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A REVIEW OF THE APPLICATIONS OF MICROFLUIDICS IN TISSUE ENGINEERING RESEARCH

OPSTI POGLED NA FENOMEN MIKROFLUIDNOSTI I NJEGOVU APLIKACIJU U ISTRAZIVANJIMA VEZANIM ZA TKIVNO INZENJERSTVO

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Ključne reči
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Abstract

Due to the scale of tissues, and the need to supply them with nutrients, the field of tissue engineering often crosses into the realm of microfluidics. This paper covers some of the recent tissue engineering research and development that has made notable use of the properties of fluids at the microliter scale. The review covers the following topics: an infection and cell adherence study, a cell separation system using ligands, a microfiber manufacturing method for tissue scaffolds, a hydrogel microwell structure for stem cell engineering, and lab-on-a-chip fabrication.

INTRODUCTION

Microfluidics is the field of reducing fluid processing volumes to take advantage of scaling laws for improved performance. It refers to the length scale of the process, regardless of the size of the entire system—which can include power supplies, pumps, and computers. It is a multidisciplinary field which finds applications in engineering, chemistry, and biology. Aside from uses in printer heads and pressure sensors, microfluidics technology is being used for drug discovery, proteomics, and medical diagnostics, among other medical applications [1]. Because they often allow multiple complex reactions in a single microchip-sized piece of PDMS, a microfluidics device is sometimes called a lab-on-a-chip (LOC) or a micro total analysis system (μTAS [2]). LOCs can carry out mixing, combining, and sorting of multiple chemicals simultaneously. [3]

Microfluidics chips are commonly made using polydimethylsiloxane (PDMS) and photolithography. This method is popular because chips can be made in 24 hours or less. The surface free energy of PDMS is low, about 22 mN/m, but good adhesion can be obtained between two PDMS layers due to the smoothness and elastomeric properties of the substance. Relief structures of chips are made on silicon wafers with a transparency photomask. The chips are optically transparent for wavelengths larger than about 230 nm, they have a refractive index of 1.43, and their thermal conductivity is 0.15 W/mK. PDMS channels are hydrophobic. [4]

What is microfluidics?

Microfluidics is the scaling down of processes and experiments involving liquids and gases to take advantage of properties that emerge at the nanoliter scale. Microfluidics technology has existed for over two decades, and has mainly developed from manufacturing methods for microelectromechanical systems (MEMS). For example, inkjet technology uses microfluidics, and it can also be used to deposit living cells as well as ink. In addition, microfluidics is a multidisciplinary field, and chips often have MEMS built into them to run experiments with thermal, optical, and chemical properties. For example, chips could incorporate optical and electrical engineering with micrometer-sized lasers and waveguides, photodiodes, piezoelectrics, and embedded electrodes. Microchemistry and drug discovery can be carried out in microreactors with controlled mixing and diffusion. [5]

Fluid channels are typically constructed with widths around 10 to 100 μm. Compared to traditional fluid work, this allows for better fluid control, better selectivity, reduced reagent costs, parallel sample processing, increased portability, easier temperature control from reactions, and easier handling of dangerous chemicals. At this smaller scale, surface forces are increasingly important due to the increased surface area to volume ratio. Due to this effect, fluids do not flow well on their own, but can be driven by electric voltage or heat, either directly or by adjusting surface tension, or by applying external fluid pressure. This results in a very laminar flow due to low Reynolds numbers, since the viscosity is large compared to the velocity and fluid length. In basic systems, this results in very slow, predictable diffusion through Fick's Law, which can be useful when fluid flow rates and volumes are controlled. There are, however, also techniques that promote faster mixing in microfluidics systems, such as etching characteristics into channels to cause rotational secondary motion, splitting and recombining fluids, or using vortex mixers to interleave fluids. [5]

Examples:

Lee et al [6] studied early-stage bacterial biofilm propagation in orthopedic implants using microfluidics. When bacteria adhere to implants, they form biofilms, which are not only difficult to target but which can also interfere with tissue growth onto the implant surface. An in vitro model was created using microfluidics to create a 3D tissue-like structure from cultured osteoblasts, and multiple phenotypes of *staphylococcus epidermidis* were added to examine their effects on the tissue-like structure.

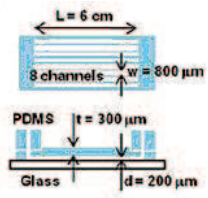

Fig 1. Illustration of the chip used by Lee et al. [6]

Poly (dimethylsiloxane) (PDMS) was used with soft lithography to create a microfluidic housing consisting of 8 channels, each with a volume of 10 μL, shown in Fig 1. Four channels were treated with fibronectin and filled with *S. epidermidis* for an hour before being washed and filled with osteoblast suspension. Staining was used to differentiate between living and dead bacteria, and to assess calcium deposition. M-αMEM was moved through the channels while they cultured, and 3D nodular structures formed from the cells and continually remodeled for the remained of the culturing period.

The remaining channels also had the antibiotic rifampicin added to the culture media, and showed that the concentration and timing of the antibiotic delivery could control the *S. epidermidis* growth into either slow-growing planktonic bacteria in the medium with biofilm on the surfaces, an antibiotic-resistant, sessile biofilm on the channel surfaces, or small clusters of mostly dead bacteria on the surfaces. It was found that osteoblasts could form 3D tissue-like structures where a bacterium was quickly killed, while a small amount of planktonic bacteria significantly challenged osteoblast growth.

The results of the study suggest future work correlating antibiotics and drug delivery, wound healing, and bio-film infection development. The microfluidic system is highly reproducible and has proved to be a useful system for developing mineralized structures by osteoblasts and observing real-time interactions between osteoblasts and bacteria. [6]

Plouffe, Radisic, and Murthy [7] studied cell filtration and depletion from heterogeneous mixtures using PDMS on glass slides. They engineered channels with large surface areas to provide either constant or variable shear stress (Hele-Shaw flow) on the fluid and to non-destructively capture cells. The chip surfaces were coated with ligands for cell adhesion, and the variable shear channels were used to study the ideal relationship between adhesion and stress with endothelial cells, smooth muscle cells, and fibroblasts, shown in Fig 2.

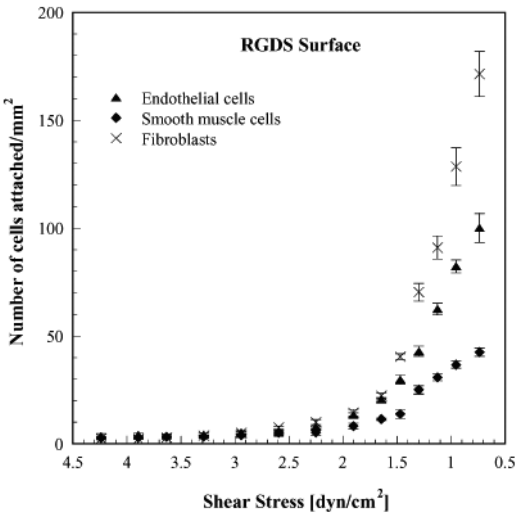

Fig 2. Adhesion of various cells with shear stress in RGDS-coated microfluidic surface. Experiments were conducted with only one cell type, and bars denote standard error from 10 repetitions. Concentration was 330 x 103 cells/mL for each type. [7]

The three tetrapeptide ligands used to coat the channel walls were arg-glu-asp-val (REDV), val-ala-progly (VAPG), and arg-gly-asp-ser (RGDS). REDV is known to bind to endothelial cells, and VAPG to smooth muscle cells, while RGDS binds with those

and also binds readily to fibroblasts. The ligands were arranged in series, and the total setup removed more than 96% of the three cell types. Results showed that, for RGDS adhesion, fibroblast attachment increased the most as shear stress went up, while smooth muscle cells showed the least attachment. Following the separation, a live/dead viability assay showed that less than 3% of the cells were dead.

The paper also notes that the constant shear channels were better at depleting specific cell types but not necessarily at obtaining specific cell capture on the channel surface. They suggest that the slow flow rate and larger channel length in constant shear channels allows for more settling than channels where Hele-Shaw flow occurs. Aside from filtration, this easily designed experimental model could potentially be tailored to better sort specific cells, or to isolate specific cells of interest by stripping away all other cells in a mixture.

Another area where microfluidics and tissue engineering research have crossed is in scaffold design. Hwang et al Š8Ć studied the formation of fibrous scaffolds of Poly (D, L-lactic-co-glycolic acid) (PLGA) using a microfluidics chip and phase inversion. PLGA is approved by the FDA and can be fabricated through various methods. The microfluidics phase inversion method, however, is fast, inexpensive, and can reproducibly create fiber diameters on the order of tens to hundreds of micrometers.

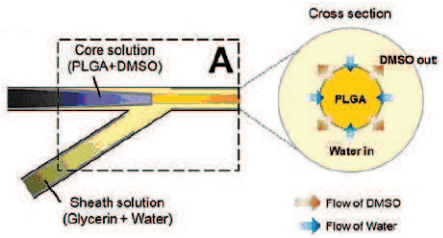

Fig 3. Diagram of phase inversion used by Hwang et al. [8]

A small pipette is placed within a microfluidics channel and PLGA in DMSO is pushed through the core of the channel. A side channel flows a glycerin and water mixture past the pipette tip to act as a sheath flow to the core PLGA flow (Fig 3). As the two liquid layers flow through the channel, a phase inversion occurs and DMSO diffuses out of the core and water diffuses in. The diameter and structure of the final PLGA microfiber depends on the flow rates of the sheath and core layers, and the fiber was spun around a cover glass for collection.

The fiber size is based partly on the diameter of the glass tip. Also, increasing core flow rate increases the fiber diameter, while increasing the sheath flow reduces the diameter. The microfibers themselves consisted of three layers (Fig 4), which are, from outside to center, 1) a thin, dense, smooth polymeric skin layer, 2) a middle region containing small voids, and 3) a central region with irregular large voids. This was due to the diffusion-controlled process: the rapid short-range exchange of DMSO and water makes a skin layer which slows diffusion further into the core, and slower diffusion creates larger voids.

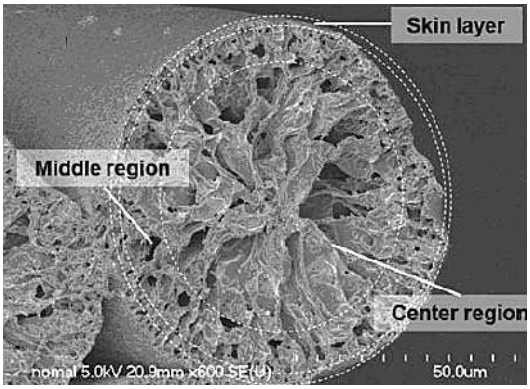

Fig 4. Layers formed in microfibers by PLGA phase inversion. [8]

The final stage t of Hwang et al's study was testing the fiber as a tissue scaffold. Several cytokines and peptides are soluble in DMSO and can be deposited onto the fibers during phase inversion. Fibroblasts were cultured onto either bare PLGA fibers, or onto PLGA fibers coated with fibronectin. It was observed that cell proliferation was similar between the two types of fibers. It was also shown that the fiber diameter affects cell alignment; most cells aligned axially on fibronectin-coated fibers with diameters of 20 μm, while orientation was random when the diameter was 80 μm. When neuronal cells were seeded on bare microfibers, they aligned parallel to the fiber, suggesting possible future use in neural tissue engineering.

Another use of microfluidics in tissue engineering is the control of cellular environments, and was discussed by Wheeldon et al [9]. Changing the mechanical and chemical environment that cells grow in can affect their development and gene expression. The main focus of the paper was on poly(ethylene glycol) (PEG) microwells. Cells can be adhered to microwells and, depending on the depth and diameter of the wells, the cells can be collected and prevented from washing away, and will subsequently be subjected to different shear stresses from fluid across the wells. For embryonic stem cells, pathway differentiation can be controlled, as well as cell aggregate size and homogeneity.

Embryoid bodies (EBs) were formed from embryonic stem cells cultured in microwells of different sizes. More EBs around 450 μm in diameter spontaneously beat and expressed the cardiac marker sarcomeric alpha actinin, while beating and sarcomeric alpha actinin occurred much less in EBs around 150 μm in diameter. Instead, the smaller EBs showed more signs of endothelial cell behavior, expressing marker CD31.

Microwells could also be used to explore other aspects of stem cell differentiation based on size and signaling, although some aspects of control are difficult.

Wheeldon et al, (2010) also explored bottom-up assembly of photocrosslinkable hydrogels on the micron scale. They created a microfluidics system that can continuously make gels in a variety of shapes for 3D assembly (Fig 5). A shutter was used with stop-flow lithography to expose a set volume of hydrogel to light as it moved through the microfluidic channel, creating a crosslinked block. Blocks could be self-assembled by placing the hydrogels in oil; the oil pushes them together, and adding another interface with a wetting substrate allows the gels to wrap and form 3D structures. There are size and structure limitations to the method, and research needs to be done to determine other materials and liquids that can perform the task better.

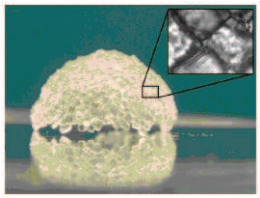

Fig 5. PEG hydrogel microgels assembled into a shell by Wheeldon et al. [9]

Lastly, the group suggested a top-down approach with natural polymers to make hydrated vascular networks to encapsulate cells. Hepatocytes were embedded in an agarose microchannel. Diffusion from the channels into the walls occurred, allowing for perfusion into the hydrogel. A ring of viable cells was visible around the channel after 3 days. This technology could potentially be used to create tissue-like constructs that mimic native vasculature, with cells and channels already built into them.

Principles of Molecular Analysis on a Chip

The Agilent electrophoretic assays are based on traditional gel electrophoresis principles that have been transferred to a chip format. The chip format dramatically reduces separation time and sample consumption. The system provides automated sizing and quantitation information in a digital format. On-chip gel electrophoresis is performed for the analysis of DNA, RNA, and proteins (Agilent Technologies, Santa Clara, CA).

Charged biomolecules like DNA or RNA are electrophoretically driven by a voltage gradient—similar to slab gel electrophoresis. Because of a constant mass-to-charge ratio and the presence of a sieving polymer matrix, the molecules are separated by size with smaller fragments migrating faster than larger ones. Dye molecules intercalate into DNA or RNA strands or Protein-SDS micelles. These complexes are detected by laser-induced fluorescence. Data is translated into gel-like images (bands) and electropherograms (peaks). With the help of a ladder that contains fragments of known sizes and concentrations, a standard curve of migration time versus fragments size is plotted. From the migration times measured for each fragment in the sample, the size is calculated. Two marker fragments (or only one marker fragment in the case of RNA) are run with each of the samples bracketing the overall sizing range. The “lower” and “upper” markers are internal standards used to align the ladder data with data from the sample wells. This is necessary to compensate for drift effects that may occur during the course of a chip run. For DNA and protein assays, quantitation is done with the help of the upper marker. The area under the upper marker peak is compared with the sample peak areas. Because the concentration of the upper marker is known, the concentration for each sample can be calculated. Besides this relative quantitation, an absolute quantitation is available for protein assays, using external standard proteins. (10)

Principle: fluid transport effected in such a way that it can serve as a measure of cellular and molecular size and concentration. This technology uses semiconductor-like microfabrication techniques to translate experimental and analytical protocols, developed in software, into chip architectures consisting of interconnected fluid reservoirs and pathways.

a) For cells: hydrodynamic focusing, e.g., controlled movement of cells on the chip generated by the pressure–driven flow.

b) For molecules (RNA, DNA, proteins): fluid transport is affected by strategically located electrodes which create electrokinetic forces capable of driving fluids through selected pathway

Agilent 2100 bioanalazyer detection is based on laser-induced fluorescence of an intercalating dye, which interacts with the molecule/gel complexes.

Physical Background:

• In electrophoresis, ions in a fluid medium are propelled toward an oppositely charged electrode by means of a voltage gradient. Partitioning occurs because of differential migration rates under these conditions.

• In electroosmosis, charges in the transport channel wall create a layer of mobile ions in the adjacent fluid which moves the entire fluid column and solutes contained therein (plug flow) in response to the imposition of a voltage gradient.

Alternative Display Option:

• Gel-like image-laddering
• Electrophoregram
• Fluorescence (tabular graphics or format)

Quantification of proteins is relative, not absolute if the standard curve does not contain target molecules (30% variations). For quantification of DNA, the standard curve is within the chip. (10)

Important Issues:

• Agilent is good for assay brought up to definite level, where the liquid component (background) is the same and the target molecule varies in concentration

• All wells have to be filled

• One chip can be used only for samples with an identical background (molecular composition in solution)

• This combination/the steady state of this combination is necessary for precise detection which is based on microfluidic movement of the molecules in question. Otherwise, the detection of different movements in one chip will be integrated into false curve-laddering, affecting the result.

• Minimize variations between samples within one chip.

• Our final samples for final assay would contain only relevant buffer and nucleotides.

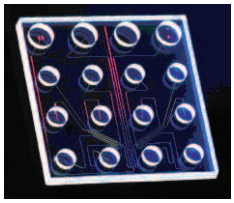

Fig 6. Agilent glass microfluidic lab-on-a-chip. Channels are gel-filled and electrodes control sample movement [10].

The chip (Fig 6) accommodates sample wells, gel wells and a well for an external standard (ladder). Micro-channels are fabricated in glass to create interconnected networks among these wells. During chip preparation, the micro-channels are filled with a sieving polymer and fluorescence dye. Once the wells and channels are filled, the chip becomes an integrated electrical circuit. The 16-pin electrodes of the cartridge are arranged so that they fit into the wells of the chip. Each electrode is connected to an independent power supply that provides maximum control and flexibility.

Charged biomolecules like DNA or RNA are electrophoretically driven by a voltage gradient—similar to slab gel electrophoresis. Because of a constant mass-to-charge ratio and the presence of a sieving polymer matrix, the molecules are separated by size. Smaller fragments are migrating faster than larger ones. Dye molecules intercalate into DNA or RNA strands or protein-SDS micelles. These complexes are detected by laser-induced fluorescence. Data is translated into gel-like images (bands) and electropherograms (peaks) (Figs 7 and 8). With the help of a ladder that contains fragments of known sizes and concentrations, a standard curve of migration time versus fragments size is plotted. From the migration times measured for each fragment in the sample, the size is calculated.

Two marker fragments (or one marker fragment for RNA) are run with each of the samples bracketing the overall sizing range. The "lower" and "upper" markers are internal standards used to align the ladder data with data from the sample wells. This is necessary to compensate for drift effects that may occur during the course of a chip run. For DNA and protein assays, quantitation is done with the help of the upper marker. The area under the upper marker peak is compared with the sample peak areas. Because the concentration of the upper marker is known, the concentration for each sample can be calculated. Besides this relative quantitation, an absolute quantitation is available for protein assays, using external standard proteins. For RNA assays, quantitation is done with the help of the ladder area. The area under the ladder is compared with the sum of the sample peak areas. The area under the "lower" marker is not taken into consideration. For total RNA assays, the ribosomal ratio is determined, giving an indication on the integrity of the RNA sample. [10]

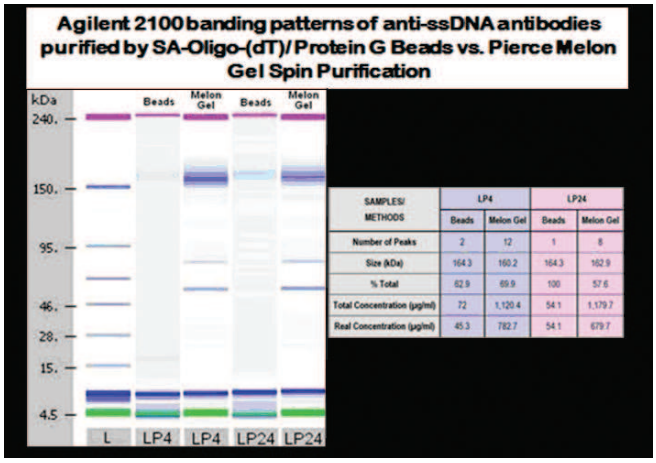

Fig 7. Agilent 2100 banding patterns of anti-ssDNA from lupus patient sera after applying two isolation methods. The superiority of magnetic beads over melon gel separation is observed through the absence of bands in the beads sample.

With permission of Dr Mirjana Pavlovic and Anna M.Kats, MS/FAU, Boca Raton

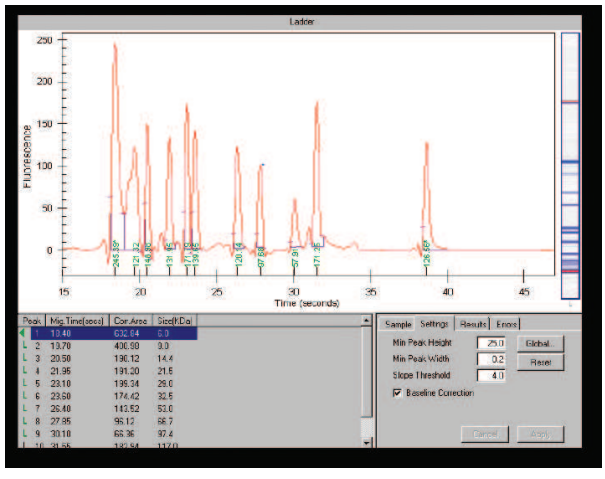

Fig 8. Example electropherogram from Agilent [9].

DISCUSSION

The use of established microfluidics technologies gives tissue engineering researchers more options for creating models, scaffolds, and in vitro experiments. Using a basic PDMS microfluidics chip, Lee et al [6] could test the interactions of biofilm bacteria and cells for implant infection models. Amounts of adhesion could be measured, as well as live and dead cells. The flow rates and environments could be individually controlled, including the shear stress, and this allowed for a constant supply of nutrients and waste removal. The microfluidics model also allows for control of the delivery of antibiotics and bacteria, allowing for a wide variety of studies to be conducted in parallel without cross-contamination.

Plouffe et al [7 used microfluidics to maintain sensitivity with small volumes, allowing for cell sorting and filtration. The fluid channels allowed for control of shear stress, which was either constant or variable, depending on the shape. Since the flow rate was controlled, calibration was used to determine the maximum number of cells that could flow through the device. The non-specific adhesion of cells to glass and PDMS could also be calculated. In the study, over 96% of the three cell types were captured, and Plouffe suggests the simple device could be used for negative filtration of desired cell types in future works. The binding properties of desired cells could be found experimentally using this device, but for rarer cells, an approach like this may be difficult unless it is known *a priori* that the desired cells will not bind to the filtering ligands.

The PLGA microfiber technique that Hwang et al [8] presented allows for fibers that are small and do not require heat. The diameter is based on the flow rates, so it is easy to reproduce, and molecules, cytokines, and peptides which are soluble in DMSO can be loaded into the fibers. The control over fiber diameter and molecule loading should allow for scaffolds to be made for a variety of specific needs. This control looks like it is also increased due to the tendency of cells to align at a certain angle depending on the fiber diameter.

Another method of creating scaffolds with microfluidics is using photocrosslinkable hydrogels and stop-flow lithography. This technique can create blocks of any desired two-dimensional shape by changing the shape of the filter that allows light in to cure the hydrogel. From there, Wheeldon et al[9] was able to use liquid interfaces to assemble these hydrogels into three-dimensional tissue-like scaffolds. Hydrogel microstructures can also be used with microfluidics for top-down tissue engineering, where microchannels are built into hydrogels. While this is a good technology, since the hydrogels allow diffusion and can mimic vasculature, there need to be many channels, and the channels must branch profusely, which is difficult to fabricate.

Microfluidics can be used with microwells to control stem cell culturing [9] . By altering the diameter microwells, shear forces affect cells inside them differently, and embryonic stem cells tend to differentiate in more predictable ways. It isn't a perfect technique, but future work with different shear stresses and microwell properties could lead to better control of stem cell differentiation. Additional benefits of the technique are to control the size of the aggregates, and therefore the embryoid bodies, and cell aggregates are protected from shear stresses except at the surface of the wells. On the downside, Wheeldon notes that the technique is unable to affect individual microwells that aggregate growth and size could be restricted by the microwells during long-term culturing, and that retrieving aggregates from the wells is currently challenging.

Lastly, microfluidics as a main principle used in Agilent bioanalyzer can greatly contribute to precise analysis of small molecules or macromolecules such as DNA, RNA and proteins such as antibodies [11, 12, 13]. The presence and low quantities of the macromolecules can be detected and determined and their functional activity (such as hydrolysis) identified. This is important for low concentrations or small samples of target molecules. The broad application of the microfluidic principles in bioengineering as emerging and rapidly developing field of research is of tremendous importance.

CONCLUSIONS

As biochemical and genetic research gets smaller and more precise, improvements in microfluidic technology becomes more important. Tissue engineering has benefited from microfluidic processes for biofilm infection studies [6] , cell filtration [7] , stem cell differentiation and controlled scaffold building with hydrogels [9] and PLGA strands [8] . Microfluidics is also used in existing products for analysis of DNA, RNA, and proteins[10] . Similar processes are used in this field to create, sort, modify, and analyze macromolecules, while keeping reagent use down and mimicking in vitro environments. There is still room to improve in vitro simulations with microfluidics, particularly for larger-scale systems, but the research this paper reviewed shows promise at the small scale, and a basic benefit of microfluidics is that more complex systems can be fabricated with relative ease. From the abovementioned topics, future research in microfluidics in tissue engineering could involve the shaping of scaffolds and creating paths within it for nutrient diffusion, biological filtration, and controlled cellular growth in larger systems.

Apstrakt:

Zbog široke skale tkiva i potrebe da se ona snabdeju hranjivim materijama, polja tkivnog inženjeristva i mikrofluidnosti se često ukrštaju. Ovaj članak pokriva neka sasvim nedavna okrića u istraživanju i razvoju tkivnog inženjerstva koja su dovela do značajne upotrebe i korišćenja osobina fluida na mikro-skali. Pregled pokriva sledeće novine: studije infekcije i ćelijske adherencije, sistem ćelijske separacije upotrebom liganda, manufakturnu microfiber metodu za tkivne scafolde (držače), hydrogel structure u mikro-okcima za inženjerstvo matičnih ćelija, i fabrikaciju poznatu kao Lab-na čipu.

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