Chapter

HyStem®: A Unique Clinical Grade Hydrogel for Present and Future Medical Applications

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Abstract

Medicine needs targeted, minimally-invasive delivery of protein-based and cell-based therapeutics to increase efficacy and reduce occurrence and severity of side effects. Local delivery requires a matrix to sequester and protect the medicine until its effect can be realized. The problem is, unlike stable small molecule drugs, proteins and cells cannot be co-packaged with a matrix in a prefilled syringe—they must be mixed with their matrix at the point of care. HyStem hydrogels fix this problem: They are arguably the first commercially available, GMP-qualified biodegradable hydrogels both with the ability to formulate with either proteins or cells in the hospital/surgical suite and with a history of safe use in humans. HyStem is designed to be protein, cell-friendly and *in situ* crosslinkable, permitting homogeneous mixing of therapeutics. One HyStem formulation is 510(k) cleared and another the subject of two European clinical trials. Key applications include localized delivery of therapeutic growth factors, antibodies, and cells. In the future, we envision HyStem's flexibility and clinical use history forming the basis for a new generation of therapeutics. Two examples described here include HyStem's use for patient-derived organoid culture to develop new drugs as well as for bioprinting to manufacture new organs.

Keywords: HyStem, GMP, hyaluronic acid, extracellular matrix, delivery, organoid, bioprinting

1. Introduction

A hydrogel is a crosslinked, water-rich network that can be fabricated from a wide variety of hydrophilic, biological or bio-inspired polymers. Human beings are in fact largely hydrogel: cellular cytoplasm is a hydrogel as is the extracellular matrix that surrounds them [1, 2]. Many body substances are also hydrogels (e.g. mucus [3], blood clots [4]). It can be argued that hydrogels in the human body carry out many of their functions as a result of being water swollen and that the functionality of manufactured hydrogels also relies heavily on this capability They can heal through hydrating and protecting wounded surfaces, fill empty spaces where there used to be tissue in younger days or prior to disease or injury, seal surgical wounds that cannot reintegrate on their own, and deliver therapeutics to places that cannot easily be accessed from systemic routes. A variety of animal and plant-based hydrogels (e.g. gelatin, pectin, carrageenan) are also used in our food supply.

Despite their restorative potential, relatively few hydrogels are currently marketed for human medical use. This is because commercialization is neither cheap nor easy: product development demands years of effort to design a stable product that is user-friendly and cheap enough to sustain commercial requirements [5]; cGMP manufacture is expensive, especially if aseptic vial fill filling is required. Commercialization also requires the requisite quality system to support the cGMP manufacturing as required by FDA 21CFR820. The regulatory path can also be onerous: while hydrogels are generally considered medical devices (Class II or III) by the US FDA, obtaining FDA approval in combination with therapeutics (drugs or cells) can increase the complexity and length of the regulatory journey due to the need for oversight by multiple FDA agencies [6].

A growing focus is in local administration of therapies to not only deliver disease-modifying drugs but also to rebuild damaged tissues [7–9]. Given the arduous path to the clinic, it is tempting to short-circuit the normal manufacturing/quality/regulatory/clinical path by improvising by using marketed hydrogels approved for different indications and derived from commonly-used biological polymers (see **Table 1**). While this approach may be attractive in the short-term, there is little gained in the long term. This is because hydrogels must be re-optimized for each therapeutic. In addition, the regulatory and clinical paths will not be circumvented: these medical devices, like drugs, must be approved independently for each new indication [10–12].

If a short-term fix is required, what is the harm in selecting a hydrogel from **Table 1** to locally deliver a therapeutic? Upon closer inspection, it becomes clear that a non-optimized hydrogel adds additional risk to the success of the therapeutic. HA-only hydrogels (Dermal Fillers, Viscosupplements, Opthalmic and Gynecology hydrogels), must be excluded for anchorage-dependent cells like mesenchymal stem cells (MSC)—they do not provide the requisite cellular attachment sequences required to prevent anoikis [13]. Many hydrogels (Dermal Fillers, Viscosupplements) are also pre-crosslinked within its sterile syringe and are dubbed monolithic [9], preventing homogeneous mixing with drugs or cells. Tissue sealants made from fibrin actually fulfill two key criteria: they contain fibronectin, an effective cellular attachment protein for many cell types [14]. They also form a gel after its components are combined, allowing homogeneous mixing of cells. These sealants however suffer from their lack of staying power:

Class	Example	Key polymer	Marketed state	Cellular attachment sites present
Wound management	Biozel®, Integra® Wound Matrix	Hyaluronic acid (HA), collagen	Uncrosslinked	Yes
Tissue sealants	Evicel®, Tisseel®	Fibrin	Uncrosslinked	Yes
Dermal fillers	Juvederm®, Restylane®	HA	Crosslinked	No
Viscosupplements	Synvisc®, Hyalgan®	HA	Crosslinked	No
Opthalmic	Healon®, Provisc®	HA	Uncrosslinked	No
Gynecology	HyaloGyn®	HA	Uncrosslinked	No

Table 1.
A sample of currently marketed hydrogels.

the endogenous plasmin degrades the hydrogel within a week, providing only brief shelter for the therapeutic [15]. Fibrin is also difficult to use for indications outside of tissue sealing. This is because it is formulated to gel in 3–5 min [16], placing a short and rigid timeframe for mixing with other therapeutics and injecting in the operating room. Collagen-containing matrices (wound management) have collagen which provides requisite cellular attachment sites [15]. The challenge with some wound management devices (e.g. *Integra* flowable wound matrix, **Table 1**) is that the collagen can be granulated. These insoluble collagen particles will not homogeneously mix with its matrix, providing suboptimal cellular attachment. Many also do not form covalent crosslinks, deteriorating their staying power [15].

What is needed is a biodegradable, biocompatible hydrogel platform that is not only customizable but also provides the requisite foundation for streamlined regulatory approval. The HyStem hydrogel platform, a clinical grade customizable hydrogel matrix, fulfills these criteria. While a discussion of key aspects to hydrogel selection and commercialization was described previously [17], this review serves to pick up where it has left off. This book chapter will have three parts: First, an introduction to the history and basics of the technology; Second, a description of the successful uses of the HyStem platform based on our customers and collaborators' published experiences for the past 10 years for both drug and cell delivery. Third, an introduction and description to recent studies in what we believe will provide the springboard to future therapies: HyStem's use for preparing tissue-specific and patient-derived organoid culture to develop new drugs as well as HyStem's use as a bioink for bioprinting to manufacture new organs.

2. Background

2.1 History

The HyStem technology was developed in the laboratory of Dr. Glenn Prestwich in the Department of Medicinal Chemistry at the University of Utah. The goal was to develop a hydrogel platform designed to recapitulate the minimal composition necessary to obtain a functional extracellular matrix [ECM] while using specific design criteria for both function and future commercialization [9]. Since the first publication in 2002 [18], over 200 articles have been published describing novel uses of the technology for drug and cell delivery. Glycosan BioSystems (Salt Lake City, Utah) obtained the exclusive rights to the technology to certain fields of use for medical and research applications in 2006. Glycosan commercialized the technology in late 2006 for research use with the ultimate goal of manufacturing and providing clinical grade material to a growing cell therapy market. BioTime (Alameda, CA) acquired glycosan in 2011, manufactured cGMP-qualified HyStem, and subsequently performed the requisite ISO-10993 biocompatibility experiments and stability experiments in support of regulatory clearances and approvals. In 2014, one HyStem formulation was 510(k)-cleared for wound management (tradename Premvia™; 510(k) number: K134037). In 2013, BioTime also embarked on two European clinical studies using another HyStem formulation (tradename Renevia®) as a delivery vehicle for autologous fat-derived cells to treat HIV-associated facial lipoatrophy (HIVLA). BioTime successfully completed its pivotal clinical study in 2017 and met its primary clinical endpoints.

2.2 Composition and reaction mechanism

The elegance of the HyStem platform is in its three building blocks. They covalently bind to each other like Lego® blocks and they can be used to make highly customized and complex matrices: these blocks are called Glycosil®, Gelin®, and Extralink® (**Figure 1**). Each has a specific role in the platform (**Table 2**). The most basic form of the platform is called HyStem-C where the concentration (w/v) ratios of Glycosil:Gelin:Extralink are equal in concentration (w/v) (**Table 2** and **Figure 2**). Upon mixing, Extralink's acrylates react with the former two components' thiol groups via click chemistry (Michael addition reaction) [13, 19]. Crosslinks form in trans (e.g. Glycosil molecules can link to Gelin as well as to neighboring Glycosil molecules). In addition, given Glycosil's large molecular weight and its ability to adopt semiflexible random coil configurations, it can likely loop back on itself and bind in cis (**Figure 3**) [20]. The final clear, transparent, viscoelastic hydrogel forms at physiologic pH and temperature in approximately 20 min and is greater than 98% water. This time frame allows an investigator to both customize the hydrogel with drugs or cells and to load and deliver the mixture through a cannula.

Also like Lego® blocks, these three base blocks can be used to make highly customized and complex matrices in large part due to its thiol chemistry. First, the number of blocks can be changed. For example, the addition of thiolated or thiol-reactive species allows a user to fundamentally change the character of the matrix. One case is the addition of thiolated heparin which provides a negatively charged component crosslinked into the matrix. This added character can aid in significantly increased sustained release of proteins non-covalently incorporated into the matrix prior to gelation [21, 22]. The marketed version of this mixture is called HyStem-HP (**Table 3**). In addition, the incorporation of either acrylate or maleimide-tagged molecules allows facile covalent linkage directly to the thiolated species [23–25]. Sometimes, however, less is more: removal of the Gelin component from HyStem-C provides an *in situ* crosslinkable adhesion barrier [26] (**Table 3**).

Second, the levels of each component can be adjusted to change the hydrogel properties. HyStem is a soft gel and can be tailored to stiffnesses comparable to tissues such as endoderm, nerve, liver, and smooth muscle (G' 20–3500 Pa) (**Table 3**). [17, 27, 28]. In more basic terms, HyStem can be made to be as soft of flavored gelatin (G' 35 Pa) or moderately stiffer than Greek yogurt (G' 1900 Pa) (B. Lohman and T. Zarembinski, unpublished). Its stiffness is modulated primarily by increasing Glycosil and/or Extralink concentrations [28]. Its pore size (less than 15.9 nm based on the hydrodynamic radius of trapped polymers (**Table 3**)) can also be modulated

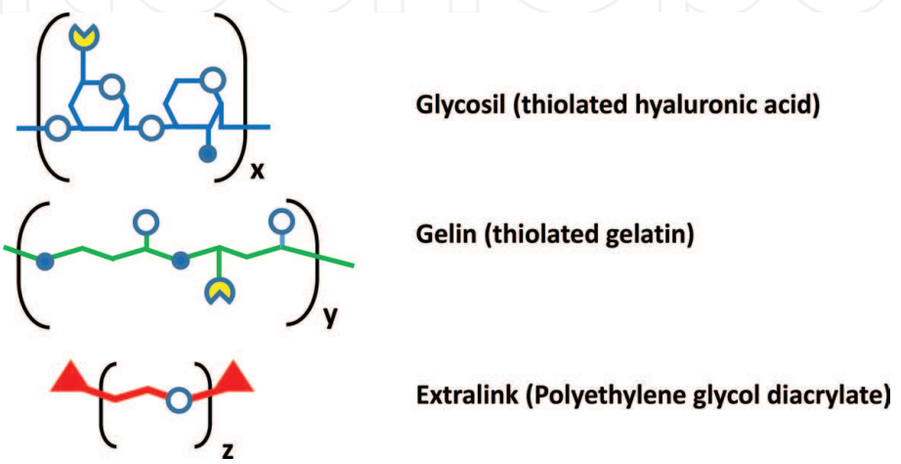

Figure 1.
HyStem-C building blocks. Shapes are as follows: unfilled circles, oxygen; blue circles, nitrogen; yellow partial circles, sulfur; red triangle, acrylate.

	Glycosil	Gelin	Extralink	References
Identity	Thiol-modified hyaluronate	Thiol-modified porcine gelatin	Polyethylene glycol diacrylate	[18, 19, 85]
Purpose	Backbone of gel	Cellular attachment sites	Crosslinker	[18, 19, 85]
Substitution density	30% of HA repeating units	42% of available carboxylates	>65% bis-acrylation	[85]
Mw (kDa)	160	Heterogeneous	3.4	[19]
Final concentrations in gel (mg/ml)	4	4	4	Unpublished

Table 2.
HyStem-C component characteristics.

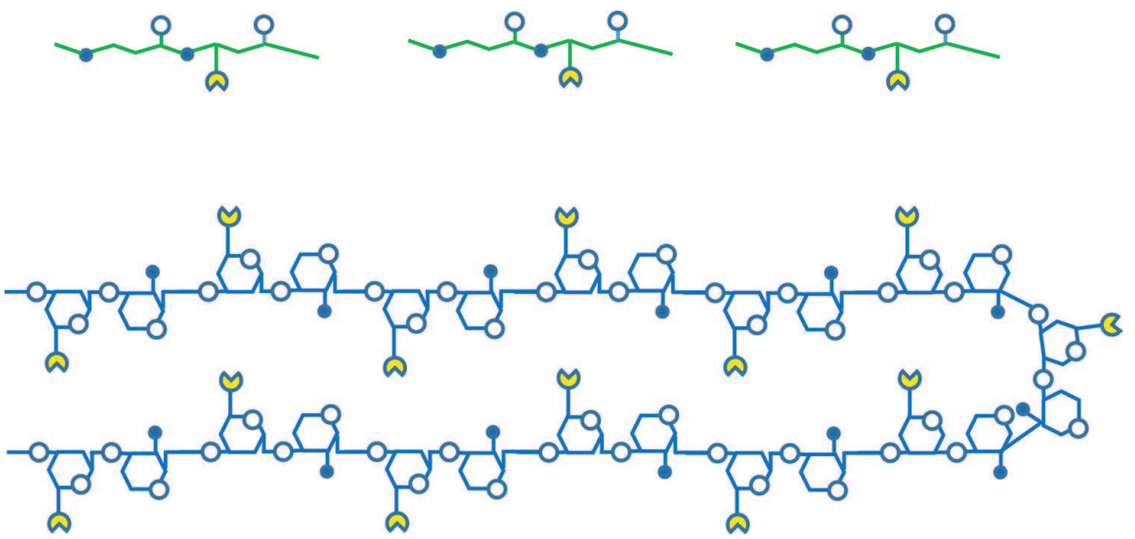

Figure 2.
Glycosil and Gelin prior to gelation. Shapes are as same as those for **Figure 1.**

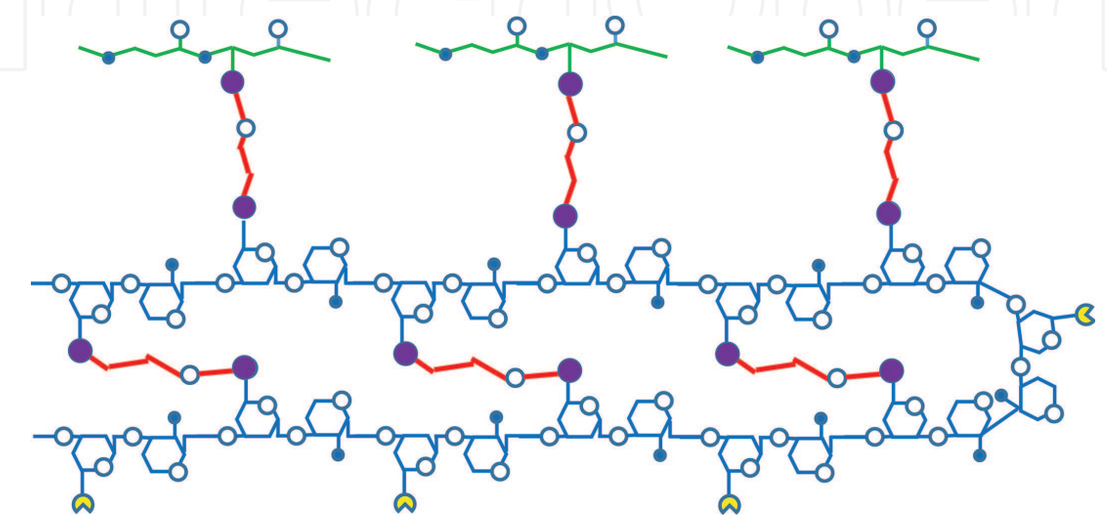

Figure 3.
Covalent bonding in HyStem after gelation upon addition of Extralink. Shapes are same as for **Figure 1** *with addition of purple circles (new bond resulting from Michael addition reaction).*

	Attribute	References
Appearance	Clear	[19]
Gelation time	Approx. 10–20 min	[19]
Versions	1. HyStem-C (Glycosil, Gelin, Extralink) 2. HyStem-HP (Glycosil with thiolated heparin, Gelin, Extralink) 3. HyStem (Glycosil, Extralink)	[9]
Stiffness (G' Pa)	20–3500	[28]
Pore size (nm)	< 15.9	[29]

Table 3.
Final hydrogel characteristics.

from by increasing or decreasing the density of crosslinks of the HyStem-C hydrogel [29, 30]. Third, HyStem composition can be easily altered by adding in any component (e.g. drug, protein) by incorporating it prior to full gelation. This is possible since HyStem has a medium rate of gelation (10–20 min) compared to other *in situ* crosslinking hydrogels [17] (**Table 3**). This non-covalent addition of any therapeutic cargo paves the way for drug and cell delivery to be discussed next.

3. Drug delivery

Sustained release of drugs of both small molecules and protein drugs occurs when they are incorporated into HyStem. While a compilation of drug release results and conclusions for individual HyStem formulations is presented below, this is not the point. The key is HyStem's flexibility: it can be leveraged to adjust release. This modulation can be done by either changing its crosslinking density by increased Extralink or Glycosil concentrations or by including different polymers with affinity for the drug such as heparin [21] or increased Gelin (M. Onorato, unpublished). In the end, this ability to optimize formulation can be crucial for maximizing efficacy *in vivo* [31].

HyStem utility for drug delivery was first described in 2005 with the sustained release of human serum albumin (HSA, 66 kDa) and human basic fibroblast growth factor (bFGF, 16 kDa) from the prototype to the HyStem-HP product (containing thiolated heparin crosslinked into the matrix). bFGF was released over 35 days at different velocities depending on amount of thiolated HP present [21]. This publication was important not only because it was the first illustration of HyStem's capabilities in drug delivery but it underscores the complexity of drug release: several variables affect speed and extent of release. These include identity of protein, molecular weight, base gel polymer (hyaluronic acid (uncharged) or chondroitin sulfate), presence of hyaluronidase (HAse), and release solution composition [21]. Sustained drug release can also occur by using HyStem to immobilize MSC engineered to express and secrete therapeutics like diabodies [32] or by covalent linkage of a small molecule to HyStem to further slow its release [33].

Generally, drugs release from hydrogels is diffusion-driven [34]. HyStem hydrogels are no different *in vitro*: they typically display first order release directly proportional to the drug concentration in the hydrogel [8]. There are two exceptions: Celecoxib and BMP-2 which display near zero order release independent of concentration [8, 31]. Celecoxib is poorly water soluble and the possibility exists that molecule may have precipitated or crystallized in gel [35]. In this event, the molecule's release may be dependent gradual disintegration of the solid [36]. Slow

Drug	Matrix	Release time (days)	MW (kDa)	Final concentration (mg/ml)	Area of research	Reference
1. Lenalidomide	HyStem	0.25	0.26	1.33	Cancer	[8]
2. R848	HyStem	0.25	0.31	1.33	Cancer	[8]
3. Celecoxib	HyStem	18	0.38	10	Cancer	[8]
4. 2′3′-cGAMP	HyStem	0.25	0.67	0.67	Cancer	[8]
5. BMP-2	HyStem or HyStem-HP (no Gelin)	28	26	0.17	Orthopedic	[31]
6. IL-15sa	HyStem	2	29	0.06	Cancer	[8]
7. Anti-PD1	HyStem	5	150	2	Cancer	[8]

Table 4.
Listing of drugs released from HyStem hydrogels (in vitro).

Drug	Matrix	Release time (days)	MW (kDa)	Final concentration (mg/ml)	Area of research	Reference
1. Dexamethasone	HyStem-C*	3	0.39	10	Auricular	[86]
2. IGF-1	HyStem-HP	28	7.6	0.02	Stroke	[87]
3. BDNF	HyStem-C	21	27	0.1–0.17	Stroke	[88, 89]
4. TrkB-Fc	HyStem-HP	5	125	0.005	Stroke	[88]
5. Ascorbic acid and platelet-rich plasma	HyStem-HP	n/a	Multiple	0.05 (ascorbic acid)	Myocardial infarction	[90]
6. miR-29B	HyStem	n/a	7.5	1.0	Myocardial infarction	[41]

**A version of HyStem-C that was used with four-fold lowered Gelin concentration.*

Table 5.
Listing of drugs released from HyStem hydrogels (in vivo).

release of BMP-2 has been described before from HA-based hydrogels and the mechanism for its release is unclear [37].

Another general rule is the smaller the molecule, the faster the release. Small organic molecules less than 1 kDa are usually fully released from hydrogel after 6 hours and larger proteins like antibodies are released on the order of days (**Tables 4** and **5**; **Figure 4**), compare data points on left and right of vertical dotted line. Red data point 1 (dexamethasone) and blue data point 3 (Celecoxib) are outliers for reasons described in this section). The relationship is not linear; this is particularly true of proteins whose release from HyStem hydrogels shows little correlation between molecular weight and release time (**Figure 4**, right of vertical dotted line). This result suggests that their different shapes and surface charges may affect rate of release. There is also a difference in release depending on whether it is measured *in vitro* or *in vivo*; Proteins *in vivo* release rate tend to have slower release rates (**Figure 4**, blue compared to red data points). This increase could be due to the increased resistance to release in the protein-rich host fluids and tissue. In the end, the optimal release rate for *in vivo* efficacy must be empirically determined since it is difficult to predict. For example, BMP-2 is released faster in HyStem compared to HyStem-HP, leading to a more complete release for HyStem at day 28 (84%) compared to the latter (68% for HyStem-HP).

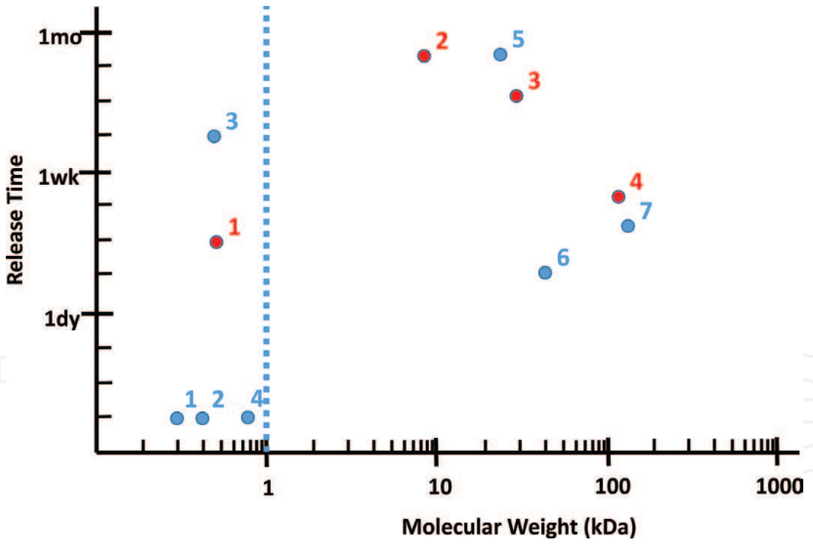

Figure 4.
Release rate of different sized molecules from HyStem hydrogels. Numbered, blue dots refer to in vitro molecules listed in **Table 4***; numbered, red dots refer to in vivo released drugs in* **Table 5***). Blue dotted vertical line separates low molecular weight small molecules (left) from proteins.*

Unexpectedly, faster release corresponds to better efficacy since the former yields 50% more ectopic bone formation [31].

In addition to small molecules and proteins, future opportunities for HyStem will be for the sustained release of a new class of polymer therapeutics, nucleic acids. In 2016, 69 antisense oligonucleotides and 37 siRNAs were in clinical trials [38]. These numbers have grown in 2018 to 87 and 57 trials, respectively (search terms: antisense oligonucleotides and siRNA in clinicaltrials.gov). HyStem can add value especially by protecting the nucleic acids from the host immune system [39]. So far, delivery of the agomir version of miR-26A and of miR-29B have been reported [40, 41]; in addition, double-stranded phosphorothioated DNA oligonucleotides have also been released slowly over 7 days at 10 mg/ml and higher; this rate of release can be modulated by adjusting the levels of Gelin in the final HyStem hydrogel (M. Onorato, unpublished).

4. Cell delivery

HyStem is a versatile tool for the implantation of cells. Beyond the ease of use mentioned previously, the biomaterial constituents provide two key functions that can be used to improve cell-based therapies. First, because these biopolymers are normally found in ECM and can be easily remodeled [19], HyStem can provide a more habitable environment for implanted cells and thereby improve engraftment. Second, HyStem's base biopolymers, hyaluronan and collagen, are ubiquitously found in tissues and the implant composition and structure is not considered foreign. This characteristic plus the small pore size of the hydrogel excluding host cells allows HyStem to shroud the implanted cells from the host's immune system while providing cellular attachment sites [29, 42]. Both of these aspects used together, provides a versatile and tailorable platform for implantation of cells into many tissues for a variety of indications. The combination of these attributes likely explains HyStem's ability to support cell survival post-implantation and to ultimately provide better *in vivo* efficacy in rodent models (**Table 6**) [43, 44].

Herein, HyStem will be presented in two different contexts for cellular delivery. (1) HyStem as a synthetic ECM for cellular remodeling and tissue regeneration

Cell type	Matrix	Last data point (days)	Area of research	Reference
Endothelial progenitor cells	HyStem-C	14	Nephrology	[42]
Neural progenitor cells	HyStem-HP	14	Stroke	[45]
Placenta-derived adherent cell	HyStem-C	56	Orthopedic	[91]
NSC and MSC expressing sTrail	HyStem-C	28	Glioblastoma	[44]
Cardiosphere-derived cells	HyStem-C	1	Myocardial infarction	[43]
Neural stem cells	HyStem-C	14	Imaging	[92]
Retinal progenitor cells	HyStem-C and variations thereof	7	Ophthalmic	[93]
MSC expressing oHSV	HyStem-C	12	Glioblastoma	[94]
NSC expressing Pseudomonas exotoxin	HyStem-C	21	Glioblastoma	[95]
MSC/GDF5 (growth differentiation factor 5)	HyStem-C	42	Bone formation (dental)	[96]
Islet beta-cells	HyStem-C	560	Diabetes	[29]
Cardiomyocytes	HyStem-C	28	Myocardial infarction	[97]

Table 6.
Listing of cells delivered in HyStem hydrogels (in vivo).

(e.g. implantation and remodeling into native tissues) and (2) HyStem as an encapsulating matrix to maintain cellular fitness, localization, and isolation (e.g. paracrine effects of implanted cell and/or immuno-isolation of implanted cells).

The first publication describing the utility of HyStem hydrogel for cell delivery appeared in 2004 [19]. Hydrogels seeded with T31 human tracheal scar fibroblasts implanted in nude mice showed two important milestones 8 weeks post-implantation: the cells were proliferating and also producing their own extracellular matrix. By these measures, the cells had made a home for themselves and were going about populating the space provided by the hydrogel. This work has since been extended to a variety of cell types such as cardiac, neural, mesenchymal based cells [32, 43–45].

While most animal experiments so far reported were performed for several weeks to show improvement in *in vivo* function across a variety of indications (**Table 6**), the possibility exists that this improved efficacy can further be amplified with lengthened time points. One case in point is the transplantation of pancreatic islets in diabetic rats using HyStem-C [29]. Surprisingly, the diabetic rats maintained normal glucose levels and remained insulin-independent for at least 80 weeks (1.5 years) and may have been studied over still longer term had the animals not succumbed to old-age related diseases. The longevity of cellular response is attributed at least in part to reduce fibrosis which is well known to occur in the alginate-based biomaterials more popular for this application.

Successful use of HyStem in animals portended successful use in humans. A major challenge in HIV patients is the disappearance of facial fat resulting from highly active antiretroviral therapy (HAART) and is known as HIV-associated lipoatrophy [46, 47]. One approach to treat this HAART complication is to transplant a patient's own fat-derived cells (stromal vascular fraction cells, SVF)

subcutaneously in the facial deficits. A clinical version of HyStem, Renevia, was used to deliver these cells harvested by liposuction and reinjected subcutaneously. The pivotal trial was a European, multi-center, randomized, evaluator-blinded, delayed-treatment-controlled study of the effectiveness and safety of Renevia in combination with the autologous SVF. The primary endpoint in this 56 patient trial was the change in hemifacial volume at 6 months in treated patients compared to patients in the delayed treatment arm as measured by 3D photographic volumetric assessment. Renevia successfully met the primary endpoint with treated patients retaining 100% of transplanted volume at 6 months. In addition, treated patients retained 70% and 64% of the transplanted volume at 12 and 18 months, respectively. All Renevia transplants were shown to be well tolerated and there were no device-related serious adverse events noted during this trial (investor.biotimeinc.com).

5. Future technologies

5.1 Introduction to organoids

In the current drug development pipeline, preclinical testing of novel drug compounds in 2D cell cultures is well established, but is not always accurately predictive of clinical outcomes in human subjects. [48]. This result is not surprising since cells grown in 2D using plastic dishes experience drastically different surface topography, mechanical properties, cell–cell interactions, cell-matrix interactions, and nutrient diffusion properties compared to a 3D architecture.

Organoids (also called organ-specific 3D cultures) are three-dimensional constructs comprised of tissue-specific cells with the intention of recapitulating the cellular microenvironment and function of their originating tissues. While organoids can be formed through self-aggregation to form spheroids, they are also formed using biomaterial hydrogels that suspend cells in 3D within polymer or protein-networked matrices (refer to the different methods shown in **Figure 5**). Biomaterial-based approaches have an advantage used over spheroid-based approaches as they allow for heightened control of the organoid and organoid microenvironment composition with regard to cellular, biochemical, and physical parameters, such as stiffness, addition of ECM components, and spatial organization of cell types [49, 50]. Organoids often contain multiple cell types that are representative of those typically found within their target tissues [51]. Importantly, these differences result in significant phenotypic and gene expression changes that is much more reflective of their *in vivo* origin. For example, when grown metastatic colon carcinoma cells are cultured in 2D culture, they exhibit an epithelial morphology and expression profile. In contrast, when the cells are introduced into a 3D liver organoid environment supported by HyStem, they "switched" to adopt a mesenchymal and metastatic phenotype [52, 53].

5.2 Novel platforms for making and using organoids

HyStem has been employed to create a wide variety of 3D tissue constructs and organoid form factors since its composition and gelation time can be customized for each model. An example of composition customization is in a recently-developed liver model: primary human hepatocyte spheroids are embedded into HyStem hydrogels modified to include liver-specific ECM extract [54]. The inclusion of liver ECM increases long-term hepatocyte viability, stabilizes albumin secretion, and supports cytochrome p450 activity [55]. These organoids have been tested in environmental toxin screening [56], and are currently being used to screen a range

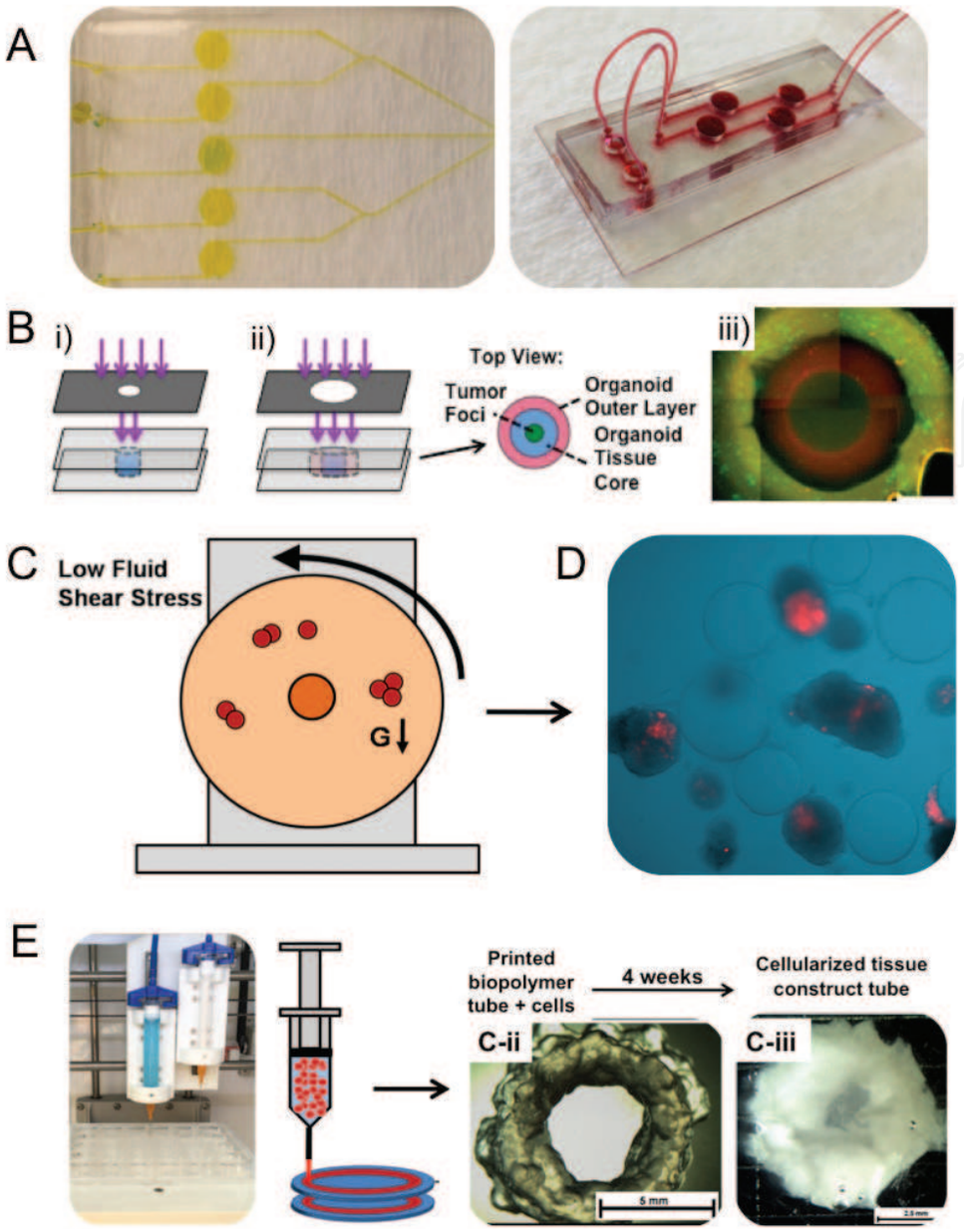

Figure 5.
Organoid biofabrication methods and form factors. (A) Examples of microfluidic devices that house organoids that are (B) photopatterned in situ through hydrogel precursor exposure within the devices through photomasks to UV or blue light, initiating crosslinking. (C) Rotating wall vessel bioreactor culture of (D) tumor organoids. (E) Extrusion bioprinting of cell-laden laden hydrogel bioinks.

of previously recalled drugs as further validation. This model can also be integrated into multi-tissue organoid, body-on-a-chip systems to test drug or toxin kinetics in the context of multiple organs [54, 57]. Lastly, organoids can also be formed by bioprinting technologies (discussed below).

Customization of HyStem's gelation time also enables novel approaches to generating and using organoids. Two examples include using bioprinting technologies (**Figure 5E**) to be discussed in the next section) and organ-on-a-chip technology. Organ-on-a-chip is a nascent 3D-based technology which employs microfluidics to more accurately model *in vivo* tissues by simulating its hydrodynamic flow as well as its placement of multiple cell types in close proximity with respect to one another [58]. This technology can also be used to make organoids by customizing HyStem's crosslinking for spatio-temporal control of gelation. For example, the recent creation of *in vitro* cancer models employ a range of biofabrication approaches, all which employ HyStem in a variety of methods. Since many tissue- and tumor-on-a-chip

platforms are based on closed microfluidic devices with no direct access to locations in which tissues will reside, introduction of cells is performed generally through fluid flow channels. To introduce 3D tissue and tumor constructs within such sealed microfluidic devices (**Figure 5A**) 3D hydrogel photopatterning strategies were developed. By adding light activated photoinitiator molecules (e.g. Irgacure 2959), HyStem is able to form solid structures by shining light through photomasks to yield defined shapes and locations *in situ* within microfluidic devices (**Figure 5B**) [59]. By harnessing control over the extracellular matrix components and adding healthy cells, the resulting organoids can have more complex stroma and extracellular matrix architectures, which provide additional components that contribute to overall tissue-tumor physiology [60]. Additional complexity can be realized by creating multiple tissue and tumor organoids and combining them in a single closed system. This facilitates study of phenomena such as metastasis, where events take place in two locations—a primary tumor site and a downstream site of metastasis. We recently demonstrated a metastasis-on-a-chip platform to model metastasis of colorectal cancer cells from a gut organoid to a liver organoid [53]. We have been able to encapsulate dissociated tumor populations from these biospecimens in 3D using HyStem or its derivatives, forming patient-derived tumor organoids for precision medicine applications for patient specific diagnosis and treatment [61, 62].

5.3 Bioprinting

The shortage of donor organs for implantation in patients has been a significant problem for years, [63, 64] and does not appear to be improving quickly [65]. For example, as of July of 2017, over 117,000 patients were still on waiting lists for donor organs, and there are only 8096 currently identified available donors [66]. Bioengineered tissues and organs have the potential to address this need for implantable tissues for patients waiting on donor lists. Bioprinting technologies have advanced in recent years, holding immense potential to 1 day be employed for biomanufacturing of transplantable tissues and widely adopted human testbeds.

Bioprinting is a multi-disciplinary technology that has emerged in recent years as a tool with immense potential in regenerative medicine and tissue engineering that combines 3D printing, biomaterials, and cell biology. [67–70] Bioprinting is a relatively new field within biotechnology and biomedical research (less than 2 decades old) that can be described as robotic additive biofabrication with a goal to create viable and functional 3D organ or tissue structures [71–75]. A number of bioprinting approaches have been recently explored, encompassing use of inkjet-like printers, extrusion devices, and laser-assisted devices [72, 73, 76]. Biomaterials play an integral role in bioprinting, as they act as the "glue", figuratively and literally, that connect the fabrication approaches with the biological components [72, 73]. Currently, few biomaterials exist that both integrate seamlessly with bioprinting hardware and are optimally compatible with living cells. Most biomaterials used in bioprinting employ terminal covalent or physical bond formation during sol–gel transitions, requiring precise timing or control over chemical reactions to facilitate printing. Put simply, if one bioprints too soon, the result is a puddle of the bioink material and cells; if one bioprints too late, the bioink is too stiff and clogs the printer. Being able to control this transition to enable successful printing of 3D structures has been a major focus in bioink development.

HyStem and its individual components have played important roles in the development of bioinks for bioprinting since the early days of bioprinting, where our group published several of the first studies in which novel biomaterial bioinks were developed specifically for 3D bioprinting [77, 78]. The key is in its compatibility with the bioprinting process. More specifically, the same characteristics that enable organoid

Crosslinker/crosslinking strategy	Key attributes
Gold nanoparticles	1. Slow gelling (24–96 hours) 2. Reversible
Four-armed PEG acrylate	Stiffer gels
Addition of photoinitiator	Rapid crosslinking using light initiation
Addition of tetraorthosilicate	Thixotropic quality

Table 7.
Different crosslinking chemistries with HyStem for bioprintin.

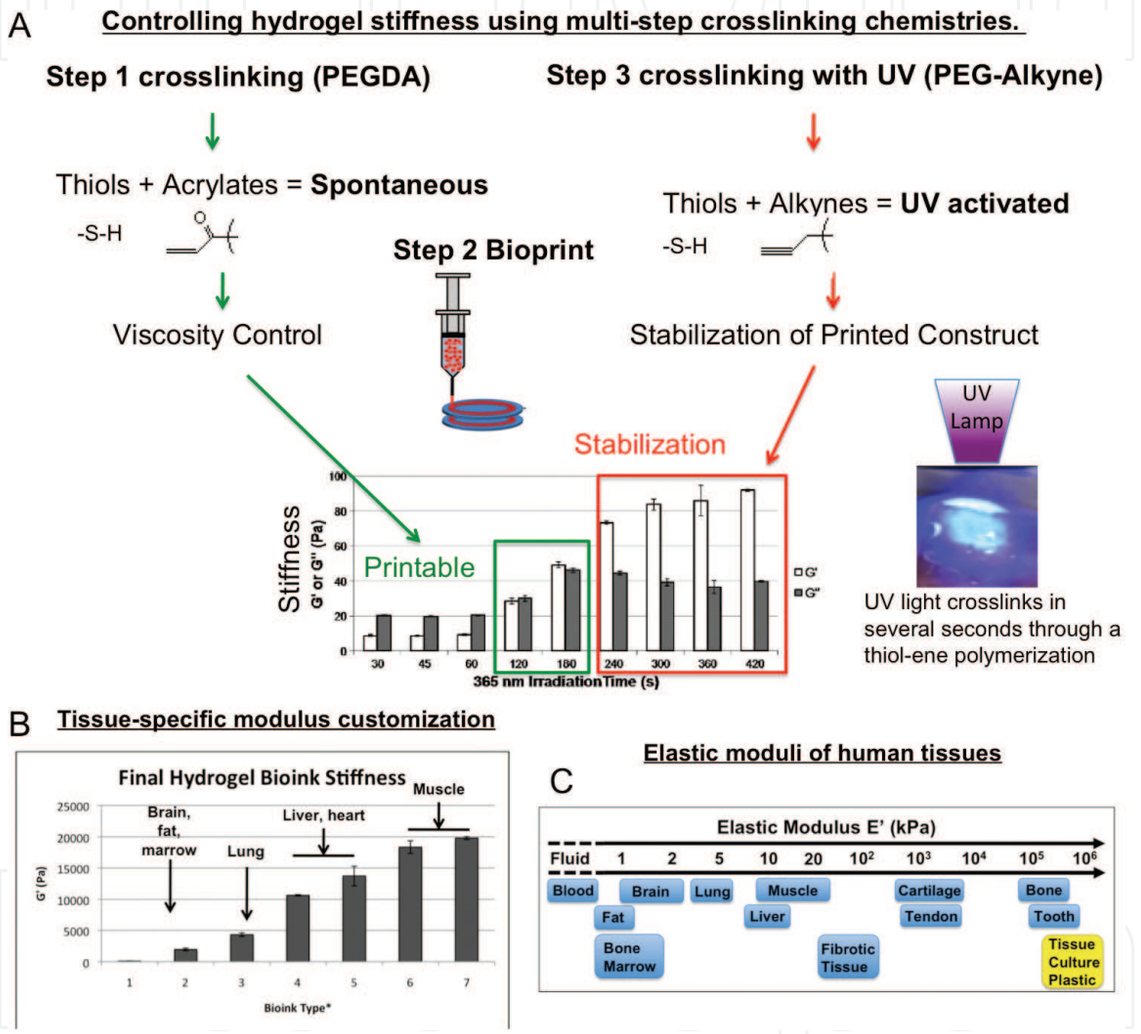

Figure 6.
Description of a multi-step crosslinking, HyStem-based hydrogel bioink. (A) Controlling G' and G" using a multi-crosslinking approach to facilitate extrusion bioprinting of thiolated HA and gelatin-based hydrogels through thiol-acrylate and thiol-alkyne reactions. (B) Data from rheological testing of bioink formulations, demonstrating the capability to mimic the elastic modulus of many soft tissues in the body. (C) Tissues occupy different ranges of elastic moduli.

culture apply here to bioprinting: HyStem can be customized easily while providing a cell-friendly matrix. For example, HyStem forms very soft hydrogels that are not structurally robust, and the crosslinking methods employed do not facilitate effective extrusion bioprinting. This result can be a limitation in terms of scalability. To address these problems, new crosslinking and molding techniques were developed which increase the breadth of the bioprints formed (**Table 7**). These technologies include the ability to form supporting matrix allowing the printing of fragile structures and which can be removed (gold nanoparticles, AuNPs). The use of multi-armed PEG

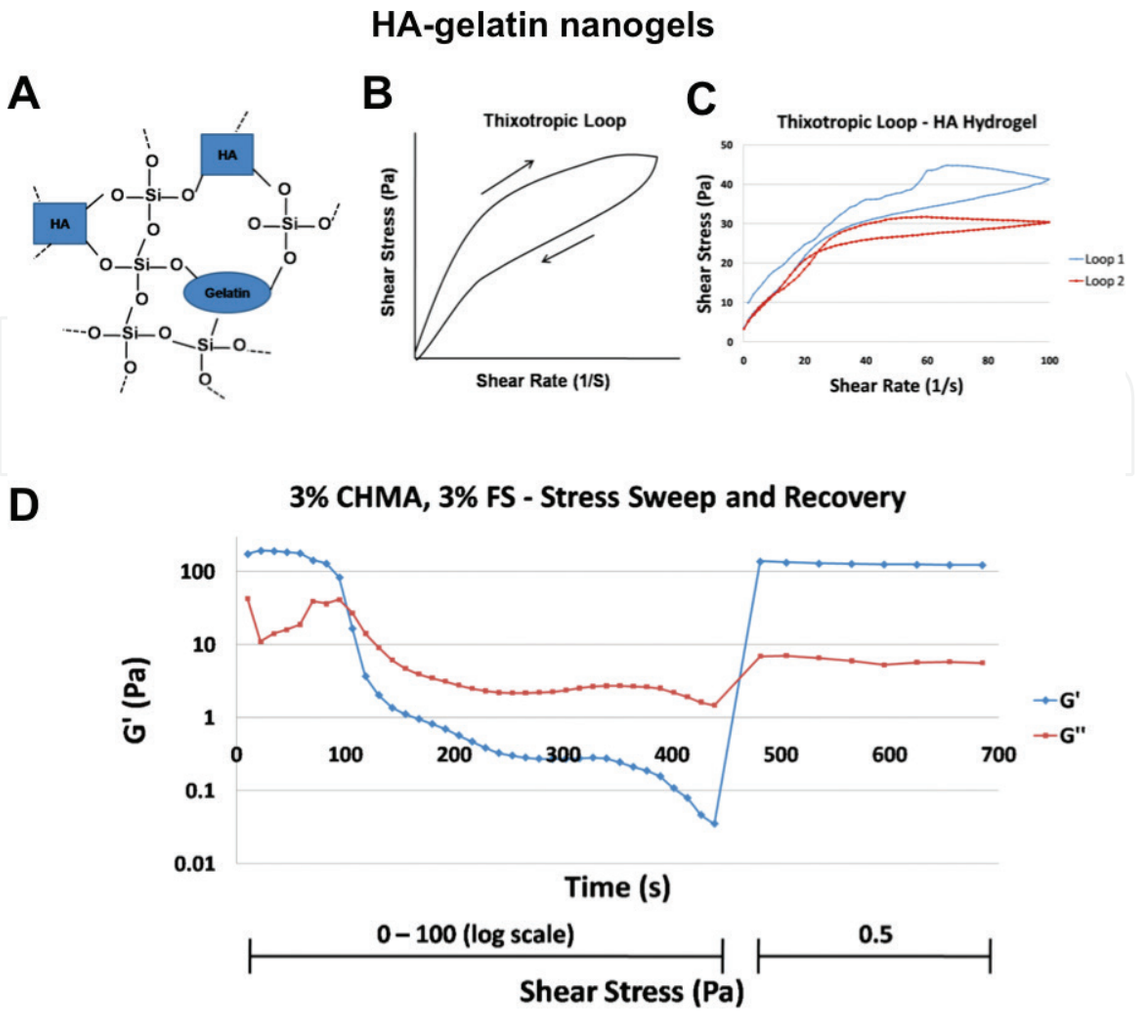

Figure 7.
Thixotropic HA hydrogels. (A) Hyaluronic acid and gelatin thixotropic hydrogel, formed using complexing with tetraorthosilicate (TEOS). (B) A depiction of a thixotropic loop mechanical test and (C) the HA-gelatin thixotropic hydrogel under such a test, indicating thixotropic characteristics. (D) A stress sweep and recovery demonstrating thixotropy.

acrylate crosslinkers to stiffen the gel [78, 79]; multistep spontaneous and light-initiated crosslinking to spatio-temporally regulate crosslinking (**Figure 6**, **Table 7** [80, 81]); and inclusion of tetraorthosilicate to render HyStem thixotropic (allowing extrusion due to reversible liquefaction of the hydrogel (**Figure 7**)).

6. Conclusion

In summary, HyStem's strength is in its clinical roots, its protein and cell compatibility, and its flexibility to enable optimized drug and cell delivery. These attributes make HyStem well-suited for present applications such as local delivery of protein and autologous cells as well as future applications are also such as organoid culture for better drug discovery and development as well as bioprinting of tissue and organs. in the longer term, we envision HyStem to be a platform for using these new technologies to develop the next generation of made-to-order therapies and tissues for an increasing tissue and organ starved human community, whether it be an individual requiring an organ transplant due to disease, a tissue transplant due to injury, or the multiple needs of the wounded warrior.

We also envision HyStem eventually becoming a standard building block for a wide variety of future therapies so that it is clinically available for a physician as an

off-the-shelf, general delivery vehicle. This is because new technologies based on 20–100 nm nanoparticles for drug delivery and for theranostics will require a delivery vehicle for local and/or sustained delivery [82, 83]. In addition, local cellular delivery of nucleic acids will grow in need especially with the rapid development of CRISPR/Cas9 technology for delivery to specific organs and tissues [84].

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Conflict of interest

Dr. Zarembinski is an employee of and owns stock options in BioTime, Inc.

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