g. HA) [30, 31] or contrast agents (e.g. manganese ions for alginate) [32] will help guide further development of space conforming [32] and biodegradation [30] as well as the precision of injection into the stroke

cavity. Most preclinical stroke studies use healthy young male adult rodents whereas human stroke is usually complicated by age and multi-morbidities in both sexes [33] in which host tissue-hydrogel interactions are underexplored (**Table 1**). In addition, considering the volume of a human stroke cavity could be ~1000 fold larger than that of rodents (for instance, 50 cm^3 versus 50 mm^3, respectively), to fully fill a human size stroke cavity, the volume and possibly concentration of hydrogels would need be scaled up. Consideration would need to be given to the impact on viability of cellular payloads due to injection shearing [29] and limited passive diffusion of oxygen [34] (**Table 1**). The effect of the larger volume of fluid in the human cavity on gelation kinetics remains unexplored and the fluid would likely need to be drained to allow gelation [35]. The feasibility of drainage from the cavity could therefore become a determinant for which stroke patients should receive hydrogel-based therapeutic interventions. Tuning formed hydrogel stiffness to mimic that of brain tissue (0.5 – 1.5 kPa) [36] produces regenerative hydrogels that can better direct and support differentiation of stem cells [37, 38] and maximize host cellular responses [27, 39] to induce neurorepair. Hydrogels are therefore capable of exerting biological effects through inherent material properties, by delivering conventional drug payloads and through extracellular matrix-mimicking support of **endogenous** (e.g. host) and **exogenous** (e.g. payload) cells. A key advantage that hydrogels have over suspension-based technologies is the ability to amalgamate these effects into a combination therapy, wherein regeneration is achieved through the synergistic actions of material, drug and cellular factors.

Despite this, no clinical trials have been successfully commissioned to explore the potential of regenerative hydrogels for the treatment of chronic stroke [2]. The wider neurological regenerative attempts are similarly disappointing with the exception of NeuroRegen **Scaffold**™, a collagen-based hydrogel for chronic spinal cord injury which has been successfully used to deliver human umbilical cord **mesenchymal stem cells** into surgically

resected spinal cord injury lesion sites (NCT02352077 [21]; NCT02688049; [40]). Spinal cord injury presents a similar pathology to that of the stroke lesion including tissue **necrosis** and glial scarring. However, the surgical approaches used in the NeuroRegen Scaffold^{TM} trials act to remove the glial scar from the injury site, and so cannot be used to inform on collagen-tissue interactions in the chronic stroke brain. Nevertheless, the knowledge gained of interactions between hydrogels and white matter tracts may be useful for stroke as such information is sparse due to limited white matter in rodent brains (**Table 1**), and may be important for patient stratification given that 15-25% of all stroke subtypes include white matter damage.

Translation of leading stroke-specific hydrogel products into phase I clinical trials remains the best opportunity to evaluate their biosafety and therapeutic potential in humans and the current lack of any hydrogel studies represents a bottleneck in the development of regenerative therapies for chronic stroke. Clearly there is a need for exceptional preclinical hydrogel technologies to warrant translation into clinical investigation. More importantly, there are high stakes in any future hydrogel technologies that enter clinical trials as their performance will ultimately influence the uptake (or rejection) of similar regenerative technologies. Indeed, these difficult challenges may explain the slow progress that has been made with regard to clinical translation. To assess and help improve the readiness of regenerative hydrogels for stroke, here we review the current state-of-the-art in preclinical regenerative hydrogel technologies by selecting five leading materials that are supported by robust preclinical validation: (1) decellularized extracellular matrix hydrogels, (2) hyaluronan-based hydrogels, (3) silk-**fibroin** hydrogels, (4) alginate-based hydrogels, and (5) chitosan-based hydrogels. Basic and historical details of each hydrogel can be found in **Boxes 2-6** so that their recent technological developments in stroke are central in this review.

3.1 Decellularized Extracellular Matrix Hydrogel Technological Considerations

In chronic stroke, extracellular matrix hydrogels are stiffness tuneable within brain physiological conditions (0.5 – 1 kPa) and *in situ* forming due to thermoresponsive gelation at 37°C [35, 41]. When implanted in the lesion of **middle cerebral artery** occlusion (MCAO) in rats, extracellular matrix hydrogels were found to promote rapid invasion of **microglia** and neural **progenitor stem cells** [42]. In the same study, hydrogels with a high extracellular matrix content were found to recruit a significantly higher proportion of brain-derived cells, namely neural progenitors, oligodendrocytes, microglia and endothelial cells, when compared to lower concentration hydrogels and had an anti-inflammatory polarising effect on infiltrating microglia that could indicate potential for inflammatory reprogramming of the stroke lesion. When implanted into the lesion of middle cerebral artery occlusion (MCAO) in rats, extracellular matrix hydrogels were found to promote rapid invasion of microglia and neural progenitor stem cells [42], with high extracellular matrix content being more effective than lower concentration hydrogels. An anti-inflammatory polarising effect on infiltrating microglia that could indicate potential for inflammatory reprogramming of the stroke lesion [42]. Slow biodegradation of extracellular matrix hydrogels in the brain results in long term filling of the stroke cavity and a reduction in lesion size, albeit this was not accompanied with improvements in behavioural recovery [43]. The resorptive potential of extracellular matrix hydrogels is particularly exciting for chronic stroke applications as they could have implications for material-induced tissue restoration *in situ* through host cell repopulation of the stroke cavity [26]. Indeed, another biodegradation study evaluating extracellular matrix hydrogels found that neurogenesis was induced in the absence of a therapeutic payload and this was attributed to the recruitment and modulation of host cells by the hydrogel due to slow biodegradation [27]. Whether these host cell-hydrogel interactions differ in human tissue (**Table 1**) and whether they are specifically linked to any improved functional outcome are yet to be established. In addition, *in vivo* **electrophysiology** would be needed to verify if newborn cells become

functioning neurons and synapses. Extracellular matrix hydrogels are also proficient in local intracranial delivery of cell-based payloads such as neural stem cells, either within the hydrogel itself [44] or encapsulated within biodegradable poly(ethylene) glycol microspheres [45]. Importantly, microsphere encapsulation was found to improve cell survival and distribution within extracellular matrix hydrogels *in situ* in the rat MCAO model [45], and this novel approach at blending natural and synthetic hydrogels could have implications for the success of emerging regenerative hydrogels in the treatment of chronic stroke.

3.2 Hyaluronan-based Hydrogel Technological Considerations

For preclinical chronic stroke applications, hyaluronan is typically blended with methylcellulose, another naturally occurring polymer, to impart thermoresponsive properties that enable *in situ* forming following injection into the brain [9]. Hyaluronan-methylcellulose (HAMC) hydrogels have been primarily pioneered by the Shoichet laboratory specifically for applications in chronic stroke and meet all of the technical considerations with regard to biocompatibility, material optimisation and stroke- and payload-dependent suitability. Biocompatibility of HAMC hydrogels has been validated extensively *in vitro* and *in vivo* using numerous mouse and rat stroke models [46] and additional spinal cord injury studies indicated that they may exert anti-inflammatory effects on microglial cells due to their hyaluronan components [47, 48]. This could have profound implications for material-based immune-**reprogramming of the glial scar**. In addition, the *in situ* forming capabilities of HAMC hydrogels and minimal swelling following gelation make them ideally suited for chronic stroke applications [9]. In addition, their physical properties and biodegradability are optimised to ensure they mimic the elasticity of neuronal tissue and undergo controlled degradation and resorption in the months following implantation. HAMC hydrogels have a particularly successful preclinical record with drug and biological payload release in rodent stroke models,

including delivery of epidermal growth factor [49] and PLGA-encapsulated **brain derived neurotrophic factor** [50] in mice and rats, respectively, that promoted endogenous neural stem cell proliferation and induced functional recovery. Combination therapy is also feasible, as evidenced by **ciclosporin**- and **erythropoietin**-loaded HAMC hydrogels that worked synergistically to improve motor recovery in the sub-acute phase in a rat stroke model [51]. Ciclosporin is typically dose-limited in stroke due to systemic toxicities, yet HAMC-mediated delivery enabled successful local administration whilst restricting passage into the peripheral blood through exploitation of the blood brain barrier [52]. These toxicity restricting effects could have significant implications for the chronic stroke treatment by enabling "smart" delivery of otherwise systemically contraindicated drugs, for example, by using of potent immunosuppressive in combination with the primary payload to mediate secondary immune reprogramming of the glial scar.

As well as vehicles for drug delivery, HAMC hydrogels are suited for stem cell-based payload delivery and have been validated using rodent stroke models. In a study investigating HAMC hydrogels as stem cell carriers for retinal and neurological cell delivery, neural stem cell survival was significantly higher in stroke mouse models when compared to a suspension based implantation in cerebrospinal fluid [53]. The same study found that HAMC-mediated neural stem cell delivery induced a significant increase in recovery when compared to suspension-based implantation that also coincided with increased tissue penetration 4 weeks post implantation. In addition, HAMC hydrogels have been shown to support the survival of **induced pluripotent stem cell** (iPSC)-derived neuroepithelial progenitor cells in rat stroke models following early *in vitro* differentiation [54]. In a subsequent study, iPSC-neural stem cell loaded HAMC hydrogels were shown to promote neuronal survival in a differentiation dependent manner [55], with early differentiated stem cells performing better than stem cells at a later differentiation stage. This study also noted mild functional recovery in stroke rats

treated with HAMC hydrogels in the absence of a cell-based payload when compared to untreated stroke controls. This observation could suggest a regenerative advantage of HAMC hydrogels due to their inherent material properties.

3.3 Silk Fibroin-based Hydrogel Technological Considerations

Silk fibroin hydrogels are clinically approved for use in structural restoration in patients with vocal fold paralysis and have been studied extensively in preclinical research for applications in drug, biological and cellular delivery, including for applications in chronic stroke [56, 57]. In addition to low cell-binding properties, silk fibroin hydrogels can be tuned to physiological brain requirements, show viscoelastic mechanics [58], exhibit non-swelling behaviour and support the survival and distribution of stem cell based payloads [29]. Good space conformity of variable rat lesion sizes [22] bodes well for the variable human lesion sizes, albeit would take some optimising of concentration and volume of hydrogel (**Table 1**). The biocompatibility of silk fibroin hydrogels has been validated in the absence of a therapeutic payload in healthy [59] and MCAO [22] rodents. Both studies confirmed microglial accumulation around the hydrogel injection site, yet no adverse material immune-reactions were observed in the either study [22, 59]. Silk fibroin hydrogels induced endogenous cell proliferation in the ischemic brain [22], which has yet to be validated using *in vivo* electrophysiology. When loaded with mesenchymal stem cells, silk hydrogels induced functional recovery in the mouse MCAO model [60] that could be due to high anti-inflammatory transforming growth factor beta 1 release from the payload [61]. Preclinical studies that examined the impact of injection shearing of hydrogel:cell constructs on cell survival [29] need re-examined using scaled up cell dose and biomaterial volume for humans (**Table 1**).

3.4 Alginate-based Hydrogel Technological Considerations

Alginate microcapsules encapsulating mesenchymal cells (GLP-1 CellBeads®, NCT01298830) and encapsulating choroid plexus [62] have been used in clinical and preclinical stroke, respectively. Though on a lesser scale, alginate-based hydrogels have also been evaluated for neural regeneration. Alginate-based hydrogels loaded with trophic factors can stimulate angiogenesis and neural plasticity in murine models of CNS injury with varying levels of success [63, 64]. In the study by Ansorena [63], this regeneration was associated with recovery of motor function whereas in the study by des Rieux [64] functional recovery was not observed. Alginate-based hydrogels have also been shown to facilitate neural differentiation of hiPSC derived neurospheres [65], but clinical studies investigating the regenerative capacity of this is lacking. These preclinical studies suggest that, with optimisation of trophic factors and the type of regenerative cells, alginate-based hydrogels hold potential as a payload delivery system for neural recovery following CNS injury, including stroke.

3.5 Chitosan-based Hydrogel Technological Considerations

Chitosan's potential in stroke has been highlighted in the form of mesenchymal stem cell/chitosan-collagen scaffold composites [66] and rutin-encapsulated chitosan nanoparticles [67] and has proven anti-inflammatory effects [68]. The ability of chitosan-based hydrogels to promote neural differentiation of encapsulated progenitor cells [69] and induced pluripotent stem cells [70] highlight the potential use of chitosan-based hydrogels in neurodegenerative therapy. After traumatic brain injury, MSC-loaded chitosan-based hydrogel reduced cell death and stimulated the secretion of neurotrophic factors, which promoted the survival and proliferation of endogenous neural cells and simultaneously increased MSC neural differentiation [71]. These changes culminated in the recovery of brain structure and neurological function following traumatic brain injury [71] and similar studies have been

shown in spinal cord injury [72] in rats. This potential may also extend to neurological recovery post-stroke and highlight the potential chitosan-based hydrogels hold in neural regeneration.

4. Concluding Remarks and Future Perspectives

Following on from the successes of the ACTIsSIMA and PISCES trials, hydrogel-based therapeutic intervention is a novel strategy to enhance the efficacies of first-generation therapies by improving stem cell payload survival, re-scaffolding the stroke cavity and synergistic reprogramming the glial scar through inherent material properties. We have highlighted five hydrogel platforms that show significant promise for translational clinical studies, and tried to identify gaps in knowledge yet to be addressed. Hyaluronic acid is a good performance benchmark to reach prior to translation as it ticks many of the essential and desirable properties (Table 1), is already a leading healthcare material especially for aesthetic applications and is ideal for large scale manufacturing, albeit at higher costs [73]. By providing insight into key avenues for future research, this review helps push forward current thinking to help guide clinical translation for stroke and overcome potential barriers. Specifically, we hypothesize that hydrogels must first be optimised to ensure that characteristics and performance are retained on human tissue; with common stroke co-morbidities; in white matter damage and in larger stroke cavities. Therefore, we suggest that the next round of experiments should include testing hydrogels in female in addition to male aged, co-morbid (obese, hypertensive, diabetic) rodents; on human resident brain cells; and on white matter pathology. So far, intracerebral delivery in such a vulnerable patient population has not presented a barrier (e.g. ACTIsSIMA and PISCES trials), albeit feasibility of fluid drainage needs to be considered for hydrogels unless their gelation kinetics are unaltered by the large volumes of fluid found in human cavities [35]. Therefore, in preparation for sizing up to a human stroke cavity, larger hydrogel volumes (e.g. 50 ml) need to be tested for gelation kinetics and *in vitro* space conformity. Thereafter concentrations need to be optimised for cytocompatibility, injectability and distribution of scaled up cell doses. Degradation in human tissue would need to be

optimised. Whilst optimal degradation half-life for nerve regeneration is 2-3 weeks [74], this is yet to be validated for hydrogels in the stroke brain. Optimal degradation would be based on slow enough to allow tissue support and cellular effects to take place and fast enough to provide vital space for axonal growth and neuronal connectivity. Preclinical *in vitro* electrophysiology measurements of functional connectivity due to host cell infiltration in hydrogels should be combined with correlation studies between functional outcome and number of host cells recruited by hydrogels (see 'Outstanding Questions box'). Taken together with generic challenges for large-scale implementation of hydrogels, including regulatory affairs, covered elsewhere [73], these experiments are important steps in the translational framework to help improve technology before clinical trials.

Therefore, we urge a delay in commissioning clinical trials to minimise the risks of poor performance at early trial stages. Whilst this is frustrating considering the overall slow progress in development of stroke therapies, the importance of successful hydrogel-based clinical trials cannot be understated. If unsuccessful, premature hydrogel-based clinical trials could have devastating effects on the future of these technologies, both in stroke and in wider regenerative applications. Therefore, in order to maximize the future of regenerative hydrogels and fully realise their potential, there is a requirement to take a cautious approach to ensure their success when they ultimately enter the clinic.

Figure 1, Key Figure. Reprogramming the Stroke microenvironment. **(A)** The inhospitable stroke microenvironment consisting of fluid, debris, inflammatory cells and mediators, pathology-provoking DAMPs and PAMPs (damage and pathogen associated molecular patterns), limited vascularization and the prohibitive glial scar. **(B)** Reprograming of the stroke microenvironment to be receptive to regenerative repair processes by hydrogels due to their innate anti-inflammatory properties, good space conformity, and interface with the glial scar. Dotted line delineates vulnerable and viable tissue. Created with Biorender.com.

380 **Table 1:** A blueprint for future clinical translation of representative hydrogel platforms.
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Hydrogel properties required for chronic stroke	Decellularized extracellular matrix hydrogels	Hyaluronan-methylcellulose hydrogels	Silk-fibroin hydrogels	Challenges for clinical translation
Essential properties				
In situ space conformity without swelling	*In situ* forming due to thermoresponsive gelation at 37°C [41] with 4 mg/mL achieving a 92% coverage and the more solid 8 mg/mL gel resulting in 89% of the cavity being filled with ECM [35].	Thermoresponsive properties of methylcellulose are used to blend with hyaluronan to enable *in situ* forming following injection into the stroke brain [49]	Hydrogels (4% w/v) were evenly spread and filled the entire stroke cavity in vivo [22] with no swelling during the solution-gel transition [29]	Scale up volume and concentration of hydrogel to fill human size lesion
Tunable to brain tissue stiffness (0.5 – 1 kPa)	8 mg/mL hydrogel (~0.5 kPa) was significantly higher than the 4 mg/mL hydrogel (~0.08 kPa) [35].	Tunable according to HA:MC content [76].	Substrate elasticity increased from 0.17kPa for 2% w/v silk hydrogels to 5.46 kPa for 5% w/v silk hydrogels [29]	Elasticity similar in rodents and humans
Interface with glial scar	Less concentrated hydrogels (3 and 4 mg/mL) exhibited tighter interface than more concentrated and stiffer hydrogels (8 mg/mL) [35]		Hydrogel (4% w/v) interspersed in the surrounding glial scar after stroke 22]	Human host tissue-hydrogel interactions
Biocompatible	Extensive preclinical biocompatibility testing performed for neuronal regeneration [75].	Biocompatibility in the CNS is well established (e.g. [76].	Hydrogels (4% w/v) induced no acute or chronic inflammatory *in vitro* or *in vivo* after stroke [22, 59].	Human host tissue-hydrogel interactions
Biodegradable	By 90 days, less concentrated hydrogels (3 and 4 mg/mL) degraded by 95%; more concentrated and stiffer hydrogels (8 mg/mL) degraded by 32% [27].	HAMC hydrogels are highly biodegradable *in vivo* with *in vitro* studies showing 90% degraded at 14 d [77].	No visible signs of hydrogel degradation by 50 days in the stroke cavity showing good retention [22].	Human host tissue-hydrogel interactions
Effective in aged/co-morbid stroke models in both sexes				Preclinical efficacy studies in aged animals with comorbidities such as hypertension,

				diabetes, and hypercholesterolemia using females as well as males
Effective against white matter injury				Preclinical efficacy studies using white matter tracts and human host tissue-hydrogel interactions with white matter
Desirable properties				
Anti-inflammatory properties *per se*	An anti-inflammatory polarising effect on infiltrating microglia that could indicate potential for inflammatory reprogramming of the stroke lesion [42]	Anti-inflammatory actions of HAMC shown in CNS (e.g. reduced IL-1alpha levels after spinal cord injury [44]) and could be dependent on molecular state of HA [47, 48]		Larger amount of inflammation in human stroke cavity
Support survival of stem cells	Provides transplanted cells with structural support and excellent survival, retaining them in the graft [44]	Significantly improved NSC survival when transplanted in HAMC versus aCSF in stroke mice [53, 54]	Can be fine-tuned to support the survival and distribution of stem cell based payloads [29]	Scale up dose of cells and volume of biomaterial
Impact of injection shearing of hydrogel:cell constructs on cell survival			Pre-gelled state is better than post-gelled state [29]	Scale up to dose of cells and volume of biomaterial
Promote neurogenesis/host cell proliferation	High extracellular matrix content recruited significantly more brain-derived cells [42]; their resorptive potential may aid host cell repopulation of the stroke cavity [26]; and may lead to neurogenesis [27].	host cell infiltrate into HAMC after stroke need to be confirmed without HAMC modifications with cell adhesive or growth factors [52].	Hydrogels (4% w/v) induced endogenous cell proliferation in the ischemic brain [22].	Human host cell-hydrogel interactions

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Text Box 1: Pathology of the Stroke Lesion

In brief, acute ischemic stroke occurs when a cerebral artery occlusion disrupts oxygenation to a region of the brain, leading to local oxidative stress, **excitotoxicity**, cerebral inflammation, neuronal injury and release of **damage and pathogen associated molecular patterns (DAMPs and PAMPs)** [78]. Temporary permeabilization of the blood brain barrier occurs, enabling immune cell infiltration from the periphery and accelerating the inflammatory and **apoptotic** cascades. This uncontrolled cascade forms a positive feedback loop that exacerbates the damage caused at the stroke site, causing mass destruction of neuronal cells, and vascular **endothelial cells** (**Figure 1A**).

In the weeks following the acute phase, the brain undergoes spontaneous remodelling to limit the spread and extent of damage at the stroke site. **Astrogliosis** occurs when reactive **astrocytes** and microglia surround the stroke site and deposit **glial fibrillary acidic protein**, interacting with the extracellular matrix to form a dense, cell-loaded fibrous network called the glial scar. The purpose of the glial scar is primarily that of neuroprotection, with reactive astrocytes eliminating excitotoxic glutamate [78], reducing reactive oxygen species levels through **glutathione** expression [78], and coordinating clearance of apoptotic neuronal cells through **chemokine ligand 2** (CCL2) dependent **macrophage** recruitment to the stroke site [78]. Reactive glial cells are also critical at initiating repair of the blood brain barrier in the aftermath of the acute phase and are essential to prevent prolonged peripheral immune cell infiltration into the brain [78]. Despite the advantages of reactive astrocytes in the sub-acute phase, the inflammatory profile of the glial scar changes in the months following the ischemic event. In chronic stroke the glial scar is considered to be one of the major forces driving the formation of the stroke lesion (**Figure 1A**). Surrounding the ischemic site, the glial scar releases neurotoxic factors that kill neuronal cells [79] and the dense fibrous network prevents the formation of new blood vessels into the ischemic site to effectively seal it off from the rest of the brain. Ultimately, this results in necrosis and cavitation of the ischemic site, leaving a fluid- and debris-filled region and an inhibitory regenerative microenvironment (**Figure 1A**).

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Text Box 2: Basic and Historical Details of Decellularised Extracellular Matrix Hydrogels.

The extracellular matrix occurs naturally in virtually all organs and is a network of interconnected proteins, proteoglycans and glycosaminoglycans (including hyaluronan) that form a three-dimensional scaffold to support cells and tissue structure [80]. Cells attach and interact with the extracellular matrix through integrin receptors that also modulate cell signalling, differentiation and survival in response to extracellular mechanical changes. Extracellular matrix is perhaps the best suited material for hydrogel-based therapeutic applications as its composition has evolved naturally to support tissue growth and development. However, the complexity of the extracellular matrix limits replication using synthetic manufacturing techniques, and decellularization of existing tissues using physical, chemical and enzymatic processing is used to obtain the material [80]. Extracellular matrix hydrogels are typically produced through physical delamination and chemical immersion of porcine urinary bladder, followed by lyophilisation and reconstitution under pH and enzyme controlled conditions [80]. In addition, they have been subjected to extensive preclinical biocompatibility testing for applications in cardiac [80] and neuronal [70] regeneration, and have undergone clinical trials for applications in rotator cuff repair (NCT00456781, GRAPHTJACKET™ Regenerative Matrix - complete), hernia repair (NCT04282720, Surgimend Mesh – in progress) and breast reconstructive surgery (NCT01781299, SurgiMend® PRS™ - complete).

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Text Box 3: Basic and Historical Details of Hyaluronan-based Hydrogels.

Hyaluronan is a naturally occurring glycosaminoglycan component of the extracellular matrix that plays an essential role in **CD44**-mediated cell signalling [83], wound healing [83], tissue regeneration [83], and extracellular matrix composition [83]. The material has been studied for healthcare applications since its discovery in the 1950s and benefits from extensive biocompatibility testing in humans and an industrial supply chain [83]. Hyaluronan is already clinically approved by the many countries including U.S. Food and Drug Administration (FDA) for use in cosmetic surgery, ophthalmic procedures and for osteoarthritic knee pain [83]. In preclinical research, hyaluronan is a leading healthcare material under investigation for applications in cancer [83], soft tissue engineering and neurological disorders [19].

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Text Box 4: Basic and Historical Details of Silk-based Hydrogels.

Silk has been used for millennia in human medical applications as a suture material for wound care [84], and silk-based products persist in the clinic today in the form of FDA approved silk fibroin surgical meshes, sutures and hydrogels for load bearing and tissue support applications. Unlike extracellular matrix hydrogels or their hyaluronan components, silk fibroin-based technologies are derived from the silk of the *Bombyx mori* silkworm cocoon and perform favourably in animal and human biocompatibility testing [85]. Therefore silk materials are a blank slate that can be design to serve diverse biomedical needs (for review see [84]).

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Text Box 5: Basic and Historical Details of Alginate Hydrogels.

Alginate is a natural polysaccharide that is typically extracted from brown algae. Alginates were initially used as an edible coating to preserve food [86], but more recently have emerged as a promising polymer in biomedical engineering due to their excellent biocompatibility.

Alginate-based hydrogels are clinically approved for use in wound dressings [87] and have shown promising results in early clinical research as a delivery vehicle for anti-cancer drugs [88] and for applications in cardiovascular regenerative medicine [89]. Coupled with their absorptive capacity [87], these properties make alginate-based hydrogels very effective as wound dressings, with the ability to absorb wound exudate and promote tissue repair [89]. In addition to being non-toxic [32, 87], alginate-based hydrogels do not elicit an inflammatory response *in vivo* [32] and their easy administration make alginate-based hydrogels a promising and effective payload delivery vehicle for pharmacological compounds and regenerative stem cells [90].

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Text Box 6: Basic and Historical Details of Chitosan Hydrogels.

Chitosan in generated by the deacetylation of chitin, a natural polysaccharide obtained from the exoskeletons of crustaceans, insects and fungi [91]. Chitosan is a biocompatible and highly adaptable polymer, meaning its use is varied and extensive. Chitosan has been used in water purification [92], in wound healing, as a payload delivery system and in tissue engineering [91].

Chitosan-based hydrogels are clinically approved for use in wound dressings and, like other hydrogels, have shown potential as a payload delivery mechanism and in tissue engineering [93]. Chitosan-based hydrogels are biocompatible and can be manipulated to alter the characteristics of the hydrogel, such as its solubility, adhesion, and the rate of biodegradation [93]. However, unlike other hydrogels, chitosan-based hydrogels can be engineered to exert antimicrobial activity, though the antimicrobial mechanism itself is not clear [93]. Furthermore, the positively charged chitosan stimulates haemostasis by recruiting red blood cells and platelets to the wound site [94], accelerates inflammatory cell infiltration and promotes wound closure via collagen maturation [94]. These capabilities make chitosan-based hydrogels an excellent candidate for wound dressings and, as such, several have been approved for use [95]. Preclinical and clinical studies have shown that these chitosan-based hydrogels significantly reduce coagulation time, improve wound closure and post-operative recovery, and reduce post-operative pain [96], highlighting the efficacy of chitosan-based hydrogels in wound repair and tissue regeneration.

Chitosan polymers are highly versatile owing to their high hydroxyl and amine content [93]. The complex and multifunctional hydrogels that they form can incorporate bioactive molecules, transforming them into sophisticated payload delivery systems [93]. Chitosan-based hydrogels has previously been used to successfully deliver drugs (and cellular payloads in preclinical studies [93].

Glossary

Acute phase: up to 48 hours

Allogeneic: from individuals of the same species

Angiogenic: properties that help the formation of new vasculature

Apoptotic: programmed cell death

Astrocytes: provide blood brain barrier and synaptic support and control of blood flow.

Astrogliosis: abnormal increase in the number of astrocytes due to the destruction of nearby neurons

Biomaterial: can be introduced into body tissue to replace an organ or bodily function

Blood brain barrier: barrier between blood and brain tissue made of endothelial, pericytes and smooth muscle cells amongst other cells.

Brain derived neurotrophic factor: helps produce newborn cells in the brain

CD44: involved in cell–cell interactions, cell adhesion and migration

Chemokine ligand 2 (CCL2): recruits cells to sites of inflammation

Chronic phase: 30 days or more

Ciclosporin: suppress the body's immune mechanisms**,**

Damage associated molecular patterns: molecules released from damaged or dying cells that are a component of the innate immune response

Electrophysiology: measures electric activity in neurons

Endogenous: originating from within an organism.

Endothelial cells: line blood vessels

Erythropoietin: increases the rate of production of red blood cells due to reduced oxygen

Excitotoxicity: massive release of the excitatory amino acid l-glutamate into the extracellular space that causes cell death

Exogenous: external origin.

Extracellular matrix: Tissue that surrounds cells that provide biomechanical and biochemical cues.

Fibroin: a protein which is the chief constituent of silk.

Glial fibrillary acidic protein: expressed by astrocytes

Glial scar: dense, cell-loaded fibrous network

Glutathione: involved in oxidation–reduction reactions

Hematopoietic: found in the peripheral blood and the bone marrow

Hemorrhage: escape of blood from ruptured vessel

Hydrogel: highly water saturated 3D matrix within which cells can be encapsulated

Induced pluripotent stem cell: from skin or blood, reprogrammed back into pluripotent state

***In vivo*:** in a living organism

Induced neural cell: a cell reprogrammed to become a neural stem cell

Ischemic: blockage in blood flow due to a clot

Macrophage: removes dead cells, and stimulates the action of other immune system cells.

Mesenchymal stem cell: present in tissues like umbilical cord, bone marrow and fat tissue

Microglia: act as the primary line of immune system defense in central nervous system.

Middle cerebral artery: most commonly occluded artery in human stroke

Necrosis: death of cells due to disease, injury, or failure of the blood supply.

Neural stem cell: found in brain tissue

Neurogenic: properties that help the growth of new neurons from neural stem cells

Neuroplasticity: the ability of the brain to form and reorganize synaptic connections

Pathogen associated molecular patterns: associated with pathogen infection and serve as ligands for host pattern recognition molecules

Polymer: a substance made from a large number of similar units bonded together

Progenitor stem cell: descendants of stem cells that then further differentiate to create specialized cell types

Reprogramming of the glial scar: Interact with the glial scar to lessen the density of the glial scar without disrupting its integrity so that it is less of a prohibitive barrier to regeneration.

Scaffold: engineered to cause desirable cellular interactions that contribute to the formation of new functional tissues for medical purposes.

Space conformity: have the same shape and outline

Stem cell: has the ability to develop into specialised cell types

Stroke: lack of cerebral blood flow to part of the brain with lasting neurological deficits

Sub-acute phase: 3-9 days

Tissue plasminogen activator: clot buster used in ischemic stroke

Thrombectomy: mechanical method of removing a clot

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Outstanding Questions Box

- Can hydrogels contribute to advanced "second generation" stroke treatment strategies, overcoming some of the key pathological features of chronic stroke?
- Does age, multi-morbidity and sex affect biocompatibility, biodegradation and regenerative properties of hydrogels in stroke?
- Is scaling-up hydrogel volume and concentration possible to fully fill human-sized cavities and does this affect viability of cellular payloads due to injection shearing and diffusion limits?
- Are hydrogel-host tissue interactions different in humans and rodents and affected by the larger amount of cavity, inflammation, glial scar, debris and white matter damage as found in human stroke?
- Does hydrogel-induced tissue restoration in situ generate functioning neurons and synapses, associated with improved functional outcome?
- Does fluid in the cavity affect gelation kinetics and, if so, is feasibility of drainage from the cavity a determinant of stroke patients who should receive hydrogel-based therapeutic interventions?

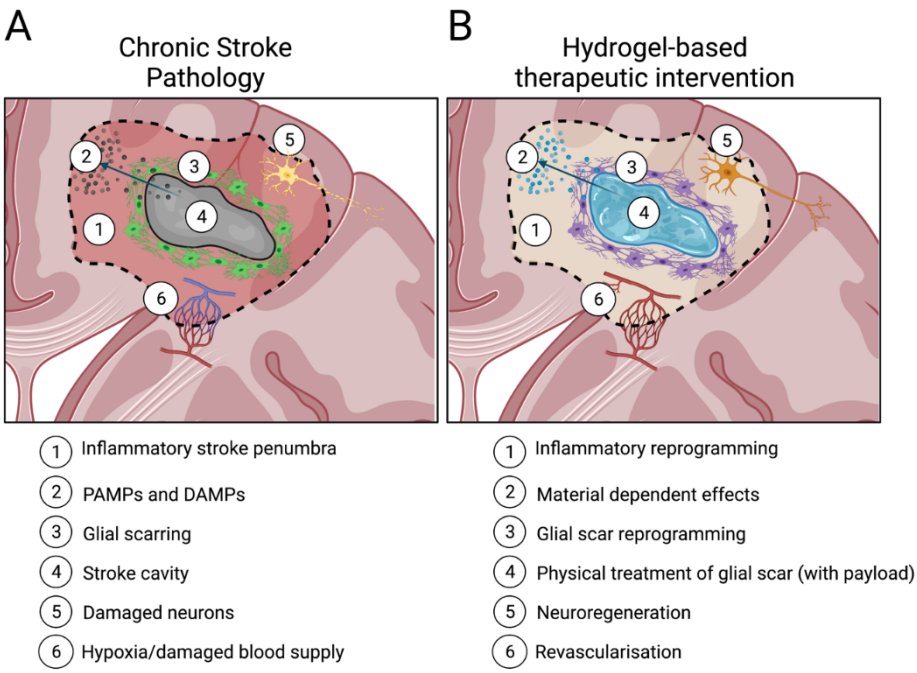
Highlights

- The stroke cavity is an ideal site for administration, being closest to a zone of neuroplasticity and able to accommodate hydrogels without compressing surrounding tissue. However, it is prohibitive to first-generation regenerative stroke therapies as it lacks an extracellular matrix; is surrounded by a glial scar; and is filled with extracellular fluid, debris and inflammatory mediators.

- Hydrogels can remodel the hostile stroke cavity to be more receptive to repair due to their innate anti-inflammatory properties, good space conformity, and interface with the glial scar.

- Hydrogel 3D structure and tuneable elasticity provide physical support for endogenous and exogenous repair processes.

- Hydrogels are used extensively in the clinic, yet no clinical trials have been successfully commissioned to explore the potential of regenerative hydrogels in the treatment of chronic stroke.


A
Chronic Stroke Pathology
B
Hydrogel-based therapeutic intervention
1 Inflammatory stroke penumbra
2 PAMPs and DAMPs
3 Glial scarring
4 Stroke cavity
5 Damaged neurons
6 Hypoxia/damaged blood supply
1 Inflammatory reprogramming
2 Material dependent effects
3 Glial scar reprogramming
4 Physical treatment of glial scar (with payload)
5 Neuroregeneration
6 Revascularisation

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