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Anti-Fas conjugated hyaluronic acid microsphere gels for neural stem cell delivery

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Abstract: Central nervous system (CNS) injuries and diseases result in neuronal damage and loss of function. Transplantation of neural stem cells (NSCs) has been shown to improve locomotor function after transplantation. However, due to the immune and inflammatory response at the injury site, the survival rate of the engrafted cells is low. Engrafted cell viability has been shown to increase when transplanted within a hydrogel. Hyaluronic acid (HA) hydrogels have natural anti-inflammatory properties and the backbone can be modified to introduce bioactive agents, such as anti-Fas, which we have previously shown to promote NSC survival while suppressing immune cell activity in bulk hydrogels *in vitro*. Although bulk HA hydrogels have shown to promote stem cell survival, microsphere gels for NSC encapsulation and delivery may have additional advantages. In this study, a flow-focusing microfluidic device was used to fabricate either vinyl sulfone-modified HA (VS-HA) or anti-Fas-conjugated HA (anti-Fas HA) microsphere gels encapsulated with NSCs. The majority of encapsulated NSCs remained viable for at least 24 h in the VS-HA and anti-Fas HA microsphere gels. Moreover, T-cells cultured in suspension with the anti-Fas HA microsphere gels had reduced viability after contact with the microsphere gels compared to the media control and soluble anti-Fas conditions. This approach can be adapted to encapsulate various cell types for therapeutic strategies in other physiological systems in order to increase survival by reducing the immune response. © 2016 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 105A: 608–618, 2017.

Key Words: hyaluronic acid, hydrogels, anti-Fas, neural stem cells, microfluidic cell encapsulation

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INTRODUCTION

Stem cell-based therapies, specifically those involving neural stem cells (NSCs), are highly advantageous to replace lost cellular populations for tissue repair after injury or disease. NSCs have a survival rate of $<10\%$[1] due to immune and inflammatory cell-induced apoptosis at the injured spinal cord lesion.[2] The low survival rate hinders successful clinical outcomes and highlights the importance of developing strategies that promote survival of the transplanted cell grafts while minimizing the negative local immune response. Hydrogels provide a protective barrier and improve stem cell survival rate,[1,3,4] and have been traditionally used for localized cellular signaling, presenting bioactive molecules,[5] and delivery of cellular grafts.[1,6] However, *in vivo* implantation of large amounts of foreign material, such as bulk hydrogel scaffolds, may elicit an acute inflammatory response in addition to the body's natural mechanism for injury repair.[7] Therefore, physical reduction of the cellular delivery scaffold is crucial to prevent further damage at the lesion site.[8]

In order to reduce the hydrogel volume, microsphere gels have been investigated. Droplet-based microfluidics has emerged as a powerful technique for high throughput, fabrication of microsphere gels with a reduced volume (10 pL − 1 nL) compared to bulk hydrogels (200 μL), making this platform technology beneficial for regenerative medicine applications.[9,10] Minimizing the hydrogel volume can lead to a reduced inflammatory response while also allowing for the delivery of clinically relevant cells and bioactive molecules to the injury site.[11–13] The fabrication of microsphere gels using microfluidics provides additional advantages, such as increased surface area for immune cell contact and enhanced diffusion. Microsphere gels maintain their porosity and permeability to small molecules, thus allowing for nutrient, gas, and metabolic waste exchange, and ultimately supporting their application as a cell reactor

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system.[14,15] Furthermore, microsphere gels with conjugated bioactive molecules have a role in directing cell behavior and fate, while providing physical isolation. Previous studies have reported multiple fabrication steps for bioconjugated microsphere gels. Allazetta et al. has reported a two-step fabrication process that first fabricates the microsphere gel carriers using a microfluidic device, and then functionalizes the surface off-chip.[14] However, our fabrication process is one-step using the pre-functionalized hydrogel precursor solution in a single continuous stream without any additional steps on a microfluidic platform,[15] thus providing a facile approach to fabrication of bioactive microsphere gels for use in cell delivery and immuno-isolation.

In this study, we focused on the encapsulation of NSCs as a strategy to protect the cells from immune and inflammatory cells that secrete harmful cytokines. It has been demonstrated that Fas receptor (FasR)/Fas ligand (FasL)-mediated signaling pathway provides neuroprotection, thus enhancing the survivability of NSCs.[16] Anti-Fas, antibody to FasR, conjugated hydrogels have also been shown to induce apoptosis in immune and inflammatory cells, such as central nervous system (CNS) resident microglia and T-cells due to the high FasR expression on the cell surface.[17,18]

Here, a hyaluronic acid (HA) hydrogel system was used in a one-step fabrication process to generate anti-Fas conjugated microsphere gels for NSC encapsulation as a potential neural repair strategy. HA was chosen for hydrogel fabrication due to its: (1) abundance *in vivo*, especially in the CNS extracellular matrix[19]; (2) natural anti-inflammatory properties, which can aid in the reduction of immune and inflammatory cells at the site of injury[20]; and (3) net negative charge and hydrophilic nature, which increases the permeability of nutrient and waste exchange[21] to support the viability of the encapsulated cells, and minimize host cell deposition.[22–24]

The use of a bioactive molecule, such as anti-Fas, conjugated to the polymer to fabricate microsphere gels encapsulated with NSCs has not been previously reported. We demonstrated that the NSCs were viable through the fabrication of the microsphere gels and the encapsulation process. However, the T-cells after contact with the anti-Fas conjugated HA microsphere gels undergo cell death.

MATERIALS AND METHODS

Fabrication of microfluidic devices

A flow-focusing microfluidic network was designed with two inlet channels, a collection chamber, and one outlet channel [Fig. 1(A)] using DraftSight software (Dassault Systemes). One inlet was used to flow the hydrogel precursor and the second inlet was used to flow the oil resulting in a stream of monodisperse hydrogel droplets at the intersection of these immiscible fluids. A high aspect ratio channel (100 μm wide, 50 μm high, 4 mm long) facilitated droplet formation and cell encapsulation, and a wide chamber was used to decrease droplet velocity and allow for droplet stabilization and collection.[25] A master silicon wafer was fabricated using standard photolithographic techniques in clean room facilities using the photoresist SU-8 2035 (MicroChem). Polydimethyl siloxane (PDMS, Sylgard 184, Dow Corning) was used to cast the microfluidic devices. The fabricated PDMS device was plasma bonded onto a glass slide and heated at 80°C for 48 h to restore material hydrophobicity.[26,27] A bright field image of the designed microfluidic device is presented in Figure 1.

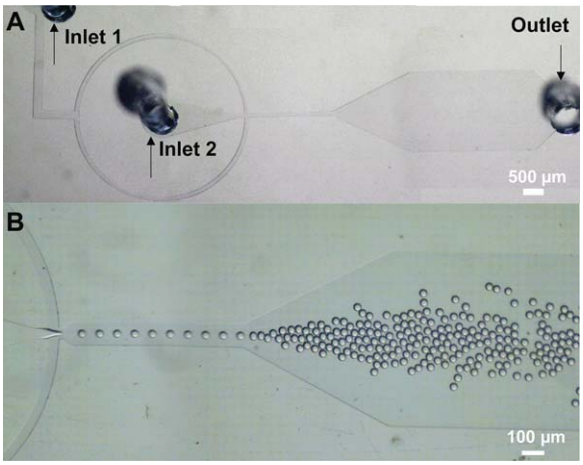

FIGURE 1. Bright field images of flow-focusing microfluidic device. **(A)** Oil flows through inlet 1. VS-HA or anti-Fas HA precursor solution containing the photocrosslinker, I2959, flows through inlet 2. Droplets are stabilized in the chamber and collected through the outlet where they are crosslinked by UV exposure. **(B)** Uniform sized droplets are collected in the chamber. Surfactant present in oil prevents droplet coalescence prior to on chip UV crosslinking.

VS-HA and anti-Fas HA microsphere gel fabrication and cell encapsulation

The hydrogel precursor solutions were fabricated using our previously published methodology.[18] Briefly, 1% HA was dissolved in 0.1 *M* NaOH and modified using divinyl sulfone (DVS) at 3 *M* excess to form VS-HA, followed by dialysis and lyophilization for storage. Anti-Fas HA was prepared by reacting the reconstituted 0.75% (w/v) VS-HA solution with 250 ng/mL of anti-Fas (BD Biosciences), dialyzed, and lyophilized for storage. The lyophilized hydrogel precursor was reconstituted in phosphate buffer (PB) containing the photocrosslinker, 0.1% Irgacure 2959 (I2959, BASF), for a final working concentration of 0.75% (w/v). Inlet 2 was infused with the dispersed phase, which consisted of the hydrogel precursor solution (VS-HA or anti-Fas HA), photocrosslinker, and cells. Inlet 1 was infused with the continuous oil phase, which consisted of mineral oil with 3% sorbitan monooleate (Span 80, Alfa Aesar) surfactant, and Solvent Blue (Koch), a dye used to distinguish the phases. Mineral oil is commonly used carrier fluid for microsphere gel formation.[11,28,29] Span 80 is a commonly used surfactant to minimize coalescence and promote stable microsphere gels.[28,30] PicoSurf™ (2% in FC-40, RainDance Technologies), was also used as an alternative oil phase for cell encapsulation.[9] Microsphere gels were fabricated using two independently programmable syringe pumps (Harvard Apparatus 33 Dual Syringe Pump, USA) to control the flow rate of both the dispersed phase and continuous phases. Adjusting the

flow rates for both phases independently controlled the diameter and production rate of the microsphere gels.

HA and anti-Fas HA degradation

Ten millimeter VS-HA ($n = 5$) and anti-Fas HA ($n = 3$) hydrogel discs were prepared for degradation studies. Both conditions were reconstituted at a concentration of 0.75% (w/v) in PB containing 0.1% I2959, and crosslinked under a 365 nm long-wave UV light source (150 mW/cm^2). The initial mass of the hydrogels discs was measured (W_d). Hydrogels discs were initially swelled in phosphate buffered saline (PBS) for 24 h and mass measurements were recorded at 3 h, 6 h, 12 h, and 24 h. PBS containing hyaluronidase (10 U/mL)[31] was added to each well without any changes in solution after 24 h. VS-HA and anti-Fas HA samples were weighed (W_s) every 24 h for 3 consecutive days. Samples sizes represent independent hydrogel batches. The following equation was used to determine the degradation percentage:

$$\%\ \text{Degradation} = (W_s/W_d) \times 100\%$$

Analysis of microsphere gel diameter

As the microsphere gel diameter is controlled by the flow rates specified for the continuous and dispersed phases, various flow rates were used to obtain specific microsphere gel diameters. The flow rates used for the continuous oil stream were 10, 25, and 100 μL/h. The flow rates of 1, 5, 10, and 25 μL/h were used for the hydrogel dispersed phase. The flow rate of the continuous oil phase or dispersed hydrogel phase was held constant while the other was varied to determine the effect on the diameter of the microsphere gels. A continuous flow of microsphere gels were imaged on the microfluidic device using a HiSpec4, high speed camera (Fastec, 1200x944, 30 fps). One image every 200 frames was selected for microsphere gel diameter measurements using ImageJ software, and a minimum of 450 microsphere gels were measured per flow rate variation.

NSC and T-cell encapsulation within microsphere gels

NSCs (Cyagen) were maintained and propagated as neurospheres in NSC media (Stem Cell Tech) supplemented with 1% B27 (Life Technologies), 1% Penicillin/Streptomycin (Pen/Strep, Life Technologies), epidermal growth factor (EGF, Invitrogen), fibroblast growth factor −2 (FGF-2, Invitrogen), and heparin (Sigma Aldrich). Neurospheres (passages 5–10) were dissociated using TrypLE (Life Technologies) prior to encapsulation. NSC expression of FasR was verified using phycoerythrin (PE) conjugated antibody to Fas (BD Biosciences, data not shown). T-cells (Jurkat cells, ATCC) were maintained in RPMI media (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% Pen/Strep. T-cell expression of FasR has been previously reported in literature,[17,32–34] thus was not verified. Single cell NSCs or T-cells were resuspended in 0.75% VS-HA or anti-Fas HA hydrogel precursor solution at a cell density 10x10^6 cells per mL. The microsphere gels encapsulated with either NSCs or T-cells were collected from the collection chamber, washed in the respective media, and placed in a 96-well plate to culture for 24 h. The Live/Dead assay (Invitrogen) was performed as specified by the manufacturer's protocol to determine viability of the encapsulated cells. Images were taken in a 96-well or glass bottom multi-well plate using a Zeiss Axiovert microscope with a Zeiss AxioCam MRm CCD camera. Images of VS-HA and anti-Fas HA microsphere gels containing encapsulated NSCs were analyzed to determine the viability by counting the total number of calcein and ethidium homodimer positive cells. The number of calcein positive cells was divided by the total number of calcein and ethidium positive cells counted in the microsphere gels imaged. Cell viability percentage was normalized to the respective hydrogel condition at the initial time point ($t = 0$ h). A sample size of $n = 3$ experimental repeats was used to quantify encapsulated NSC viability with the exception of the control VS-HA condition at the 24 h time point ($n = 2$). The viability of encapsulated T-cells in the anti-Fas HA microsphere gel was not quantified as the images represent a single experiment.

Analysis of T-cell viability in presence of HA and anti-Fas HA microsphere gels

T-cells are one of the immune cell types responsible for inducing apoptosis in transplanted NSC cells *in vivo*. In order to determine whether T-cell viability would be affected after contact with the anti-Fas, T-cells were cultured with either VS-HA ($n = 4$) or anti-Fas HA microsphere gels ($n = 3$), and normalized to the media control ($n = 3$). Additionally, a soluble anti-Fas (BD Biosciences) condition ($n = 3$) was used as a positive control in order to account for the effects of HA on survival. The suspended T-cells were placed in a 96-well plate at a cell density of 15,000 cells per well. Approximately 3000–4000 VS- HA or anti-Fas HA microsphere gels were added to each well. For the soluble anti-Fas condition, a concentration of 250 ng/mL of antibody was dissolved in complete culture media. T-cells were cultured for 48 h without any media changes, except for the soluble anti-Fas condition, in which a media change was performed after 24 h post-seeding to avoid a potential loss of antibody function. All conditions were stained using the Live/Dead fluorescent assay. After performing the Live/Dead assay, the wells were imaged using Zeiss Axiovert microscope with a Zeiss AxioCam MRm CCD camera. The wells were divided into 5 regions of interest (ROI), and a randomly selected area per ROI was imaged for a total of 5 images per well. T-cell viability was quantified by determining the number of calcein positive cells divided by the total number of calcein and ethidium homodimer positive cells, as well as the unstained cellular population. Cell viability percentage was normalized to the media control condition, and presented graphically as normalized percent cell viability. In addition, the Annexin V (Life Technologies) marker was used to identify T-cells in early-stage apoptosis ($n = 2$ per condition). The Annexin V staining was performed according to the manufacturer's protocol at a concentration

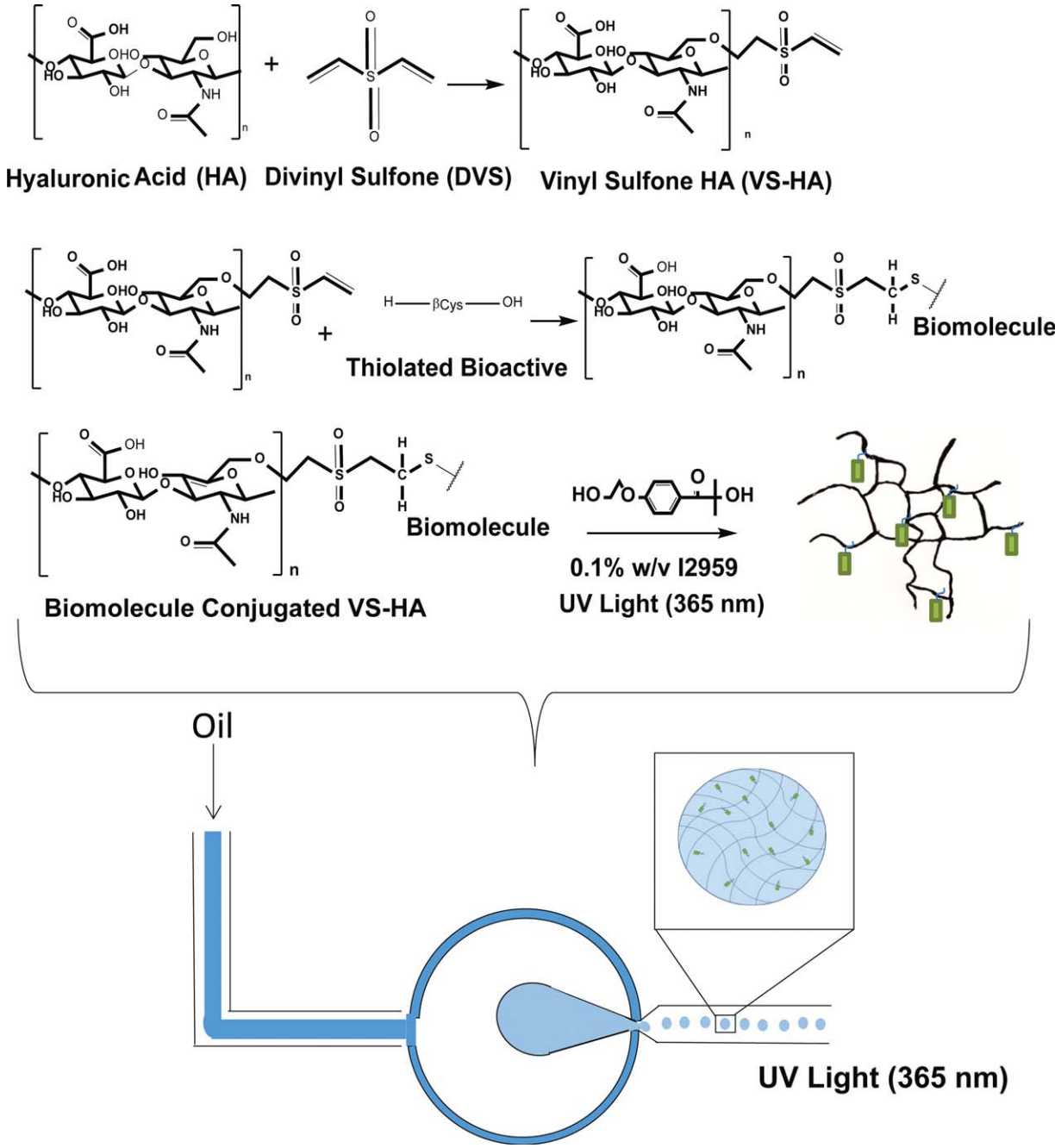

FIGURE 2. Reaction scheme for Michael-type addition of divinyl sulfone (DVS) and addition of bioactive agents. Vinyl sulfone (VS) groups allow functionalization of the hydrogel backbone with thiol containing bioactive molecules, such as anti-Fas, as well as for reaction with UV initiated radicals to form a crosslinked hydrogel. The schematic of a flow-focusing microfluidic device used to infuse the hydrogel precursor solution is shown. Microsphere gels were fabricated and crosslinked using an external UV light source as the droplets were collected from the chamber.

of 10 μL Annexin/100 μL buffer. Sample sizes represent independent experimental repeats.

Analysis of effects of the microfluidic encapsulation process on cell viability

The hydrogel photocrosslinking process exposes cells to ultraviolet (UV) light and free radicals for a short period of time, which may be toxic. To test for cytotoxicity, T-cells were plated at a cell density of 1.25×10^5 cells/mL for UV exposure characterization at 1, 5, and 10 min. There was minimal loss of viability for each exposure time (data not shown). Furthermore, to determine whether the microfluidic encapsulation components affected NSCs or T-cell viability, cells were cultured as stated above with UV conditioned cell medium containing 0.1% (w/v) I2959. Cells were incubated for a period of 24 h and characterized using the Cell-Counting Kit (CCK8, Dojindo) metabolic activity assay, as specified by the manufacturers' protocol. For the CCK8

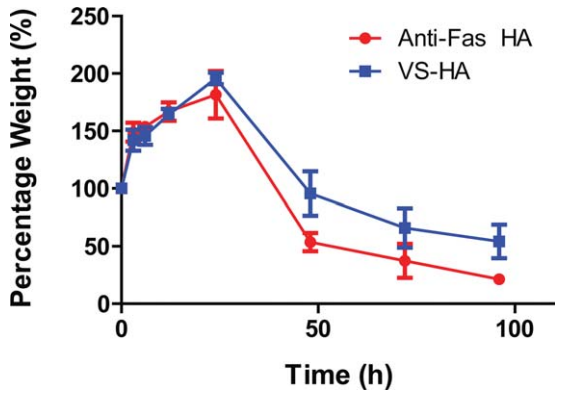

FIGURE 3. Bulk VS-HA and anti-Fas HA hydrogel degradation for UV crosslinked samples. Although a statistical difference was not observe between the two conditions, VS-HA had a higher percent weight trend at the 48 h, 72 h, and 96 h compared to the anti-Fas HA hydrogels. Sample size: $n = 5$ independent measurements was used for the VS-HA; $n = 3$ independent measurements were used for the anti-Fas HA.

assays, the I2959 treated condition was normalized to the media control to obtain normalized percent viability. An $n = 3$ independent experiments was completed for each cell line. The combined effects of the microfluidic encapsulation process, specifically shear stress, mineral oil/surfactant contact, UV exposure, and radical formation was also studied. Briefly, an aqueous stream of T-cells, without the hydrogel, at a concentration of $1x10^6$ cells/mL and 0.1% I2959 was infused into inlet 2 of the flow-focusing microfluidic device, and exposed to UV light as specified in the cell encapsulation experiments ($n = 1$). Cells were collected, stained using a Live/Dead viability assay, and imaged using previously discussed methods. Sample sizes used represent independent experimental repeats.

Statistical analysis

Statistical analysis was performed to determine significance amongst the conditions using GraphPad software. A two-way analysis of variance (ANOVA) was used to determine significance over time and hydrogel conditions for the NSC encapsulation viability, as well as for the degradation studies. A one-way ANOVA followed by *post-hoc* Tukey's test was used to determine significance for the T-cell culture experiments. A Student's t test was used to determine significance for the experiment investigating I2959 toxicity on NSC and T-cell (CCK8 metabolic activity assays). Statistical significance was considered for $p < 0.05$ among the different conditions as indicated.

RESULTS AND DISCUSSION

We previously demonstrated successful fabrication of anti-Fas conjugated HA hydrogel using click chemistry to functionalize the HA backbone and polymerize the hydrogel.[18] In this study, we investigate using a microfluidic device to encapsulate the NSCs within the HA and anti-Fas HA microsphere gels toward increasing the survival rate after transplantation as a repair strategy after neural injury.

Flow-focusing microfluidic device

A flow-focusing device with 50 μm depth and 100 μm width was used for microsphere gel generation [Fig. 1(A)]. Microsphere gel formation occurs when viscous shear stresses overcome the capillary pressure enabling the deformation of the liquid interface, and it is the elongation within the jetting stream that aides in cell encapsulation.[35,36] The flow-focusing droplet generator design is commonly used for cell encapsulation experiments, as the symmetric shearing on the dispersed phase allows for more controlled droplet formation[37] and minimizes damage to a cell laden stream. A long channel with similar dimensions to the microsphere gel and a separate wider collection chamber assists picoliter droplet formation [Fig. 1(B)].[38] In order to ensure sufficient droplet stabilization, a wide collection chamber increased residence time to ~1–3 min within the flow-focusing device.

Hydrogel chemistry, polymerization, and degradation

VS-HA or anti-Fas HA precursor solution were used for the fabrication of microsphere gels. I2959, a UV-based photocrosslinker, was used for on-chip microsphere gel polymerization. The chemical reaction schematic can be seen in the top panel of Figure 2. In the presence of I2959, the VS-HA microsphere gels were polymerized through the generation of radicals that initiate the polymerization process when irradiated with 365 nm long-wave UV light (150 mW/cm^2).[39–41] The microsphere gels were polymerized in the collection tubing (Fig. 2, bottom panel).

Photocrosslinked hydrogels have been widely used for biomedical applications as they can rapidly gel *in situ* after brief exposure to UV light. The cytotoxic effects of UV light exposure and free radical generation is minimized through short exposure times, reduced UV intensity, selection of an appropriate photoinitiator, as well as reduction in the photoinitiator concentration. Control of these parameters allows reduction in the cytotoxic effects during the encapsulation process. UV light exposure (150 mW/cm^2) up to 10 minutes had minimal effect on T-cell viability (data not shown). As the exposure time during microsphere gel fabrication is approximately 3 minutes, viability was not significantly affected by the UV light. To assess the effects of UV-formed radicals due to the presence of I2959, NSCs and T-cells were cultured with UV light exposed media containing 0.1% I2959 (w/v) prior to being cultured for 24 h. The normalized percent viability for the T-cells was >95% [Supporting Information Figure 1(A)]; while the NSCs had a normalized percent viability of 76% compared to the media control [Supporting Information Figure 1(B)]. Similar reduction in stem cell viability has been reported.[42] The reduction in NSC viability may be due to them being derived from a primary mouse cell line and therefore more sensitive to the radicals compared to the T-cells, which are an immortalized cell line.[43] The combined effects of UV, shear stress, and I2959 were also characterized on T-cells flowing in an aqueous stream within the microfluidic device. The T-cells were exposed to the UV in the presence of I2959 and were collected to perform the Live/Dead assay (Supporting Information Figure 2). Overall, it was observed that the viability of

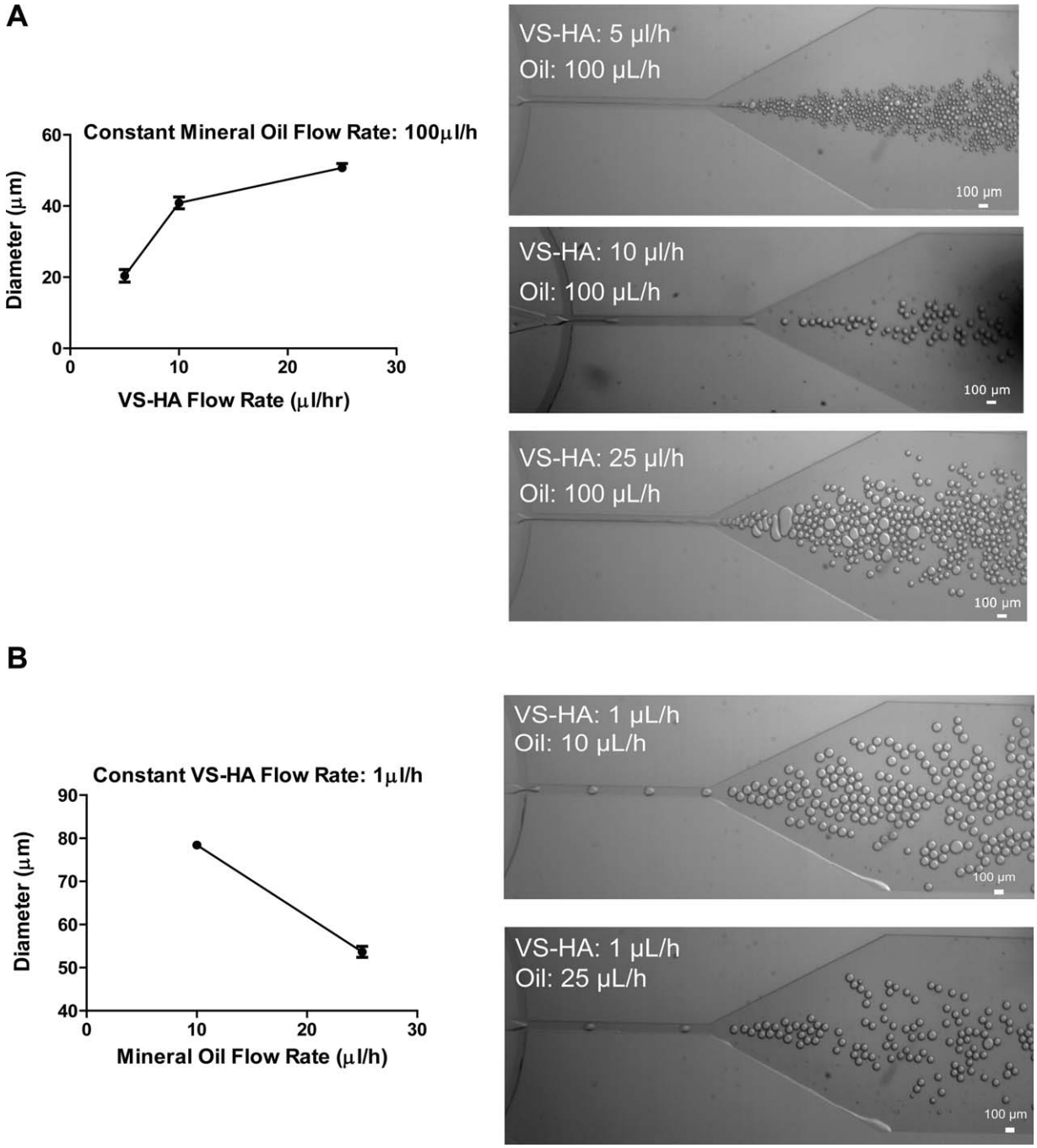

FIGURE 4. Microsphere gel diameter quantification with various hydrogel and mineral oil flow rates. Microsphere gel droplet diameters were measured manually using Image J software. **(A)** The mineral oil flow rate was held constant at 100 μL/h while the VS-HA flow rate was varied to 5, 10, and 25 μL/h. The VS-HA flow rates of 5, 10, and 25 μL/h increased microsphere gel diameters, with mean diameters of 20, 40, 50 μm, respectively. **(B)** The HA flow rate was held constant at 1 μL/h, while the mineral oil flow rate was varied to 10 μL/h and 25 μL/h. An increase in the mineral oil flow rate from 10 μL/h to 25 μL/h reduced microsphere gel diameter, with mean diameters of 78 μm and 54 μm, respectively. A minimum of 450 microsphere gels were measured for diameter size per condition. Data is presented as mean ±standard error mean (SEM).

T-cells was maintained after exposure to shear stress, surfactant, radical formation, and UV.

After the hydrogels were formed through photocrosslinking, a degradation study was performed. UV-crosslinked VS-HA and anti-Fas HA bulk hydrogels were swelled for 24 h in PBS (Fig. 3). During the first 24 h, the hydrogel discs swelled, reaching twice their initial weight, due to the influx of solvent. The enzyme was added after equilibrium was reached. Following hyaluronidase (10 U/mL)[31] treatment beginning at 24 h, both VS-HA and anti-Fas HA hydrogels degraded over a 4-day period (Fig. 3). The final percent weight for the VS-HA and anti-Fas HA hydrogels at the 96 h time point was 54% and 21%, respectively. There was no statistical significance difference between the hydrogels at the 48 h, 72 h or 96 h

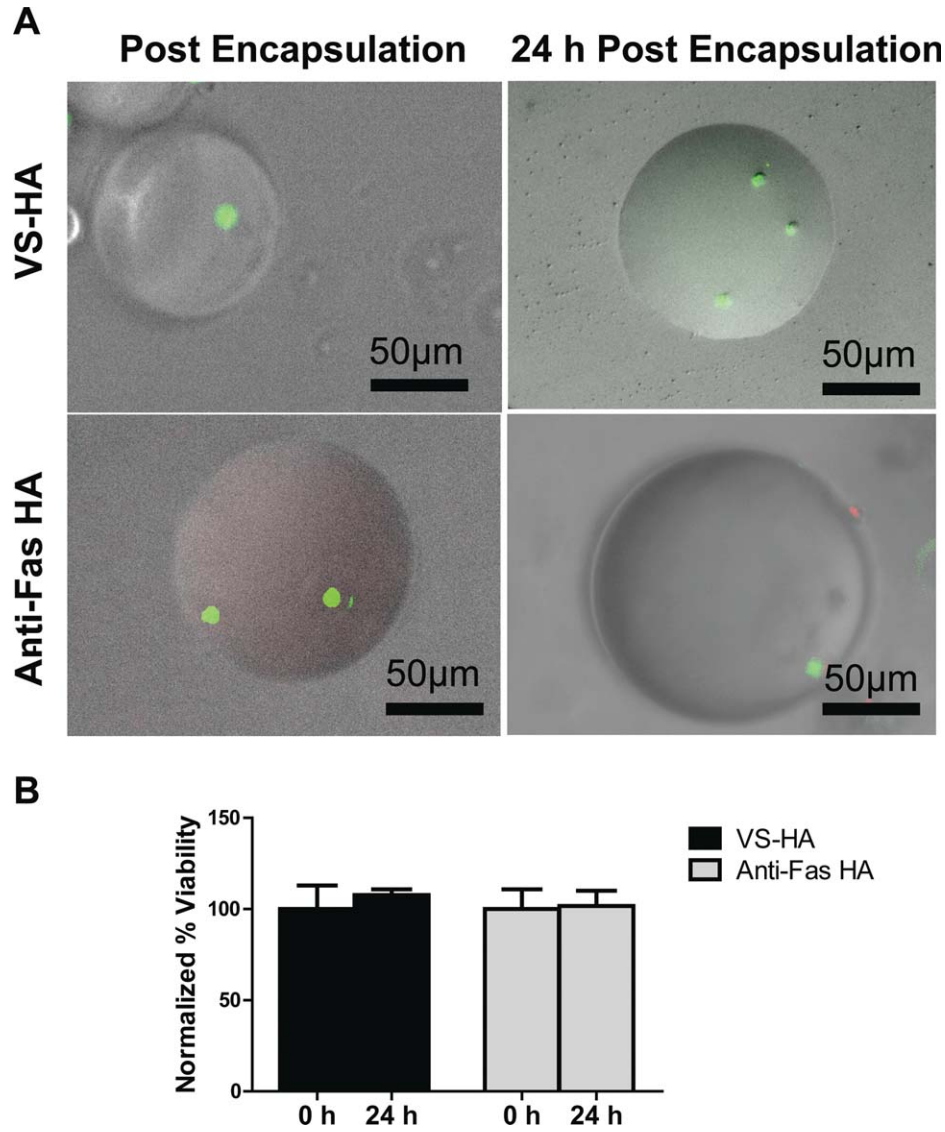

FIGURE 5. Encapsulated NSCs are viable in both VS-HA and anti-Fas HA microsphere gels. NSC viability was quantified using Live/Dead assay and imaged post-encapsulation in both types of microsphere gels, and after 24 h in culture. **(A)** Representative merged bright field and fluorescent images of NSCs in the VS-HA and anti-Fas microsphere gels. **(B)** Quantification of encapsulated NSC viability post-encapsulation (t = 0 h) and 24 h after encapsulation demonstrated that there was no statistical difference between the two microsphere gel conditions. Data presented is the normalized % viability for each independent experimental condition. A sample size of $n = 3$ was used for all conditions with the exception of the NSCs encapsulated in the VS-HA microsphere at 24 h, which was $n = 2$.

time points. It is possible that the VS-HA samples degraded the least as it had more VS-groups available to react with the UV generated radicals. Although the anti-Fas HA hydrogels had <50% of its mass after the 96 h time point, it is important to note that this is a relative change in percent weight and does not represent the *in vivo* degradation environment, as this is dependent on localized enzyme concentration.[31]

Microsphere gel size characterization

The diameter of microsphere gels is determined by device geometry, fluid viscosities, surface tension, surfactant concentration, and flow rates of the continuous and dispersed fluids.[36] When the oil/surfactant phase was infused at 100 μL/h, flow rates for the VS-HA precursor solution phase of 5, 10, and 25 μL/h generated microsphere gels with mean diameters of 20, 40, 50 μm, respectively [Fig. 4(A)]. Therefore, an increase in the VS-HA flow rate at a constant mineral oil flow rate caused an increase in the microsphere gel diameter. However, at high overall flowrates some droplets coalesced. Droplet formation did not occur at VS-HA flow rates >25 μL/h. A VS-HA at a flow rate of 50 μL/h almost ran parallel to the oil phase, and thus was too fast to produce droplets. These flow rates were also characterized by tip streaming, which is a specific type of droplet break up process where droplets rapidly form from the tip of the dispersed stream within the middle of the channel.[28] Droplet formation due to tip-streaming results in a higher frequency of droplet formation, but may affect cell viability due to the

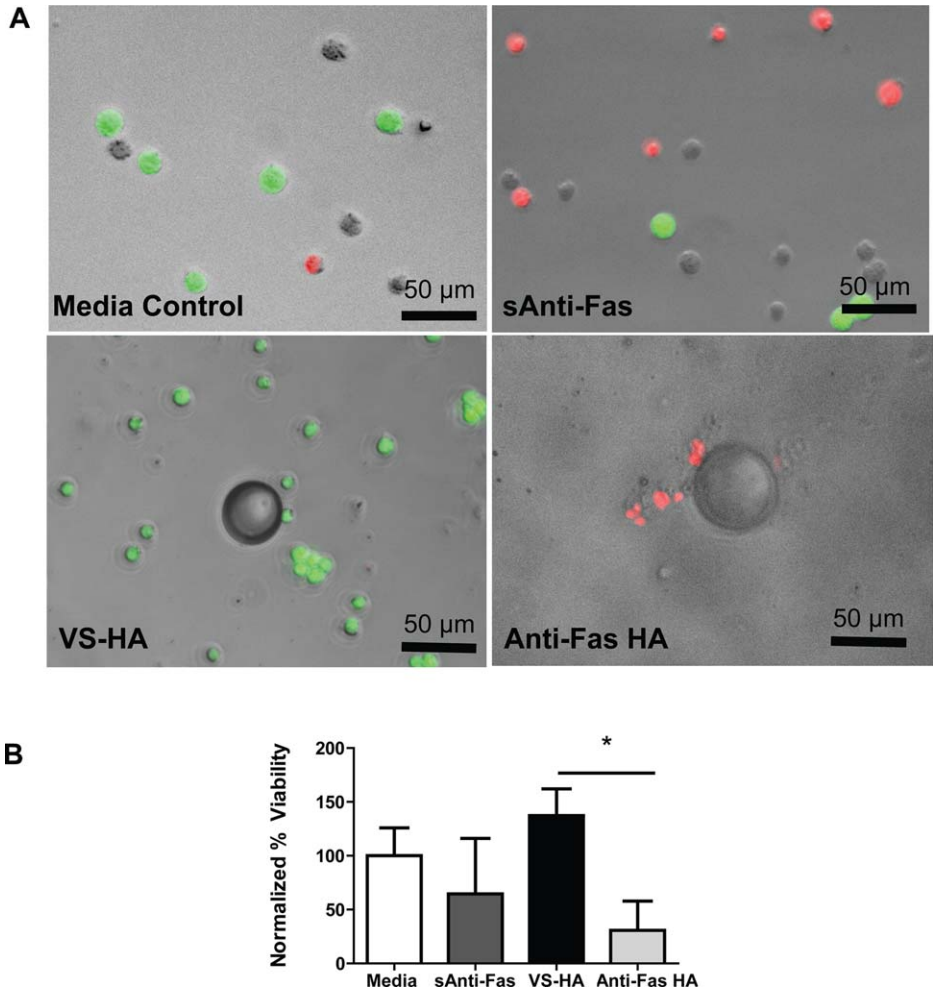

FIGURE 6. T-cell viability decreased after contact with anti-Fas HA microsphere gels. T-cells were cultured with VS-HA microsphere gels, anti-Fas microsphere gels, soluble anti-Fas (sAnti-Fas), and a media control for 48 h. (**A**) Representative merged bright field and fluorescent images for T-cells cultured in media only, soluble anti-Fas, VS-HA microsphere gels, and anti-Fas HA microsphere gels. (**B**) Quantification of T-cells cultured in soluble anti-Fas have a decreased viability (65%) compared to the T-cells cultured in media only control. Similarly, T-cells remained viable after contact with the VS-HA microsphere gels (>95%) compared to when T-cells came into contact with anti-Fas HA microsphere gels (31%). A significant difference was observed between the two conditions. A minimum of $n = 3$ independent experiments were used to determine percent viability. An * signifies statistical difference of $p < 0.05$ between indicated conditions.

shear stress imposed on a cell laden stream.[36] Alternatively, when the VS-HA precursor solution phase was held at a constant 1 μL/h, an increase in the mineral oil flow rate from 10 to 25 μL/h reduced microsphere gel diameter, with mean diameters of 78 μm and 54 μm, respectively [Fig. 4(B)]. These flow rates were utilized for all experiments as tip streaming did not occur, and frequency was lower. Furthermore, a high frequency for droplet stabilization is not required for characterization of viability *in vitro*. However, a high production rate is desired to fabricate sufficient cell encapsulated microsphere gels for *in vivo* applications. Therefore, the high-throughput mechanism for droplet formation is to increase the frequency through the incorporation of droplet generators in parallel.[44]

Cell encapsulation in microsphere gels

Single cell NSCs or T-cells were homogeneously suspended within the 0.75% (w/v) VS-HA or anti-Fas HA precursor solution with the photocrosslinker, and dispersed due to the shear stress provided by the oil/surfactant stream for independent cell encapsulation. Representative merged bright field and fluorescent images of the NSCs encapsulated within the VS-HA and anti-Fas HA microsphere gels can be seen in Figure 5(A). The NSCs were positive for calcein, which indicates viable cells. A statistical difference was not observed in the normalized NSC percent viability between the VS-HA and anti-Fas HA microsphere gel conditions (≥95%) 24 h post-seeding [Fig. 5(B)]. Therefore, the anti-Fas HA microsphere gels did not elicit a pro-death response in the NSCs. These results are consistent with our previously published studies in bulk anti-Fas HA hydrogel.[18] The cell viability percentage obtained is similar to NSCs encapsulated in microsphere gels fabricated using other techniques, such as emulsion based.[45] In contrast, T-cells encapsulated in the anti-Fas HA microsphere gels appeared to have decreased viability after 24 h (Supporting Information Figure 3).

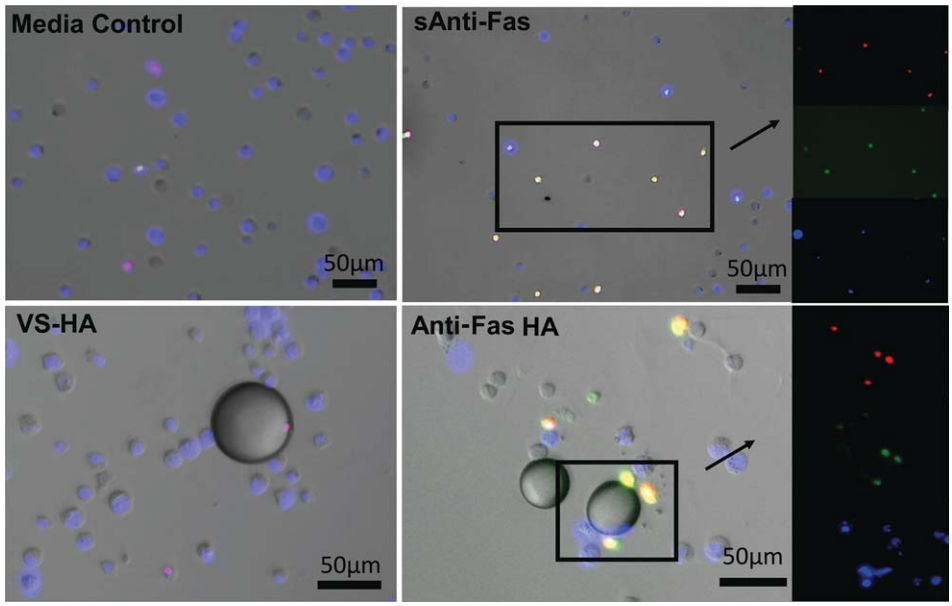

FIGURE 7. Apoptosis of T-cells after contact with anti-Fas HA microsphere gels and soluble anti-Fas. Annexin V binding was used to identify early-stage apoptotic T-cells. Representative merged bright field and fluorescent images of T-cells cultured with VS-HA microsphere gels, anti-Fas microsphere gels, soluble anti-Fas, and media only over 48 h can be seen. To the right of the merged images for soluble anti-Fas and anti-Fas HA microsphere gel conditions, the single filter fluorescent images for Annexin (green), ethidium homodimer (red), Hoechst nuclei stain (blue) can be seen. Positive Annexin stain confirms that the T-cells underwent early-stage apoptosis. For all conditions, $n = 2$ independent experiments.

To further demonstrate anti-Fas functionality of the hydrogel and its effect on cell viability in a microenvironment more representative of *in vivo* T-cell interactions with the hydrogel, suspended T-cells were cultured with either VS-HA or anti-Fas HA microsphere gels. Other studies have conjugated bioactive molecules, such as RGD peptide,[11] gelatin,[14] and fibronectin[14] that promote cellular adhesion of encapsulated cells to the surface of the microsphere gel; however, they do not elicit a cellular response from cells external to the microsphere gel. We conjugated bioactive molecules that induced a response based on external cell contact. It has been previously demonstrated that T-cells undergo apoptosis when exposed to anti-Fas functionalized polyethylene glycol bulk hydrogels.[17] A soluble anti-Fas control was included to observe the effects of the anti-Fas alone independent of the hydrogel or its conjugation chemistry. Representative merged bright field and fluorescent images of the T-cell viability for the 4 conditions can be seen in Figure 6(A). The number of calcein-positive viable cells was quantified for all 4 conditions [Fig. 6(B)]. The VS-HA microsphere gels did not induce T-cell death, as normalized percent viability was $>95\%$ and not significantly different from the media control. Although soluble anti-Fas reduced T-cell viability to an average of 65%, this result was not significant. A significant decrease in T-cell viability was observed when cultured in the anti-Fas microsphere gels (31%) compared to the VS-HA condition ($>95\%$), indicating that cell death was due to the presence of anti-Fas alone. Cell contact with the microsphere gel is required in order to elicit a cellular response as it is a receptor mediated signaling pathway. It is hypothesized that the non-viable suspended T-cells had previously been in contact with the microsphere gel, underwent apoptosis, and then detached from the microsphere gel, as it is unlikely that the covalently-bound anti-Fas leached into the media.

In addition to the Live/Dead assay, Annexin V marker (green) was used to identify T-cells undergoing early-stage apoptosis in all 4 conditions, in addition to ethidium homodimer (red), and Hoechst nuclei stain (blue). Representative merged bright field and fluorescent images can be seen in Figure 7. To the right of the soluble anti-Fas and anti-Fas HA microsphere images, the single filter images can be seen for both conditions, which demonstrate that the cells were non-viable as they were positive with ethidium homodimer and that they underwent apoptosis as those cells were positive with Annexin V staining. Positive staining for Annexin V and ethidium homodimer was not observed for the control VS-HA microsphere gel condition compared to the soluble anti-Fas control and the anti-Fas HA microsphere gels. It is possible that the presence of any Annexin V negative cells indicates that the cells are in late stage apoptosis rather than the early-stage when the staining was performed.

CONCLUSION

We have utilized a microfluidic platform for the fabrication of bioactive microsphere gels for the encapsulation of NSCs as a potential cell delivery vehicle. Microsphere gel cell encapsulation within a closed microenvironment provides isolation and protection against inhibitory cytokines secreted by immune and inflammatory cells, which is a major challenge associated with *in vivo* cell delivery. We have developed a microsphere gel that encapsulates NSCs and maintains their viability while decreasing the viability of immune and inflammatory cells after contact with the anti-Fas HA

microsphere gels. This is significant as these cells are responsible for the low NSC survival rate. Therefore, we have developed a potential immune modulating hydrogel delivery vehicle for cellular transplantation. A future direction for this project is to increase the encapsulation efficiency of cells for use as a therapeutic strategy for tissue repair.

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