

# Simple and Novel Three Dimensional Neuronal Cell Culture Using a Micro Mesh Scaffold

Sang Jin Yoo<sup>1</sup>, Jongmin Kim<sup>2</sup>, Chang-Soo Lee<sup>2</sup> and Yoonkey Nam<sup>1\*</sup>

<sup>1</sup>Department of Bio and Brain Engineering, KAIST, Daejeon 305-701,

<sup>2</sup>Department of Chemical Engineering, Chungnam National University, Daejeon 305-764, Korea

Conventional method of cell culture studies has been performed on two-dimensional substrates. Recently, three-dimensional (3D) cell culture platforms have been a subject of interest as cells in 3D has significant differences in cell differentiation and behavior. Here we report a novel approach of 3D cell culture using a nylon micro mesh (NMM) as a cell culture scaffold. NMM is commonly used in cell culture laboratory, which eliminates the requirement of special technicality for biological laboratories. Furthermore, it is made of a micro-meter thick nylon fibers, which was adequate to engineer in cellular scales. We demonstrate the feasibility of the NMM as a 3D scaffold using E18 rat hippocampal neurons. NMM could be coated with cell adhesive coatings (polylysine or polyelectrolyte) and neurons showed good viability. Cells were also encapsulated in an agarose hydrogel and cultured in 3D using NMM. In addition, the 3D pattern of NMM could be used as a guidance cue for neurite outgrowth. The flexible and elastic properties of NMMs made it easier to handle the scaffold and also readily applicable for large-scale tissue engineering applications.

**Key words:** 3D culture, scaffold, micro mesh, neuronal culture, 3D cell patterning

## INTRODUCTION

Conventional method of cell culture studies has been performed on 2-dimensional (2D) surfaces such as glass, well plate and Petri dishes. These 2D cell culture platforms are widely used because of the easy of handling, its convenience and bio-compatibility. Although the understanding of basic biology has been improved with these advantages of 2D systems, many scientists give careful attention to 3-dimensional (3D) cell culture system due to the limitation of 2D environment (Lund et al., 2009).

Two-dimensional substrates have a limitation in the replication

of 3D cellular environments and the absence of 3D factors in cellular developments could limit the biological outcome in the culture (Zhang, 2004). Moreover, cells cultured on the 2D substrate have to adapt to rigid and flat surfaces while natural *in vivo* environments have large influences from microenvironments derived from extracellular matrix (ECM) and cell-cell interactions. Recent studies showed that the elements of 2D environment that were different from 3D environment could lead to change from gene expression to cell functionality (Zahir and Weaver, 2004; Birgersdotter et al., 2005).

There have been active pursuits in developing new biomaterials for 3D cell culture scaffolds (Shin and Mikos, 2003). For example, polymer based biomaterials (Cheung et al., 2007), protein based biomaterials (Stegemann et al., 2007) and nanofiber (Yoshimoto et al., 2003) have been utilized as a 3D cell culture scaffold. And with these biomaterials, there have been many applications of

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\*To whom correspondence should be addressed.  
TEL: 82-42-350-4362, FAX: 82-42-350-4310  
e-mail: ynam@Kaist.ac.kr

3D cell culture scaffolds (Dutta and Dutta, 2009). For example, there were clinical trials that artificial tissues which were made by 3D scaffolds used for regenerative medicine (Karageorgiou and Kaplan, 2005) and *in vitro* 3D tissue modeling (Yamada and Cukierman, 2007). However, most of the 3D cell culture scaffolds require complex fabrication process and expensive equipment that is usually not accessible for biologists, which limits the extended usage of 3D scaffold systems in cell culture.

Here we propose a nylon micro-mesh (NMM) as a new 3D cell culture scaffold based on its mechanical flexibility, biocompatibility, and practicality. And we show that NMM is suitable for 3D cell culture scaffold and has some potential for 3D tissue engineering applications.

## MATERIALS AND METHODS

### Substrate preparation

Three kinds of Nylon micro meshes (NMMs) which had 20, 10, 8  $\mu\text{m}$  pore size were purchased from Spectrum Laboratories (Spectrum, CA, USA). And NMMs with 40  $\mu\text{m}$  pores were taken from a cell strainer (BD Falcon, NJ, USA).

NMMs were sonicated with acetone, isopropylalcohol (IPA) and DI water for 5min per each and dried by air stream. NMMs were coated with Poly-D-Lysine (PDL, Sigma, MO, USA) by soaking the NMMs in PDL solution (0.1 mg/ml in borate buffer, pH 8.5) at 37°C. After 6 hour, PDL solution was aspirated and NMMs were immersed in 70% ethanol for sterilization. After the sterilization, NMMs were rinsed with DI water. The NMMs were finally dried in clean-bench or incubator.

Polyelectrolyte multilayer (PEL) were coated using layer-by-layer method. The cleaned NMMs were immersed in the solution of Poly allylamine hydrochloride (PAH, 20 mM, pH 9.0, Sigma, MO, USA) for 1 min, followed by five times washing with DI water. The PAH coated NMMs were immersed in the solution of Poly sodium 4-styrenesulfonate (PSS, 60 mM, pH 9.0, Sigma, MO, USA) for 1 min, followed by five times washing with DI water. The process of PEL coating were repeated ten times and finished with the PAH coating. NMMs were sterilized by UV for 5 min and dried in clean-bench or incubator.

Agarose gel (gelling range: 36–39°C, Amresco Inc., Ohio, USA) was dissolved in 100°C DI water and 0.7% (w/v) agarose hydrogel was formed. After cooling the agarose gel to 36°C, cell suspension was mixed with the agarose and plated on PDL coated NMMs.

### Cell culture

Hippocampus tissues were dissected from E18 Sprague/Dawley rat embryo. The tissues were washed three times with Hank's

Balanced Salt Solution (HBSS) and mechanically dissociated using 1 ml pipette tips. Cell suspensions were centrifuged for 3 min at 1,000 rpm and the cell pellets were suspended again in plating medium which was composed of serum free Neurobasal/B27 (Invitrogen, CA), 12.5  $\mu\text{M}$  L glutamate (sigma, MO), 2 mM L glutamine (Gibco) and 1% penicillin streptomycin (invitrogen, CA). Finally, the cell suspend plated onto the coated NMMs at the density of 200 to 300 cells/ $\text{mm}^2$ . Cultures were maintained in an incubator (5%  $\text{CO}_2$ , 37°C). After three days from cell plating, half of the medium was replaced with fresh culture medium without L glutamine every 3 days.

### Live-dead staining

Neurons were stained by Live-Dead viability/cytotoxicity kit (Invitrogen, CA, USA). 10  $\mu\text{l}$  of ethidium homodimer and 2.5  $\mu\text{l}$  calcein acetocymethyl ester were mixed in 5 ml of phosphate buffered saline (PBS). 200  $\mu\text{l}$  of the dye solution was added in 1 ml sample and covered with aluminum foil to protect the dye from photobleaching. After the incubation (5%  $\text{CO}_2$ , 37°C) of the sample for 20 min, the solution in the sample was removed and NMMs washed with PBS.

## RESULTS

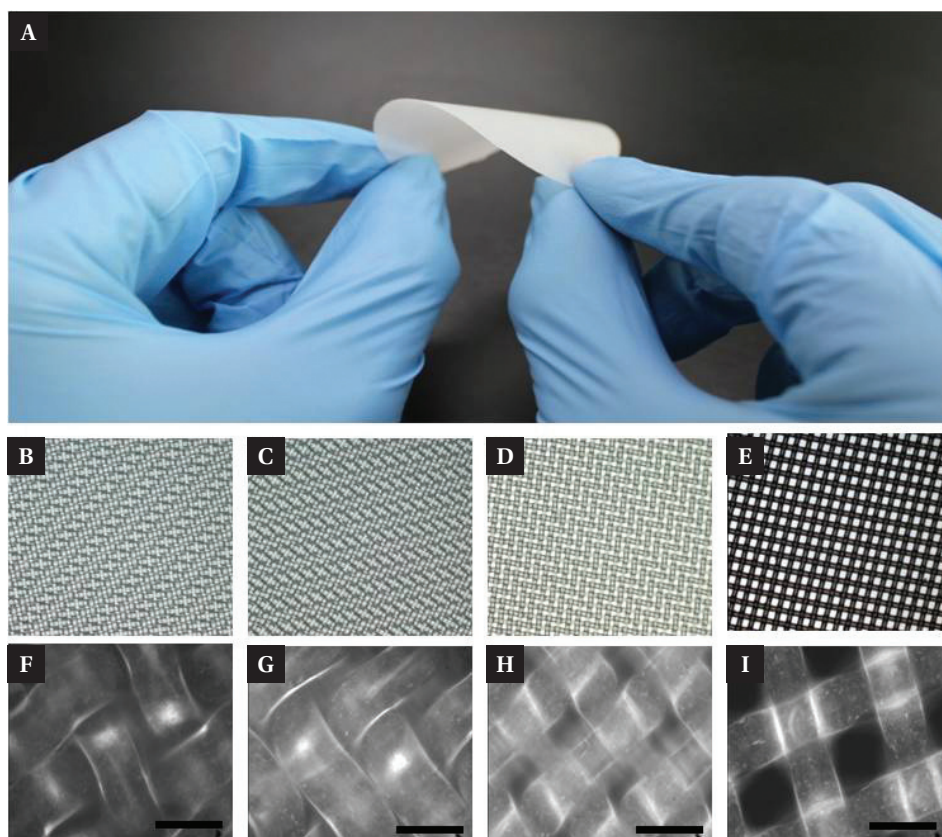
### Mechanical properties of nylon micro-mesh as a three dimensional cell culture scaffold

Appropriate mechanical properties of 3D scaffold are important in 3D tissue engineering (Lee et al., 2008). First, flexibility is required to maintain the shape of scaffold and handling. Second, micro and nano-scale structural architecture is desirable to mimic *in vivo* microenvironments (Curtis and Wilkinson, 1998; Bayliss et al., 1999). Third, some porosity is required for the diffusion of nutrients, metabolic wastes and interconnectivity of cells.

Fig. 1A shows the compliance of the NMM to the mechanical bending. It was also easy to handle by hand for forceps. Fig. 1 also shows the ordered structures of NMM in micrometer scales. The thickness of the individual nylon fiber was 30 or 35  $\mu\text{m}$  and the thickness of NMM was 75  $\mu\text{m}$ , 45  $\mu\text{m}$ , 55  $\mu\text{m}$ , 60  $\mu\text{m}$ , respectively (Fig. 1B–E). NMMs have many regular pores with the pore diameters of 8  $\mu\text{m}$ , 10  $\mu\text{m}$ , 20  $\mu\text{m}$ , and 40  $\mu\text{m}$  (Fig. 1F–I). These properties made NMMs suitable for 3D cell culture scaffolds.

### Cell Viability and biocompatibility

Biocompatibility of materials is a crucial parameter, since it can be decisive criteria as a culture scaffold. Most of the reported scaffolds were composed of ECM molecules (Badylak, 2002), or had bioactive molecules to modulate cellular activities (Zhu,



**Fig. 1.** Nylon micro-mesh. (A) Actual image of NMM. Flexibility and hardness of the NMM show that it is simple to handle and not weak as a 3D culture scaffold. (B~E) Optical microscope image of the NMMs, diameter of pore is 8  $\mu\text{m}$ , 10  $\mu\text{m}$ , 20  $\mu\text{m}$ , 40  $\mu\text{m}$ , respectively and (F~I) the magnified image of those NMMs. Scale bar: 50  $\mu\text{m}$ .

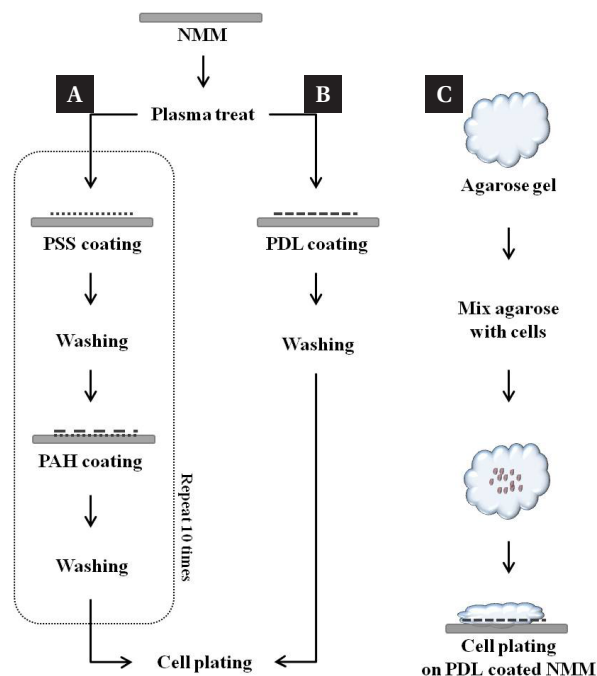
2010), or they had controllability in chemicals (Yildirim et al., 2010).

Fig. 2 shows the surface modification of NMMs using positively charged polymers. We coated NMMs with either poly-D-lysine (PDL) or polyelectrolyte multilayer (PEL). PDL is one of the most common polymer used as a cell adhesive coating material. PEL was used to modify the NMM surface with much denser positive charge layers through layer-by-layer coating technique, which turned out to be more efficient in terms of reproducibility and reliability.

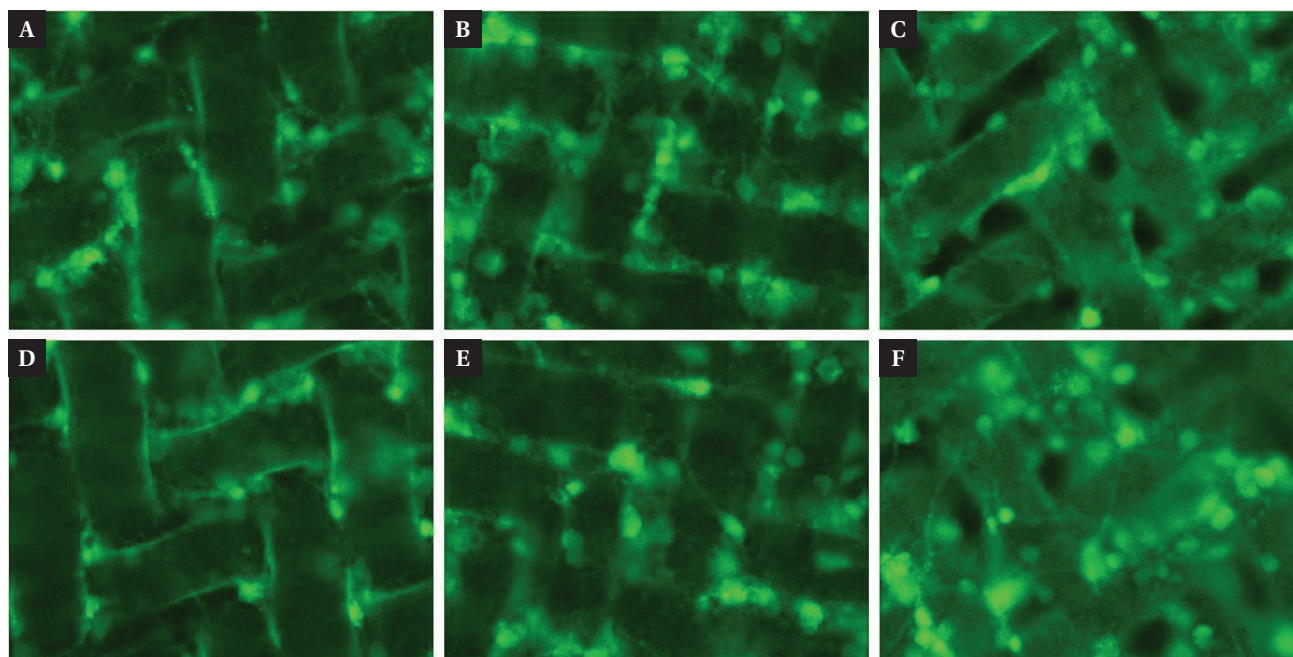
In order to investigate the effect of chemical controllability and viability of cells on the NMM, we prepared different pore size of NMMs coated by PDL and PEL and cell plate. Fig. 3 shows excellent cell viability at 9 DIV. Neurons tend to grow on and in-between the micro-fibers and pores were be filled with neuronal growth.

#### *Entrapment of cells in three dimensional space*

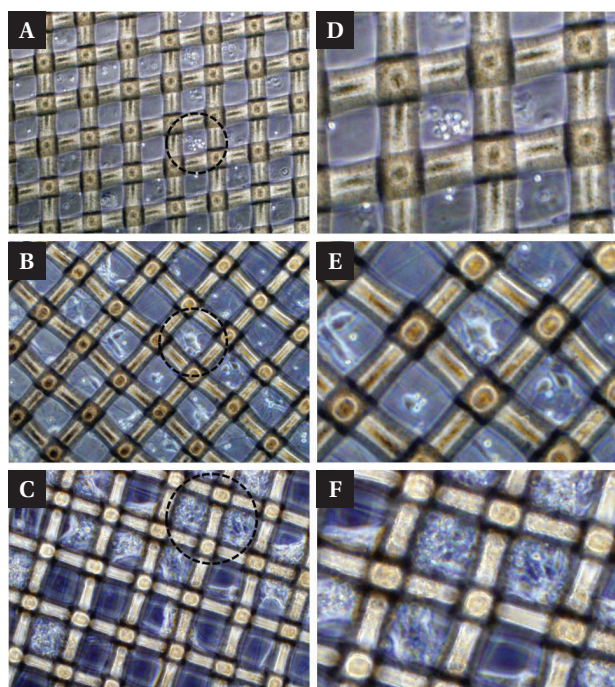
Entrapment of cells in three dimensional space has a special meaning that it provide distinct differences in cell morphogenesis, behavior, gene expression, differentiation from 2D environment (Holmes et al., 2000; Zaman et al., 2006). In other word, the recovered 3D environment cause different viability and functio-



**Fig. 2.** Schematic illustration of the chemical coating and 3D entrapment procedures for cell culture. (A) PEL coating method. PSS and PAH transform the surface charge from positive to negative and vice versa, respectively. (B) PDL coating method. PDL, highly positive charged amino acid chain, promote cell adhesion in culture (C) low temperature agarose gel was used for 3D cell entrapment.



**Fig. 3.** Neuron growth on the NMMs at 9DIV. PDL and PEL coated NMM was covered by hippocampal neurons and formed the neural tissue. Since chemical controllability and neuron viability on the NMM, it has sufficient qualifications for 3D cell culture scaffold. (A~C) Neuron growth on the PDL coated NMM. (D~F) PEL coated NMM. Diameters of pore are 8  $\mu\text{m}$  (A, D), 10  $\mu\text{m}$  (D, E) and 20  $\mu\text{m}$  (C, F). All of viable neurons (green) were labeled with Calcein AM.



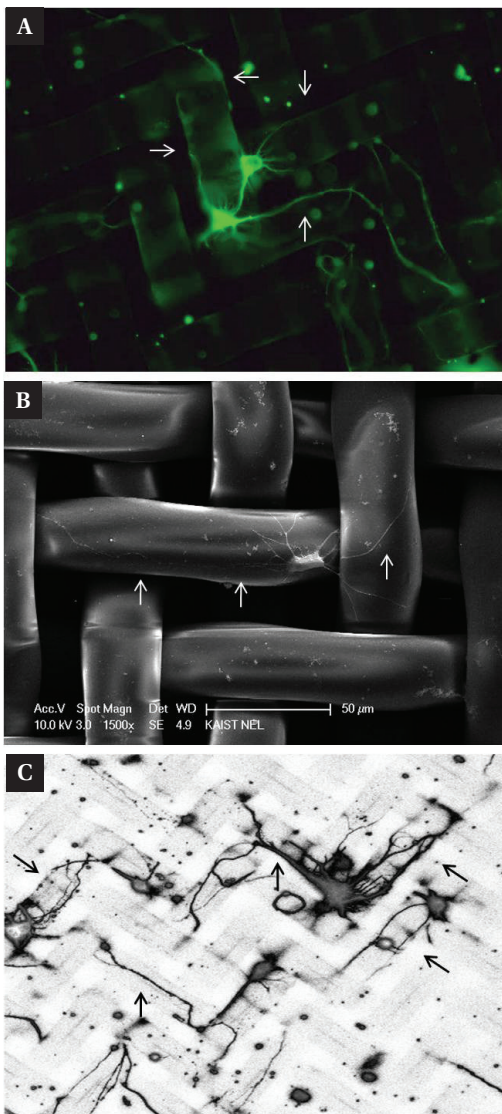
**Fig. 4.** 3D entrapment of neuron on the NMM. After PDL coating the NMM, 0.7% agarose gel was used for 3D entrapment. (A) Agarose gel positioned the hippocampal neurons on the pore and fiber of NMM. And they generate the neurites at 2DIV, (B) growth and connected each other at 4DIV. (C) Finally, many of neurons established complex network on the NMM at 14DIV. Diameter of the pore, we used in 3D entrapment, is 40  $\mu\text{m}$  (D~F). Magnified circle images in (A), (B), (C), respectively.

nality of cell.

Many of the studies used natural hydrogels or synthetic gel such as alginate (Pangas et al., 2003), collagen (Batorsky et al., 2005) and poly ethylene glycol (PEG) (Elisseeff et al., 1999) to entrap cells. We used agarose hydrogel which is a common material in biological laboratories for the cell entrapment (Fig. 2). Fig. 4 shows the neuronal growth in agarose hydrogels. Neurons were well dispersed in the gel, and neurites were formed and extended (Fig. 4B, E). They tend to get clustered in the gel after forming thick fiber bundles. As the gel was not functionalized to be cell adhesive, the clustering process was likely to occur in the matured neuronal cultures. However, neurons were still viable and maintained in 3D NMM scaffolds.

#### *Three dimensional neurite guidance effect*

As NMMs had ordered structures in micrometer scales (Fig. 1), each nylon fiber can provide the topographical guidance cues to the growing neurites. Fig. 5A shows an example of the guided neurite outgrowth along the 3D mesh fibers. The fiber thickness was comparable to the size of soma and neurites followed the edge of the fibers. This is also shown in scanning electron micrograph in Fig. 5B. Fig. 5C shows more 'patterned' neurites on the mesh. Although the fiber itself was too thick to control thinner neurites, its edges clearly had an effect on neurite guidance.



**Fig. 5.** 3D neuron patterning on the NMM. Fibers of NMM compose 3D grid pattern which is a guide-way to the neurites and these 3D structural patterns of NMM appropriate to neuronal patterning. (A) Cacein AM labeled live neurons. Arrows indicate that neurites growth along with 3D grid pattern. (B) SEM image of patterned neuron. (C) Confocal laser scanning microscope image also show the patterned neurons on the NMM. All of neurons were extracted in hippocampus and stained at 6DIV. Diameter of pore is 20  $\mu\text{m}$ .

## DISCUSSION

In summary, we proposed NMMs as a simple but effective 3D cell culture scaffold. Unlike other 3D scaffold materials that requires expensive or special technicality, NMMs are an inexpensive material that is common biological laboratories. NMM had excellent mechanical and structural properties to support neuronal cell growth and gel based cell cultures. It was also chemically modifiable to convert the nylon surface into cell

adhesive surfaces. Moreover, the micro-scale structural regularity could be potentially be applicable to 3D tissue engineering applications.

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