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Formation of ordered cellular structures in suspension via label-free negative magnetophoresis

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Abstract

The creation of ordered cellular structures is important for tissue engineering research. Here we present a novel strategy for the assembly of cells into linear arrangements by negative magnetophoresis using inert, cytocompatible magnetic nanoparticles. In this approach, magnetic nanoparticles dictate the cellular assembly without relying on cell binding or uptake. The linear cell structures are stable and can be further cultured without the magnetic field or nanoparticles, making this an attractive tool for tissue engineering.

The goal of tissue engineering is the full regeneration of functional tissues that have been damaged through disease or injury. Typically, a combination of cells, bioactive factors, and/or biomaterials are used to provide an environment that encourages regeneration.^{1, 2} Controlling the spatial distribution and organization of cells within a biomaterial matrix remains one of the key challenges in restoring the function and normal operation of healthy organs and tissues. This is especially true for larger organs that require complex vascularization to enable nutrients to reach interior cells.^{3, 4}

A range of techniques have been developed to organize cells into controlled spatial arrangements on cell-adherent surfaces⁵ or in 3-D biopolymer scaffolds.⁶ These strategies frequently include the use of lithographic techniques to pattern cell-adhesive regions onto non-adhesive surfaces.⁷⁻¹¹ In addition, inkjet printing has been implemented for the construction of 2-D cell patterns by printing cells onto a substrate in the desired pattern,¹² or 3-D organ printing in which a 3-D structure of cells is printed in layer-by-layer rapid prototype format.¹³ These techniques can be time-consuming and costly, and additionally, lithography sometimes employs toxic solvents which can potentially denature biomolecules.⁶

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SUPPORTING INFORMATION: Detailed description of the materials and methods used in this work. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The use of electric forces is a promising alternative for the manipulation and alignment of cells, ^{14, 15} but this technique has constraints, including the potential for inducing cellular electrochemical processes and localized heating. ^{16, 17} Additionally, the relatively small electrical permittivity differences between the cells and the surrounding medium result in weak assembly forces that further limit the capabilities of this approach. ¹⁸ While magnetophoresis has few of the above limitations, current approaches to manipulate cells require either the attachment of magnetic materials onto the cells or cellular uptake of the particles, ¹⁹⁻²⁴ both of which present acute and long-term cytotoxicity concerns. Thus, a system that utilizes the capacity of magnetophoresis to organize cells while avoiding the need for particle uptake or attachment would be ideal for cell manipulation.

We present here a novel approach for magnetically-driven organization of ordered cellular structures without using microfabricated substrates, potentially harmful electrical fields, or the physical attachment to or uptake of material by the cells. Inert, biologically compatible ferrofluids consisting of suspensions of protein-passivated iron oxide nanoparticles drive the organization of cells into linear structures (Figure 1). The purpose of the magnetic nanoparticles is to induce an effective magnetization in the extracellular fluid in order to shepherd the cells into highly organized structures, such as those typically observed with non-biological particles in inverse ferrofluids. ²⁵⁻²⁷ It is worth noting that the dimensions of the ferrofluid particles must be in the range of ~10-20nm in order to succeed in manipulating other submerged non-magnetic materials. Particles that are too small will lack sufficient susceptibility to magnetize the fluid in an external field, making the interaction forces between the suspended cells significantly weaker. ²⁸ Particles that are too large, such as the suspensions of >100nm diameter magnetic particles known as magnetorheological fluid, will experience such strong particle-particle interactions that the fluid will no longer behave as a magnetic continuum. ²⁹

Individual cells have previously been “trapped” at a given location via inverse magnetophoresis using paramagnetic salts and superconducting magnets, ³⁰ however the magnetic susceptibility of a typical paramagnetic fluid is more than 5 orders of magnitude weaker than typical ferrofluids ³¹ which may prevent the assembly of ordered multicellular structures using this approach. Additionally, the strong ionic concentrations required to achieve sufficient magnetic susceptibility with paramagnetic salts (approaching 1M in some cases) may adversely affect certain cellular suspensions. In contrast, the energy of interaction between two cells in ferrofluid is more than 10 orders of magnitude stronger than is possible in paramagnetic salts, making negative electrophoresis using ferrofluids an attractive method for assembling multiple cells into extended structures. The ferrofluid-based platform, which is compatible with media compositions and pH levels optimized for cell culture, represents a significant advantage over other cellular manipulation approaches.

Here, we demonstrate the ability to form chains of cells both in suspension and adhered to tissue culture plates without using special surfaces or substrates and without modifying the cells. The cellular chains were found to be intact even upon the removal of the nanoparticles and/or magnetic field. This inexpensive, straightforward technique does not utilize toxic chemicals and does not require special equipment other than a magnetic source. Overall, this approach is versatile, exhibiting great promise as a first step for generating more complex cellular structures for potential tissue engineering applications. ²⁵⁻²⁷

Nonmagnetic spherical particles such as cells immersed in a magnetized ferrofluid will behave like a non-magnetic cavity inside a magnetized medium, and therefore exhibit the field characteristics of a point dipole. ³² These non-magnetic particles experience dipole-dipole interactions resulting in the formation of linear chains oriented along the external field direction, $\vec{H}$. The maximum magnetic adhesion energy, U_{MAX} , between individual cells in the chain can be quantified using the following expression:

$$U_{MAX} = \frac{3}{8} \mu_o V_n \left(\frac{\mu_p - \mu_f}{\mu_p + 2\mu_f} \right)^2 \vec{H}^2 \quad (1)$$

Clearly, the magnetic energy depends on the volume of the particle, V_n , and the magnetic permeability difference between the particles, μ_p , and the surrounding fluid, μ_f . In classical magnetophoretic cell manipulation techniques, a material such as iron oxide is attached to the cell to give it greater permeability than the surrounding fluid $\mu_p > \mu_f$ and thus facilitate the application of force to the cell. An alternative approach is to use a fluid with a strong magnetic permeability, such as ferrofluid comprised of magnetic nanoparticles, allowing for cell manipulation without attachment or uptake of magnetic material by the cell. Magnetic manipulation can only be achieved when the magnetic potential energy greatly exceeds thermal fluctuation energy, kT . Given that the magnetic permeability of most ferrofluids is 1-2 times the permeability of vacuum, μ_o , this relation implies that nonmagnetic objects as small as 650 nm in diameter can be assembled by negative magnetophoretic force using magnetic fields as low as 100 Oe. Since cells are usually at least ten-fold larger than this lower limit, this concept is highly suitable for cell manipulation.

We explored the use of negative magnetophoresis for manipulating cells by preparing a solution of human umbilical vein endothelial cells (HUVECs) at passage 2 suspended in Endothelial Growth Medium (EGM-2 with 2% fetal bovine serum, Lonza, Allendale, NJ) containing 30 mg/mL of BSA coated iron oxide (Fe_3O_4) nanoparticles (12 nm core) (Figure 1d). These magnetic nanoparticles were previously demonstrated to be stable in media and non-toxic even at high concentrations.³³ To eliminate cell adhesion to the surface of standard glass microscope slides, we pre-treated the slides with BSA solution, thereby permitting the examination of unhindered chain formation directly in solution. Alignment of the cells was performed using a 100 Oe magnetic field applied using permanent magnets and measured using a handheld gaussmeter (Lakeshore Cyrotronics, Westerville, OH). To verify that the cells were arranged into linear structures, HUVECs stained with 10 μM CellTracker Green CMFDA (Invitrogen, Carlsbad, CA) were examined with a Zeiss LSM 510 inverted confocal microscope to obtain a series of micrographs rotated at 30° intervals (Figure 2a-d). A lower magnification view (Figure 2e) demonstrates that the linear structures are observed across the entire plane. The cell structures were also found to remain intact even after removal of the magnetic field (Figure 2f). The strong intercellular interactions, likely mediated by cell-cell adhesion receptors such as cadherins, maintain the cells in linear motif. This finding provides evidence that the cell chains are highly stable and can be implemented in tissue engineering strategies, such as for vascular engineering where it has been demonstrated that linear chains of endothelial cells can form capillary-like structures.^{8, 9}

To determine the kinetics of cell chain formation, the growth of cell chains was examined as a function of the time exposed to magnetic field. HUVECs stained with CellTracker Green CMFDA were trypsinized from the flask and resuspended in EGM-2 to a concentration of 5×10^6 cells/mL. The HUVECs were first dispersed in 30 mg/mL ferrofluid and then placed into a fluid chamber formed with two BSA-coated glass microscope slides separated by 100 μm slide spacers (Invitrogen) (Figure 3a). A 100 Oe magnetic field was then applied to the chamber by passing current through 2.5 inch iron-core solenoids (Fisher Scientific, Pittsburg, PA) placed at the perimeter of the microscope stage (Nikon Eclipse TE300 inverted microscope). The cells quickly chained into linear structures oriented along the external field direction (Figure 3b). The average chain length grew with increasing time of magnetic field exposure (Figure 3c), which is consistent with the main features of diffusion limited cluster aggregation models^{25, 34, 35} that predict a power law dependence for effective chain length, S , as a function of time, i.e. $S(t) \propto t^z$ (Figure 3d). The kinetics of the experimental chain growth were obtained through

manual analysis of photomicrographs obtained with a digital camera (QImaging Retiga 2000R). This kinetic behavior indicates that the effective length of the cell chains in suspension is controllable through length of magnetic field exposure.

One of the primary concerns regarding the addition of magnetic nanoparticles to cellular suspensions is the potentially adverse effects that suspended nanoparticles may have on the cells. Apoptosis or abnormal behavior in a cellular system can be triggered by extracellular fluid containing cytotoxic components or by uptake of toxic particles by the cells.³⁶ Ferrofluid applications are particularly challenging, since relatively high concentrations of nanoparticles are required, and many ferrofluid preparations are typically stabilized with surfactants that would induce cell lysis. To examine the cytotoxicity of this system on HUVECs, the cells were subjected to varying concentrations of BSA-ferrofluid and their viability was measured after 2 hours of ferrofluid exposure. HUVECs stained using CellTracker Green CMFDA were seeded at 2×10^4 cells/well into 96-well black-sided tissue culture plates (Corning, Corning, NY). The cells were incubated overnight to allow them to adhere and spread. Then BSA-ferrofluid was mixed with EGM-2 to produce ferrofluid concentrations of 0, 15, 30, and 45 mg/mL. EGM-2 was removed from the wells and the ferrofluid/EGM-2 mixture was added to the cells (N=3 wells for each ferrofluid concentration). The cells were incubated for 2 hours at 37 °C and 5% CO₂. The BSA-ferrofluid/media mixture was then replaced with EGM-2 and 7.5 μM propidium iodide (from LIVE/DEAD staining kit, Invitrogen). After 5 minutes of incubation, the cells were imaged under the fluorescent microscope. Digital images were taken (N=4 images in each well) with a green filter (all cells) and a red filter (dead cells). The cells remained completely viable (>95%) after submersion in the ferrofluid (Figure 4a and 4b). We further examined the behavior of the cells by conducting the experiment in a tissue culture-treated plate for a period of 2 hours, after which time the cells were well adhered to the plate. After the ferrofluid was washed away and replaced with complete medium, the cells were capable of spreading and growing to confluence (data not shown). We thus conclude that exposure for 2 hours to the BSA-ferrofluid does not have a significant impact on cell viability or on the ability of cells to proliferate normally on tissue culture-treated plastic.

The linear cell chains formed using this technique can be beneficial to tissue engineering research if the resulting structures can adhere to 2-D substrates or be immobilized in a 3-D matrix for further culturing prior to use. To demonstrate the capability of these cell assemblies to adhere to a cell culture surface following formation, tissue culture-treated 35 mm dishes (Corning, Corning, NY) were coated with a thin layer of type I collagen (PureCol, Inamed Biomaterials, Fremont, CA) at a density of 1 μg/cm² in a buffer containing 15 mM Na₂CO₃ and 35 mM NaHCO₃, pH 9.4, with overnight incubation at 4 °C.³⁷ After two washes with Phosphate Buffered Saline, these dishes were placed in an incubator at 37°C and 5% CO₂ and subject to 100 Oe magnetic fields produced by the solenoids. 50μL droplets of HUVECs in EGM-2 with BSA-coated iron oxide nanoparticles were pipetted onto these surfaces, and within minutes linear cellular chains formed. After 2 hours, the cells had settled due to gravity and sufficiently adhered to the substrates to allow the ferrofluid to be replaced by standard cell culture media without disrupting the oriented structures. The formation and adhesion of single cell-wide chains in a low concentration of magnetic nanoparticles (15 mg/mL) was observed (Figure 5a). Higher concentrations of magnetic nanoparticles (30 mg/mL) led to the formation and adhesion of multi-cell wide chains (Figure 5b) similar to macrochaining phenomena seen in aggregation of dense colloids.²⁵ Incubation of the cell structures on collagen-coated dishes generated stable assemblies, as demonstrated by the persistent presence of cell chains after 18 hours of incubation (Figure 5c). The ability of these magnetically-formed cellular structures to adhere to various cell culture surfaces while maintaining their morphology permits the subsequent removal of the nanoparticles and replacement of the surrounding media, making this approach useful in developing organized cellular structures for tissue engineering.

In summary, we have developed a new method for the magnetic manipulation of cells using freely suspended inert and cytocompatible magnetic nanoparticles. Specifically, we have shown that the magnetic nanoparticles can be used to shepherd the cells into linear, oriented structures under uniform magnetic fields through negative magnetophoresis. The dimensions of the cellular chains were found to depend upon magnetic field exposure time and nanoparticle concentrations. The linear cell assemblies are stable after removal of the magnetic field and ferrofluid, and the cells are able to adhere to standard tissue culture surfaces and can then be further cultured for cell studies or tissue regeneration experiments. Finally, the magnetic nanoparticles are shown to have no cytotoxic effects on the cells, as evidenced by the viability studies.

This novel cell assembly approach holds much promise for tissue engineering research and offers the ability to organize cells in an inexpensive, easily accessible technology platform that could be widely implemented. Future work using this technology will include investigating whether the formation of linear chains of HUVECs promotes the process of vasculogenesis as measured by tubule formation and protein expression patterns, as well as the ability to form these linear cell structures and promote vasculogenesis in three-dimensional biopolymer systems that more closely resemble the native extracellular matrix microenvironments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic of process of cell chain formation in ferrofluid. (a) Suspension of cells in ferrofluid assumes a random orientation in the absence of a magnetic field. (b) The suspended cells form linear arrangements in ferrofluid in presence of magnetic field (arrows), where the ferrofluid particles shepherd the cells into chains due to their induced magnetic dipoles. (c) Linear arrangement of cells adherent to cell-adhesive surface survive and grow upon removal of ferrofluid and magnetic field. (d) Schematic of BSA-passivated nanoparticle synthesis.

Figure 2.

Confocal microscopy images of HUVECs chained in BSA-ferrofluid. (a-d) rotated images of cells at 30° intervals under magnetic field forming oriented linear chains. The arrow indicates the direction of the magnetic field. Scale bar = $50\ \mu\text{m}$. (e) Low magnification view of cells under magnetic field. Scale bar = $200\ \mu\text{m}$. (f) View of cell chains 1 hour after removal of magnetic field. Scale bar = $50\ \mu\text{m}$.

Figure 3.

Kinetics of cell chain formation. (a) Fluorescently-labeled HUVECs without the presence of a magnetic field exhibit no orientation in BSA-ferrofluid. Following magnetic field application, (b) cells cluster and align with field after 1 second, and (c) cell chains grow in length after 5 minutes. Scale bar represents 100 μm . (d) Log-log plot of effective chain length size (S) versus time (t) ($\blacklozenge$) following a power law-dependence consistent with the form of general colloidal aggregation models (dotted line).

Figure 4. Cell viability in the presence of ferrofluid. (a) Cell viability of HUVECs is high after culture with 30 mg/mL magnetic nanoparticles for 2 hours as visualized by LIVE/DEAD staining [green = all cells; red = dead cells]. The 2 dead cells in field of view are shown with arrows. Scale bar represents 100 μ m. (b) Ferrofluid concentration does not affect cell viability.

Figure 5.

Chain structures of HUVECs on collagen-coated dishes. (a) Chains formed with 15 mg/mL and (b) with 30 mg/mL BSA-ferrofluid. Cells were exposed to a 100 Oe magnetic field for 2 hrs, during which time they adhered to the collagen-coated surface. (c) Chain structure of HUVECs on collagen substrates formed with 30 mg/mL BSA-ferrofluid, exposed to magnetic field for 2 hours, and then incubated overnight. Magnetic field was applied in the direction of the arrow in each case. All scale bars represent 100 μm .