Open Architecture System for Biomanufacturing of Scaffolds for Tissue Engineering

by

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ABSTRACT

Tissue engineering is a new interdisciplinary area which has the goal of growing tissues or organs directly from a single cell taken from an individual, potentially addressing a total market of tissue reconstruction and organ replacement. A key component in tissue engineering is the use of porous scaffolds to guide cells for attachment, proliferation and differentiation in the tissue regenerative process. Upon satisfactory in-vitro culture, this engineered living scaffold is implanted into the regeneration site of the patient to function as the tissue substitute. Conventional processing techniques for the fabrication of scaffolds often encounter difficulties in the precise control of the internal architecture, interconnectivity and distribution of pores within the scaffold. These challenges, along with the advances in biology, medicine, and information technology for tissue engineering applications, have led to using rapid prototyping systems in tissue engineering.

In this thesis, two open architecture rapid prototyping systems are described. One of them was designed and manufactured in previous work by Erdem Cerit and his colleagues. Other system was developed in this work. Both systems were developed in the Manufacturing and Automation Research Center at Koç University. Both of the systems are 3 axis machines. First generation rapid prototyping (RP) system has three servo motors and one step motor. In the second generation RP system four step motors are used to actuate the system. Since the systems are open architecture, various tool path generation algorithms developed in-house can be implemented. In this work precise algorithms were developed and implemented to both of the systems. Developed programs enable production of parts without using any CAD or CAM software. Hollow spheres and 3 dimensional scaffolds can be manufactured on first generation RP system. Other two algorithms which were implemented on the other system allow operator to fabricate equilateral quadrilaterals as well as scaffolds with different dimensions. The developed programs for both of the systems process input data and

generates electronic signals to drive the motors. Various 3D parts produced by both systems are also presented. Finally, polymeric scaffolds fabricated by using old RP system were cell cultured in vitro to determine if they could be used for tissue engineering applications. It was shown that scaffold material is not toxic and cells can attach and proliferate on it.

ÖZET

Doku mühendisliği, bireyden alınan tek bir hücreden doku ve organ büyütmeyi amaçlayan yeni bir disiplinlerarası alandır. Bu alanın uygulama potansiyeli çok büyüktür ve medikal sektörü için de oldukça büyük önem taşımaktadır. Doku mühendisliğinin anahtar bileşeni, yeniden üretilen doku sürecinde hücrelerin tutunması, çoğalması ve farklılaşması için yol gösterebilen gözenekli bir doku iskeletinin kullanımıdır. Doku iskeleti fabrikasyonu için geleneksel imal teknolojileri doku iskeletinin iç yapısı ve içindeki gözeneklerin dağılımı hassas kontrolü konusunda zorluklarla karşılaşır. Bu zorluklar, doku mühendisliği uygulamalarındaki bilgi teknolojileri, tıp ve biyoloji alanındaki ileri düzeyde ilerlemelerle beraber, bilgisayar destekli doku mühendisliğinin yeni bir alanının gelişimine neden olmuştur.

Bu tezde iki adet açık mimari hızlı prototip üretim sistemi geliştirilmiştir. Bunlardan biri daha önce Erdem Cerit ve arkadaşları tarafından tasarlanmiş ve üretilmiştir. Diğer sistem ise bu çalışmada geliştirilmiştir. Bu donanımların her ikisi de Koç Üniversitesi'ndeki Üretim ve Otomasyon Araştırma Merkezi'nde (MARC) geliştirilmiştir. Her iki system de aslında bilgisayar yardımıyla hassas ayarı yapılabilen 3 eksenli bir makinedir. Bunlardan biri sistemi harekete geçirmek için üç servo motor ve bir adım motoru, diğeri ise dört adım motoru kullanmaktadır. Sistem açık mimari olduğundan, kurum içi geliştirilmiş çeşitli araç yörüngesi oluşumu algoritmaları uygulanabilir. Bu çalışmada birkaç algoritma geliştirilmiş ve her iki sisteme de uygulanmıştır. Geliştirilen programlar CAD ve CAM yazılımları olmaksızın bazı parçaların üretimine olanak vermektedir. Önceki sistem algoritmaları boş küre ve üç boyutlu doku iskeletlerinin üretimine olanak verirken, diğer sisteme uygulanan diğer algoritmalar ise eşitkenar dörtgen gibi temel geometrilerin yanında farklı boyutlarda doku iskeletlerinin üretimine de olanak tanımaktadır. Geliştirilmiş yazılım, veri girişini ve oluşturulan elektronik sinyalleri çalışması için motorlara yönlendirir. Son olarak, üretilen bu doku iskeletleri istenilen fonksiyonlarını yerini getirip getirmediğini görmek için laboratu-

arda hücre kültürüne bırakılmıştır. Hücrelerin yaşayabirliği, proliferasyonu ve yapay iskeletinin toksikliği irdelendi.

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NOMENCLATURE

ABS	Acrylonitrile Butadiene Styrene
AM	Additive Manufacturing
RP	Rapid Prototyping
CAD	Computer Aided Design
CAM	Computer Aided Manufacturing
CATE	Computer Aided Tissue Engineering
CLSF	Cutter Location Source File
CNC	Computer Numerical Controlled
DCM	Dichloromethane
ECM	Extracellular Matrix
H&E	Hematoxylin and Eosin
MARC	Manufacturing and Automation Research Center
NIH3T3	Mouse Embryonic Fibroblast Cell Line
OARP	Open Architecture Rapid Prototyping
PBS	Phosphate Buffered Saline
PCL	Polycaprolactone
RPBOD	Rapid Prototyping Robot Dispensing
SFF	Solid Freeform Fabrication
SLA	Stereolithography
SLS	Selective Laser Sintering
STL	Standard Triangulation Language or Stereolithography (File format)
UV	Ultraviolet

Chapter 1

Introduction

Tissue engineering is a field of medicine through which damaged or missing tissue is replaced or facilitated in its regeneration. The long-term applications of tissue engineering include complete organ replacement without the logistic and burden of transplantation, such as being forced to find a matching tissue donor. It is hoped that tissue engineering will provide alternatives to existing clinical treatments and solve problems such as transplant organ shortages, chronic rejection, and cell morbidity [1].

To achieve these goals, tissue engineering researchers are working towards developing techniques in building tissue constructs of greater size and complexity than are currently possible. To grow an entire, functional 3D organ in vitro, it will be necessary to establish appropriate environment for both cell growth and long-term behavior, a vascular network within that environment to provide a steady stream of nutrients to the growing cells, and a structure capable of maintaining shape and withstanding biological forces without negatively affecting cellular processes [2]. As of yet, no single biomaterial has proven to be able to perform all of these functions.

The seeding of cells on a 3D substrate is the foundation for the scaffold-guided strategy of tissue engineering, currently the dominant approach in the field today. 3D scaffold is constructed to the desired shape and material specifications for the tissue engineering therapy. The cells are seeded upon the scaffold, and the tissue is allowed to culture in conditions favorable for cell growth. The cells grow into tissue upon the scaffold, which may or may not degrade in the process, and the new tissue construct is implanted into the patient. Because the cells belong to the patient, there is a reduced chance of rejection [3].

In this study, two algorithms were developed and implemented in the first generation open architecture rapid prototyping (RP) system. Algorithms enable to manufacture hollow spheres and grid like structures called scaffold without using any CAD or CAM software. Also main electronic wiring part between microcontroller and servo drivers was replaced with a simpler system. Developed RP system in the literature is called as rapid prototyping robot dispensing system (RPBOD); it consists of a moving X-Y table carrying the prototype and a deposition sub-system attached to a ball-screw mechanism driven by a servo motor which provides the motion in Z direction. Actually this system was designed and manufactured in a previous work [4]. Developed algorithms were implemented and different parts were manufactured with the machine. Main aim of the algorithms was to develop a design and fabrication approach for man-made tissue substitutes/scaffolds using open architecture rapid prototyping (OARP) system. As a preliminary study on implementing the algorithms in the system silicone (polysiloxane) was used as a production material. After different parts were successfully produced by using silicone, it was decided to use polymeric solution for producing bioactive scaffolds. Polycaprolactone (PCL) was selected as the polymer and dichloromethane (DCM) was chosen as a solvent for the fabrication process. Solution of PCL and DCM is capable of being deposited by the system at room temperature. However, because of the experienced problems which are discussed in Section 4.1., it was decided to develop second generation OARP system.

Second generation OARP system was designed and manufactured. This new system is lightweight, compact 3 axis machine which has modular deposition system. Deposition system enables to use different size syringes for different applications. Actually second generation system works by extruding a material onto a table through a syringe which is mounted on a motorised X and Y axis. The table can move down in the Z direction to allow for each layer that is created. Two similar algorithms were developed to manufacture different size quadrilaterals and scaffolds on the system. Those algorithms design parts without using any CAD/CAM software. Designed algorithms were implemented in the system and different parts were manufactured. Only silicone was used as a production material because of lack of time to tune the system to use other materials such as polymer solution used by first generation system.

After polymeric scaffolds were manufactured by first generation OARP system they were sterilized and cell cultured to test biocompatibility, cell proliferation and cell attachment. Thus it could be determined whether those scaffolds could be used in biological applications. In this thesis, developed RP systems and its recent outputs are introduced.

Chapter 2

Literature Review

2.1. Computer Aided Tissue Engineering

Every year just in the US nearly a million surgical procedures are performed and the need for organ and tissue substitutes to repair or replace damaged or diseased organs increases year by year [5]. The use of substitutes for damaged or diseased tissue is often necessary depending on the loss of bone tissue due to the different reasons. Nowadays the most commonly implanted materials in the surgeries which are conducted countrywide are bone substitutes, including auto grafts, allograft, and synthetic materials. Nevertheless, these substitutes are not very close to the ideal and have some problems like non-availability of donor site tissue, implant subsistence and failure, immune rejection and fixation problems. Beside that repeated surgeries are necessary due to the fact that the non-living synthetic materials in the body does not grow or adapt to the changing environments that the patient undergoes. This situation exposes an active research area which is known as "Tissue Engineering" according to the need and demand for living substitutes that can help grow the patient's own functional tissue [2].

The application of principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve tissue function is known as Tissue Engineering [6].The fact that sample cells can be cultured ex vivo, established within the scaffold in an environment appropriate for cell and tissue growth, and that the new tissue/organ can finally be implanted to restore the patient tissue function, is a major hypothesis for tissue engineering. The major component of the tissue engineering concept is to regenerate

the functional tissue or organ structure which requires a scaffold to lead the overall shape and the three dimensional growth of diverse cell types [7]. Hitherto, growing human skin [8], human urinary bladders [9], non load bearing cartilage such as the ear [10], and to a limited extent in bone tissue substitutes have been achieved by researchers [11], [12]. Growing human tissue and organ such as the bone, liver and heart valves are hoped to be available for clinical applications in the next several decades.

The unavailability of the automation and computer integrated fabrication has limited the capability of the scaffold architecture during the early years of research on tissue scaffold topic. Methods such as salt leaching [13], gas foaming [14], fiber bonding [15] which are chemical based were used in the fabrication of the scaffolds which cause random pore generation and distribution. By arranging the chemical fabrication parameters only limited architectural control was achieved. Thus, repeatable structures could not be guaranteed and it was meaningless for a structured characterization or systematic biological study process. Since 2000, chemical based techniques made way to alternate methods of fabrication that had the capability to be integrated with CAD/CAM technologies [16], [17], [18], [19]. As a result the fabrication of the scaffolds by using CAD software and rapid prototyping machines became possible. This process brings advantages such as its ability to produce reproducible structures for biological and clinical study, process capability in producing micron-size features, the ability to develop computational and simulation algorithms that could predict the effective mechanical and transport properties and the availability of a large number of candidate biomaterials that could be used as the scaffolding material [20], [1]. The current preferred approach among bone tissue engineering researchers is graphically displayed in Figure 2.1 [20]. Basically, to obtain the sliced 2D data of the defect site, non-invasive image acquisition technologies are utilized and reconstructed into a 3D model. The 3D model of the defect site is used to aid in the design of a 3D scaffold that fits within the defect site. Then for fabrication the model is transmitted to the available rapid prototyping system. The scaffold is filled with the patient's own cells, growth factors, collagen and nutrients, grown in an incubator for a period of time and is then implanted into the defect site of the patient. After healing, the scaffold degrades away leaving behind the patient's own regenerated tissue. "Design of the scaffold" is

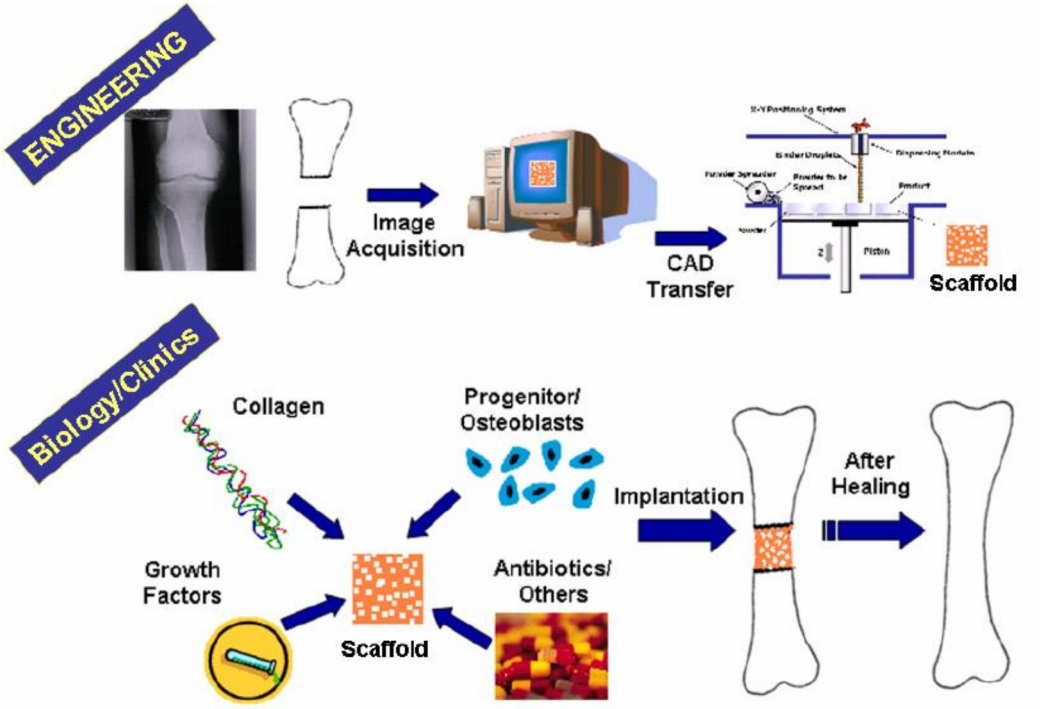


Figure 2.1: Using CAD/CAM Technologies in Tissue Engineering [20]

the main component in this approach that must meet multiple mechanical, biophysical, biological requirements before this approach could be made viable.

Scaffolds with desired biological, mechanical and transport properties can be achieved by controlling the scaffold internal pore architecture. In the same manner, matching with the geometrical fitting qualities at the defect site can be achieved by controlling the external architecture. As a consequence, in order to function as a true substitute, any man made substitute must at best mimic the natural tissue properties by providing an immediate mechanical fixation and support upon implantation, particularly for load bearing substitutes such as bone.

It is not easy to design scaffolds in current enabling CAD software. Design of mechanical components is allowed by CAD software's capabilities based on conceptual designs that are created by engineers and scientists. As a result of strict design rules defined by nature during the evolution over a million of years tissue scaffold substitutes are not man-made. A single design of a tissue scaffold could potentially encompass multi-scale features ranging from the nano-scale all the way up to the meso-scale level. For the design and fabrication the development of biomodeling process paths,

specialized computational methods and process planning algorithms are necessary for inclusion of a hierarchical multi-scale structure of features within a scaffold. Scientific community argues about how much of these natural nano and micro features must be mimicked within a man-made substitute. The process capability to reproduce and replicate multi-scale features for the success of scaffold guided tissue engineering is important since that features whether it be at the nano-scale level or at the meso-scale will influence cellular behaviour.

Recent advances in computing technologies both in terms of hardware and software have helped in the advancement of CAD in applications such as design and analysis. Nowadays in many applications such as clinical medicine, customized medical implant design to tissue engineering, CAD is commonly used. The developments made in imaging technologies and reverse engineering techniques supported equally by both hardware and software technology advancements made it possible. A new field of Computer-Aided Tissue Engineering (CATE) developed due to the usage of computer-aided technologies in tissue engineering which integrates advances in Biology, Biomedical Engineering, and Information Technology to Tissue Engineering application. The application of enabling computer-aided technologies, including computer-aided design (CAD), image processing, computer-aided manufacturing (CAM), and rapid prototyping (RP) and/or solid freeform fabrication (SFF) for modeling, designing, simulation, and manufacturing of biological tissue and organ substitutes can be defined as CATE.

2.2. Fabrication Technologies in Tissue Engineering

It was recognized that in the early stages of incubation or implantation, scaffolds require adequate mechanical strength to support and maintain the porous matrix that is required for the culturing cells. The mechanical stiffness of the scaffold should be in the range of the implant area [21]. A fabrication method which provides high level accuracy is needed to keep the consistency and repeatability for the fabrication of the scaffolds with specific features. There are several conventional techniques introduced in the recent years for scaffold fabrication such as fiber bonding, solvent casting, and particulate leaching, melt molding, gas foaming, and soft lithography [22]. The restric-

tions on the consistency of internal architecture and shape control of the designed tissue scaffolds are the main disadvantages of these techniques. Solid freeform fabrication (SFF) has no limitation to shape, control and consistency dissimilar to the conventional fabrication techniques [23]. SFF systems are directly linked to CAD software and this software simplifies the design of the scaffold in a virtual environment after the transfer of the CAD model to the systems for fabrication. The SFF system is different from the traditional machining in the way that it builds parts by selectively adding material as specified by the CAD model. Figure 2.2 shows the process by which a scaffold designed in CAD software is converted to machine instructions for part buildup [24].

To fabricate prototypes from CAD designed parts, SFF technology has been primarily used in the rapid prototyping industry since the 1990's [2]. Many different methods have been originated by the companies and the universities in the short history of RP technology [25], [26]. Many of the methods had limitations and could not become popular, some of the methods disappeared and only few of them could be commercialized. Fused deposition modeling, Stereolithography, Selective laser Sintering, Ballistic Particle Manufacturing, 3- dimensional printing, Laminated object manufacturing and Rapid prototyping robotic dispensing system are the most popular methods which are globally accepted to be successful. Various books or review papers which give detailed information on the subject have been published [25], [27]. Rapid prototyping technology made it possible for conceptual designs to be fabricated rapidly, thus design changes early in the product development period became possible. Nevertheless for the last 6 years these technologies have been used by researchers in the production of scaffolds for tissue engineering.

Obtaining precise control over material distribution, high level of design capability in the model's internal structure and the ability to fabricate highly reproducible scaffolds with a variety of composite biomaterials are three main abilities which lead to the system's extensive use in tissue engineering applications. In other words the SFF process has become the most preferred method for the fabrication of engineered scaffolds/constructs thanks to the reasons mentioned above.

3-D printing [22], fused deposition [19] and the Micro-nozzle based extrusion [17] systems are the active research areas for the freeform fabrication of tissue scaffolds

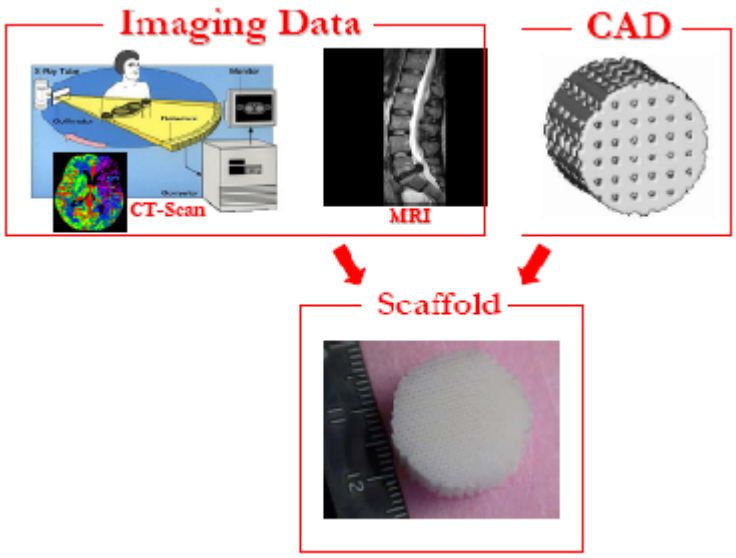


Figure 2.2: Design of a scaffold using CAD [24]

throughout the contemporary SFF methods. Also there are some other methods such as Stereolithography and Selective Laser Sintering, which are used in Tissue Engineering. However since Stereolithography (SLA) [28] and Selective Laser Sintering (SLS) [29] methods are incapable of providing bio-friendly fabrication environments and lack of candidate bio-materials, they became unpopular among researchers.

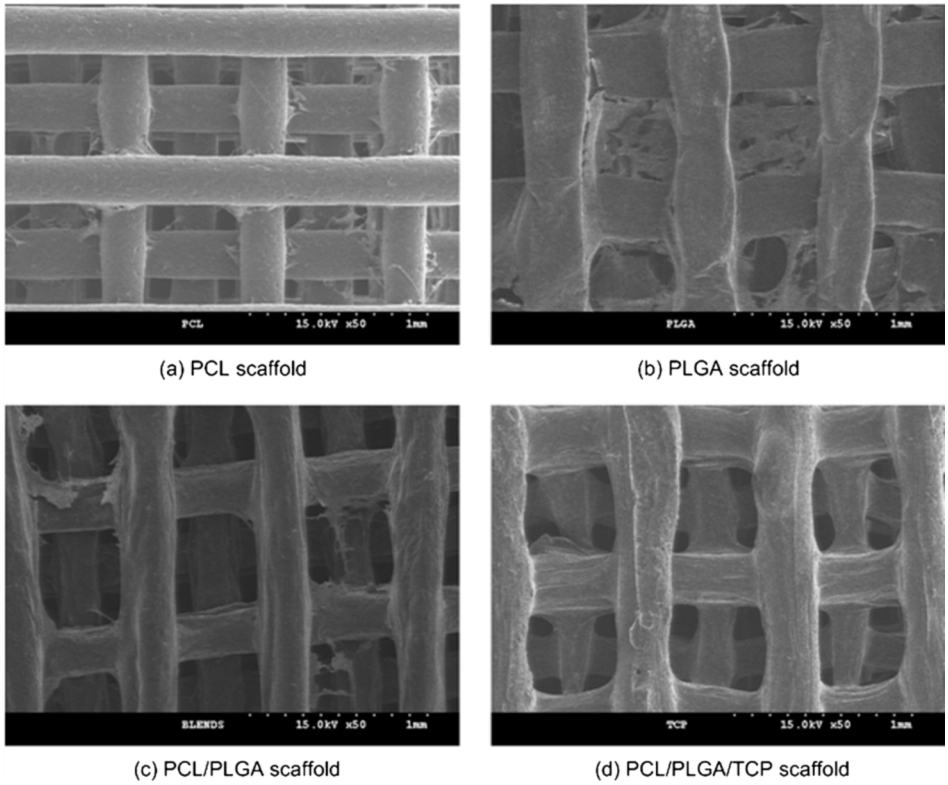
Specific requirements of scaffold properties should be satisfied for in vitro regeneration of human tissue. The physiological functions of the native cellular matrix need to be mimicked by a three dimensional scaffold [28]. An ideal scaffold design should increase the growth of cells and proliferation so that it replaces the degrading scaffold. It has been globally accepted that a biodegradable biomaterial that does not cause inflammatory reactions should be used in the production of tissue scaffolds [29]. Due to the fact that many of the polymers are biocompatible and can be processed uniquely, they have been preferred as biomaterials for tissue engineering applications. It is very common to use matrix-producing connective tissue cells, namely osteocytes and chondrocytes, to successfully regenerate bone tissues [30]. Scaffolds produced from natural or synthetic materials are used as temporary native extracellular matrix. The special three-dimensional cell distribution of complicated organs like livers, hearts, and neural tissue, leads to difficulties in tissue engineering, thus, the scaffolds are to be manufactured with specific pore sizes and internal architecture to allow and adapt to proliferation of cells. Since computer-controlled techniques known as SFF or rapid prototyping

(RP) have unique advantages over traditional methods, they have been used to cope with the fabrication of such complicated designs. Tissue scaffolds can be used in either of the two ways: to promote natural regeneration, in vivo, or to create a bioartificial organ, in vitro. The makeup of the extra-cellular matrix (ECM), pore size, biodegradability, cell adhesion, growth factors, immunological response, cytotoxicity, and the required vasculature of the tissue and scaffold are the essential parameters in the design and manufacturing of the scaffold [31]. There are two main approaches conducted in the development of the scaffolds. The scaffold needs to supply support in vivo in the first approach, whereas in the second, it supplies support in vitro until the cells are capable of supporting themselves in vivo [32].

Kim et al. [33] studied adhesion of human bone marrow stromal cells and proliferation characteristics of different scaffolds fabricated using multi-head deposition system. Moreover effects of polymer and ceramic on a tissue scaffold were studied via mechanical testing and cell interaction analysis of scaffolds fabricated using polycaprolactone, poly-lactic-co-glycolic acid and tri-calcium phosphate. Figure 2.3 illustrate the SEM image of the proliferated cells and extracellular matrix (ECM) on different scaffolds.

Hyung Kim and Son [34] introduced a new 3D plotting system with integrated piezoelectric vibration system to produce a surface-modified 3D scaffold which was inscribed with nano/microsized wavy shapes. Vibration system was implemented to increase the surface roughness of the scaffold without any additional chemical process. Actually the surface of the scaffolds fabricated without designed system was too smooth for initial required cell attachment. It was observed by cell culturing results that the interactions between chondrocytes and the PCL scaffold were much more favorable than those between the cells and conventionally plotted 3D scaffolds.

Woodfield et al. [35] present and characterize a fiber deposition technique for producing three-dimensional poly (ethylene glycol) - terephthalate - poly (butylene terephthalate) (PEGT/PBT) block co-polymer scaffolds for engineering of articular cartilage. Fiber deposition device mainly have fused deposition modeling (FDM) type deposition system with computer controlled X, Y, Z table. 3D-deposited scaffolds were produced with a range of mechanical properties by changing PEGT/PBT composition,



(a) PCL scaffold (b) PLGA scaffold

(c) PCL/PLGA scaffold (d) PCL/PLGA/TCP scaffold

Figure 2.3: An SEM images of proliferated cells [33]

porosity and pore geometry. 3D-deposited scaffolds were seeded in vitro and in vivo with bovine and human articular chondrocytes. Results showed homogeneous cell distribution with subsequent cartilage-like tissue formation. This was demonstrated by the presence of articular cartilage extra cellular matrix constituents (glycosaminoglycan and type II collagen) throughout the interconnected pore volume.

Hutmacher et al. [36] developed computer-controlled method to manufacture three-dimensional polycaprolactone scaffolds using commercial 3 axis FDM system. Also mechanical properties and in vitro biocompatibility of polycaprolactone scaffolds with two matrix architectures were studied. Scaffolds with different honeycomb - like pattern of triangular and polygonal pores were studied (Figure 2.4). In vitro studies were conducted with primary human fibroblasts and periosteal cells. As a result of cell culture study it was observed that fibroblasts and osteoblast-like cells can proliferate, differentiate, and produce a cellular tissue in an entirely interconnected 3D polycaprolactone matrix.

Heo et al. [37] manufactured polycaprolactone scaffolds using 3D robotic system with addition of nano- or micro-sized hydroxyapatite particles and to investigate the ef-

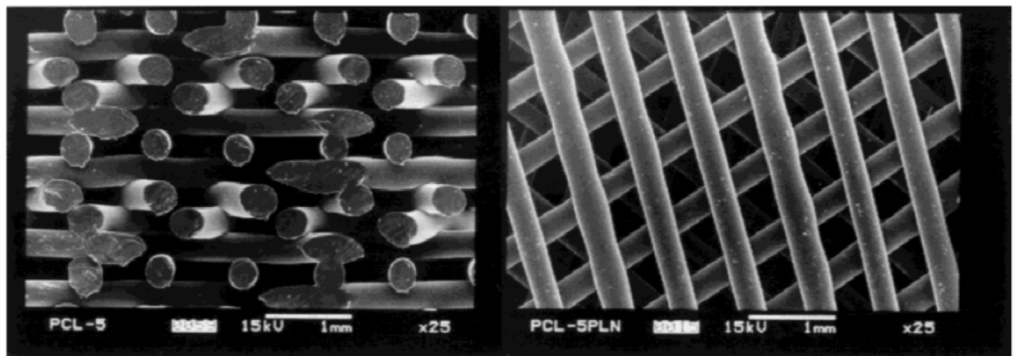


Figure 2.4: Fabricated scaffolds having honeycomb pattern with polygonal pores [36]

fects of particle size in vitro. Moreover, the superiority of layer manufacturing process (LMP) over the conventional scaffolds made by salt leaching and gas forming process (SGP) was investigated through animal study (Figure 2.5). Cellular responses to the two kinds of scaffolds were compared. Both n-HPC and m-HPC exhibited good in vitro biocompatibility. It was observed that produced n-HA/PCL composite scaffolds showed better mechanical properties, in vitro bioactivity, and cell and bone tissue responses compared with the scaffold produced by the conventional method.

Woodard et al. [38] studied relative osteoconductivity and the change in the mechanical properties of hydroxyapatite (HA) scaffolds. Scaffolds were manufactured using robocasting, a solid freeform fabrication technique based on the robotic deposition of colloidal pastes. Non-microporous scaffolds contained only macroporosity (250 - 350 μm) and microporous scaffolds contained both macroporosity and microporosity (2 - 8 μm) were compared. Recombinant human bone morphogenetic protein - 2 (rhBMP - 2) was incorporated into all scaffolds via gelatin microspheres prior to implantation into the latissimus dorsi muscle of Yorkshire pigs. Implanted and as-fabricated scaffolds were compared using histology, microcomputed tomography, scanning electron microscopy, and compression testing. It was observed that implanted scaffolds had a stress - strain response similar to that of cancellous bone. Finally, the importance of scaffold microporosity on bone ingrowth and on the mechanical behavior of HA implant materials was discussed.

Oh et al. [39] fabricated Polycaprolactone (PCL) (by the addition of Pluronic F127) cylindrical scaffolds with gradually increasing pore size along the longitudinal direction using a novel centrifugation method to investigate pore size effect on cell and tissue interactions. In vitro cell interactions were observed using different kinds of

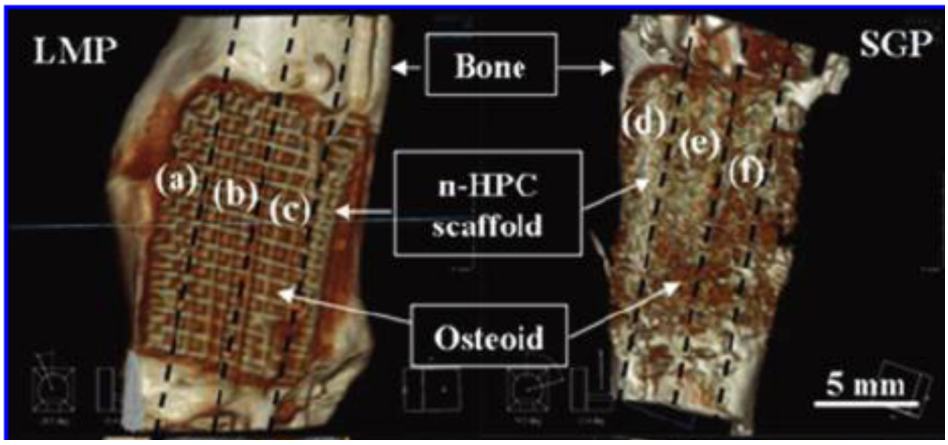


Figure 2.5: Micro-CT images of scaffolds after 4 weeks [37]

cells (chondrocytes, osteoblasts, and fibroblasts) and in vivo tissue interactions using a rabbit model (skull bone defects). It was observed that different kinds of cells and bone tissue were shown to have different pore size ranges in the scaffold for effective cell growth and tissue regeneration: 380 - 405 μm pore size for chondrocytes and osteoblasts and 186 - 200 μm pore size for fibroblasts growth. Figure 2.6 shows the surface morphologies of the selected PCL cylindrical scaffold sections.

Xu et al. [40] manufactured composite scaffolds of pearl/poly(lactic - co - glycolic acid) (pearl/PLGA) using the low - temperature deposition manufacturing (LDM). In fact LDM allows fabricating scaffolds at low temperature and keeping the bioactivity of biomaterials with designed microstructure and macrostructure. SEM, WST - 1 assay, alkaline phosphatase activity assay, immunofluorescence staining and real - time reverse transcription polymerase chain reaction were used to determine adhesion, proliferation and differentiation of marrow stem cells into osteoblasts. Some of the fabricated scaffolds can be seen on Figure 2.7.

Lee et al. [41] successfully fabricated 3D scaffolds using micro - stereolithography. As a scaffolds matetial poly(propylene fumarate) and diethyl fumarate was used. Produced scaffolds had very clear and regular shapes of the solidified lines and pores (Figure 2.8). Fibroblasts cell line were cultured in vitro on the scaffolds to observe cell adhesion and biocompatibility. Cells were fixed after 4 days, 1 week, and 4 weeks of culture and observed under SEM. Cell attachment and proliferation was observed under scanning electron microscope.

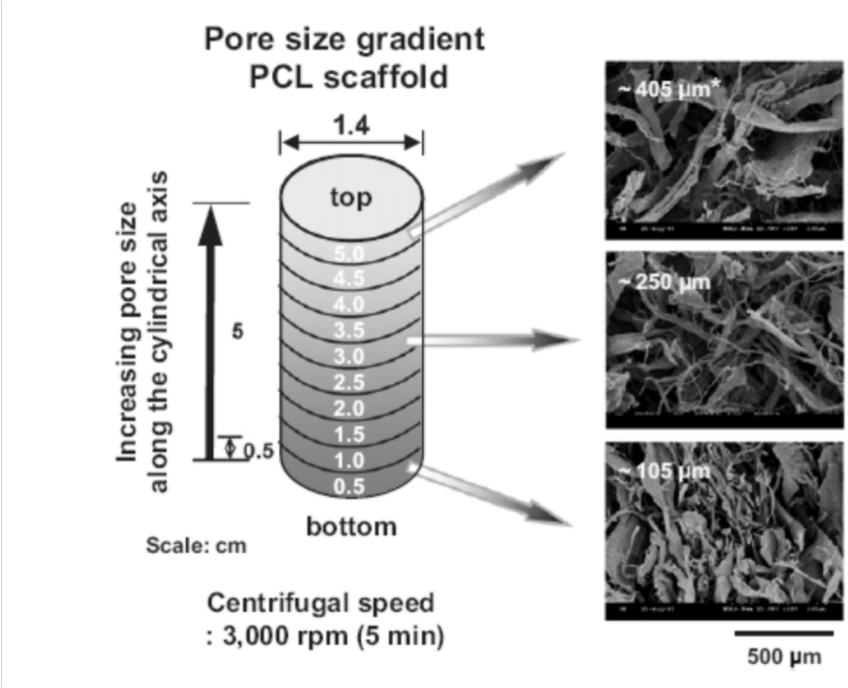


Figure 2.6: SEM photographs of scaffold sections along the longitudinal direction [39]

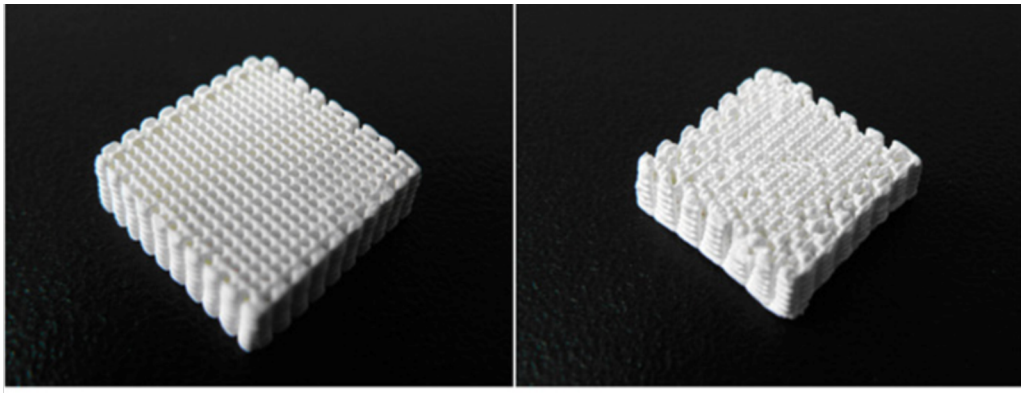


Figure 2.7: Pearl/PLGA composite scaffolds [40]

Kanczler et al. [42] fabricated scaffolds using surface selective laser sintering (SSLS) and observed their biocompatibility as templates, in vitro and in vivo, for human fetal femur - derived cell viability, growth and osteogenesis. Fetal femur derived cells were successfully cultured on SSLS -poly(D,L)- lactic acid (SSLS - PLA) scaffolds expressing alkaline phosphatase activity after 7 days. Cell proliferation, ingrowth, Alcian blue/Sirius red and type I collagen positive staining of matrix deposition were observed for fetal femur - derived cells cultured on SSLS - PLA scaffolds in vitro and in vivo. It was shown that SSLS techniques allow fabrication of biocom-

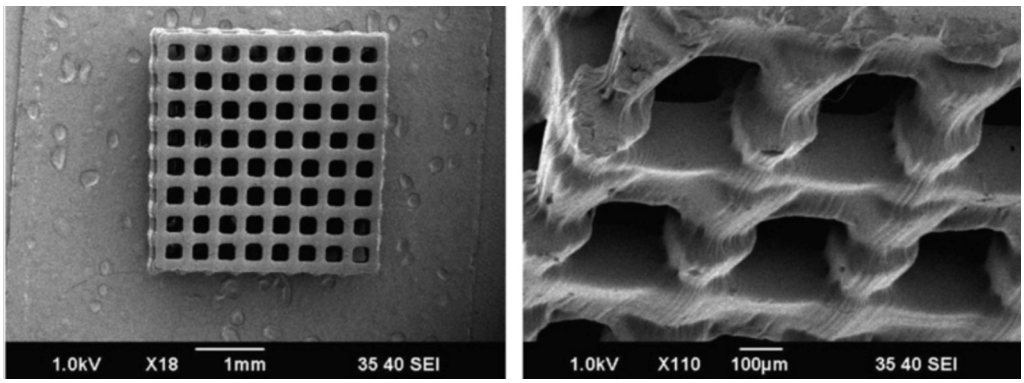


Figure 2.8: Fabricated scaffolds using micro - SLA [41]

patible/biodegradable scaffolds, computationally designed to fit any defect, providing a template for cell osteogenesis in vitro and in vivo.

Park et al. [43] fabricated polymeric three - dimensional (3D) structures characterized by nano and microfibers for use as an extracellular matrix-like tissue engineering scaffold. Figure 2.9 shows the structure of the hybrid scaffold with its unit layers containing the nanofiber matrix. While producing scaffolds a hybrid process using direct polymer melt deposition and an electrospinning method were employed. As a material in microfibers and nanofibers PCL and PCL/collagen diluted in 1,1,1,3,3,3 - hexafluoro - 2 - propanol (HFIP) was used respectively. To evaluate the levels of cell adhesion and proliferation of the fabricated scaffolds, chondrocytes were seeded and cultured within the developed scaffolds. It was observed that the polymeric scaffolds with nanofiber matrices fabricated using the proposed hybrid process provided favorable conditions for cell adhesion and proliferation.

Yang et al [44] developed technique to produce tissue scaffolds (Figure 2.10) with a uniform and well - defined structure. Photolithography was used to design and pattern a planar scaffold skeletal structure on a photoresist (SU - 8), and variety of microembossing processes were developed to transfer the skeletal pattern to the poly(DL - lactide - co - glycolide) substrate as scaffold skeletons. Subcritical carbon dioxide bonding technique was successfully used in assembly of 3D polymer scaffolds at low temperatures. Moreover, no organic solvent was involved throughout the whole process from pattern design to the final 3D scaffold. It was experienced that the scaffolds were cytocompatible and could provide a unique platform for studying scaffold effects on cell morphology and tissue development.

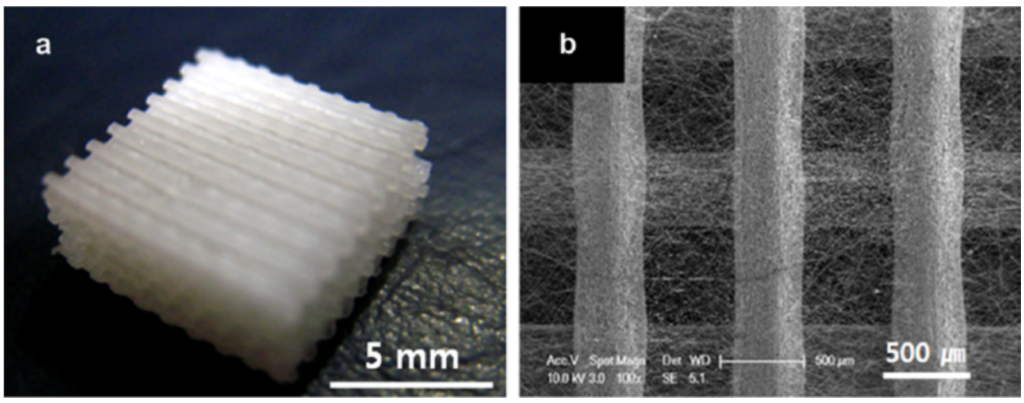


Figure 2.9: (a) Photograph of the overall scaffold; (b) Magnified images of hybrid scaffold [43]

Figure 2.10: Photograph of fabricated scaffold [44]

Moroni et al. [45] successfully fabricated electrospun (ESP) scaffolds of poly(ethylene oxide terephthalate) - poly(butylene terephthalate) (PEOT/PBT) copolymers (Figure 2.11). It was shown that depending on the solvent used and ESP paramaters, wide range of porous and nonporous fibers could be obtained. A set of ESP scaffolds with specific diameter and surface topography was selected to study their influence on cell attachment and activity. The fabrication of ESP scaffolds with incorporated dyes with different molecular dimensions was also reported and their release measured. Finally, it was observed that after 14 days, the scaffolds were almost fully covered with cells and penetrated inside the fibril network.

Sanchez- Salcedo et al. [46] have developed design of porous scaffolds by combining a low temperature shaping method with stereolithography (Figure 2.12). Firstly, using ceramic/agarose suspension hydroxyapatite/ β - tricalcium phosphate porous scaffolds have been manufactured. Then polymeric negative, obtained by stereolithog-

raphy technique, was filled with this suspension. After that, using alkaline solution at room temperature polymeric negative was eliminated. Secondly, scaffolds were dried with two different methods, using freeze-drying techniques and air dried at room temperature. Also, it has been shown that different porosity could be achieved depending on the drying technique employed. Finally, it was shown that scaffolds perfectly fit in the bone defect of the patient and could be easily utilized by the surgeon.

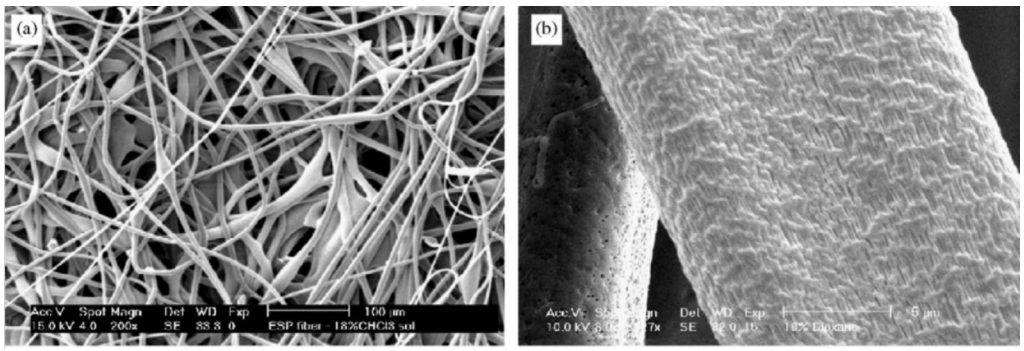


Figure 2.11: SEM photographs of ESP scaffolds (a) General view; (b) Magnified picture [45]

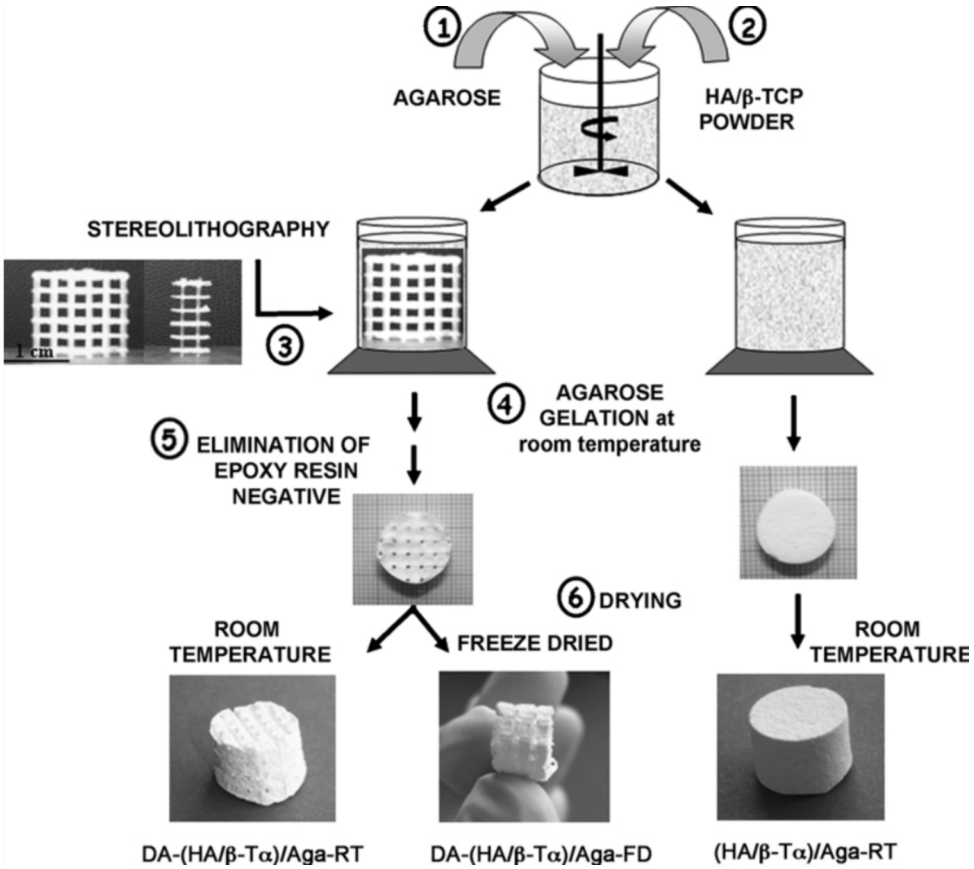


Figure 2.12: Shaping method of scaffolds [46]

Chapter 3

First Generation OARP System

3.1. Introduction

First generation open architecture rapid prototyping (OARP) system including its hardware and software was developed at Koc University in Manufacturing and Automation Research Center in previous work. The hardware includes a 3-axis machine that is precisely controlled by a PC via a servo system. Since the system is open architecture, various tool path generation algorithms developed in-house can be implemented. Developed system consists of a moving X-Y table carrying the prototype and a deposition sub-system attached to a ball-screw mechanism driven by a servo motor which provides the motion in Z direction General view of a system is given below on Figure 3.1. More detailed information about this system can be found in the Master Thesis written by Erdem Cerit [4].

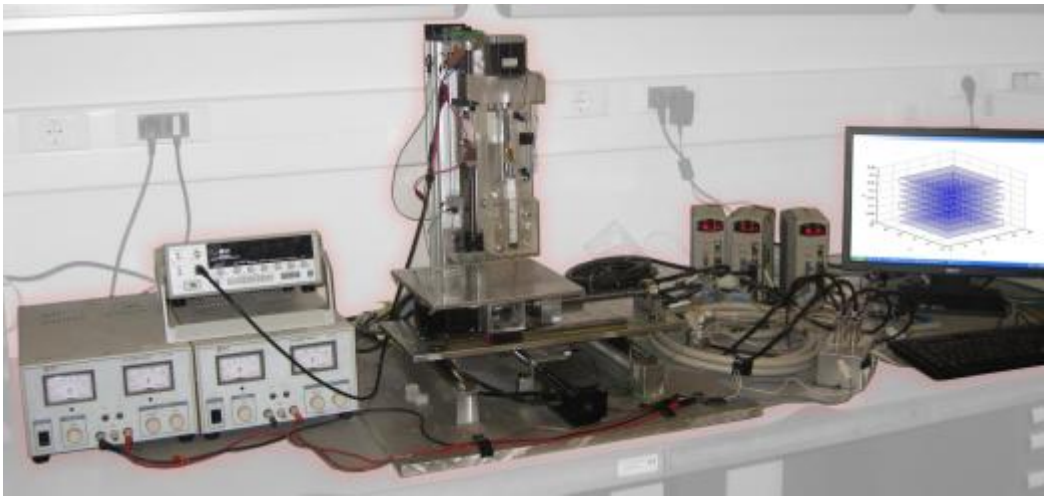
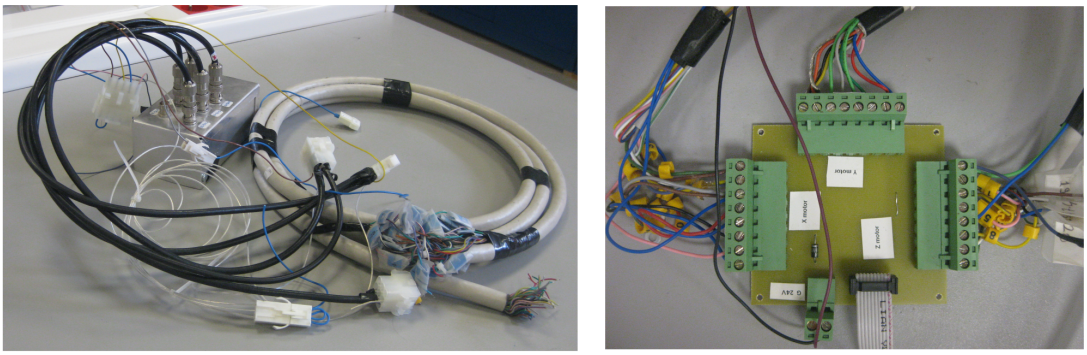


Figure 3.1: A general view of a previous OARP system

3.2. Data and Signal Processing

Micro controller was connected to the drivers of the motors over an isolated BNC connection box. However electrical wiring was complicated and useless thus it was decided to change connection by fabricating a new card as shown on Figure 3.2. Details about wiring are given in Appendix 1. Board was successfully implemented in the system. Decoded data from the microcontroller are sent successively via new board to the drivers and motors are simultaneously driven.



BEFORE AFTER

Figure 3.2: A new electrical wiring

Besides the micro controller and RS232 connection, the data can be sent over a controller board. In previous study [4], a controller board of dSPACE Inc.'s 1104 R& D controller board was utilized successfully in the developed system.

The stepper motor used in the dispensing system is driven from a function generator. In fact, the stepper motor can also be driven by creating a sixth pulse array in the program, and sending it over RS232 port and micro controller. However, in the laboratory, the tip diameter of the dispensing system is changed according to the research and the part that is produced. Depending on required diameter of fiber extruded from syringe different diameter needles have been used so far. Needles (Hayat Inc.) ranging in internal diameter from 910 microns to 240 microns were used so far. For different tip diameters, the stepper motor should be driven at different frequencies and for new tip diameters a tuning process must be performed to find the proper frequency. Therefore, it is more convenient to drive the stepper motor from a function generator.

3.3. Software

One of the main purposes of RP technology is to produce the prototype of a newly designed product as fast as possible and check its validity before the mass production, such as checking the dimensions of the part and controlling whether the part fits with other parts or not. It is also known that rapid prototyping techniques are frequently used in tissue engineering in scaffolds fabrication [47]. In previous study [4], three different algorithms have been developed to run developed RP system. MATLAB program has been used to write and run the programs, and to send the created pulses to the system. Algorithms have been written as m-files. All of the algorithms have different capabilities.

First algorithm requires a Stereolithography (STL) file which is a defacto standard data transmission format for Rapid Prototyping. By using this STL file, algorithm creates the path that the deposition head of the machine follows. Second program requires a Cutter Location Source File (CLSF) as input. This file is obtained from the manufacturing module of the Unigraphics NX program and includes the path that cutter of Computer Numerically Controlled (CNC) milling machine follows while machining a part. Third program requires operator input. The operator enters X, Y and Z coordinates of nodes that are on the desired path. That is, this algorithm lets the operator to create his/her own path and make the machine follow this path. The mentioned algorithms are explained in detail by Erdem Cerit in his MS Thesis [4].

Then two more algorithms were developed by improving the third algorithm described above for fabrication on the system. The main improvement is that it is possible to fabricate sphere and grid-like structure by entering only few data. Both algorithms ask operator to enter different dimensions of parts before manufacturing, so there is no need for any CAD or CAM software. First algorithm is used for fabrication of hollow spheres with different dimensions. Second algorithm is used for fabrication of scaffolds for tissue engineering purposes. In the next section the discussed algorithms are explained in detail.

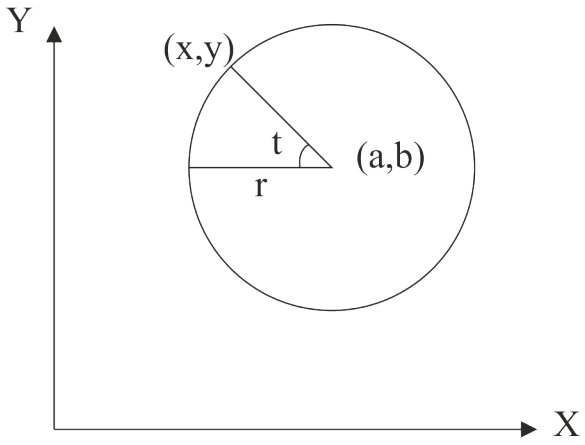


Figure 3.3: Circle of radius r in Cartesian coordinate system

3.4. Algorithm 1

The aim of the first algorithm is to allow the operator to create his/her own hollow spheres without using a CAD program. The first algorithm can be very useful if there is need only for hollow sphere for different applications, such as different types of prototypes.

Operator just needs to enter the values, increment in Z direction and diameter of sphere – in mm- to fabricate the sphere. After values are entered program evaluating coordinates of all points in each layer. And as each layer of a sphere is a circle it is very easy to calculate the coordinates of all points on it. In an $x-y$ Cartesian coordinate system, the circle with center (a,b) and radius r is the set of all points (x,y). The equation for set of points can be written in parametric form using the trigonometric functions sine and cosine as [48]:

$$x = a + r\cos(t) \tag{3.1}$$

$$y = b + r\sin(t) \tag{3.2}$$

Tool path for the system will be consisting of all set points which were evaluated before. So the more consequent points are closer to each other the more curvature of path will be closer to circle.

After coordinates are evaluated algorithm calculates the necessary number of pulses for each direction in each layer and creates pulse arrays for each layer. Finally,

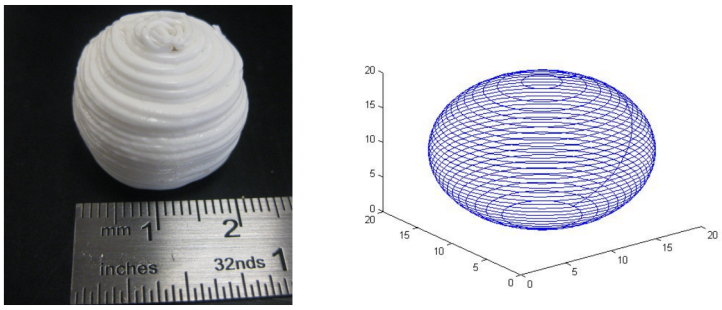


Figure 3.4: Hollow sphere before manufacturing and after

these pulse arrays are combined successively to create part. Manufactured sphere can be seen on Figure 3.4, where on left there is sliced sphere and on the right manufactured part.

3.5. Algorithm 2

Second algorithm is very useful in tissue engineering, because it can manufacture 3-D scaffolds. In this method program requires operator to enter few data about scaffold, they are: distance between struts (increment) and dimensions of a scaffold in X, Y, and Z directions, and a number of duplicated layers. Duplicated layers increase pores and strength of a fibers in X and Y. On Figure 3.5 required parameters are highlighted. After all dimensions are entered algorithm estimates the necessary number of pulses for each direction in each layer and creates pulse arrays for each layer. Also depending on a viscosity of slurry used in a dispensing unit, gear ratio of each servo driver can be adjusted accordingly to have required strut diameter. Finally, these pulse arrays are combined successively to fabricate a scaffold. Scaffold can be seen before and after manufacturing on Figure 3.5.

3.6. Tested and used materials

A wide range of materials were tested on the system to obtain the best result. Developed system has allowed so many materials to be used, because it is an open architecture system that has allowed different deposition systems to be tested. Some thermo plastics such as ABS, starch with different types of glue, silicone, chocolate and polymer clay have already been used before. The most frequent successfully used

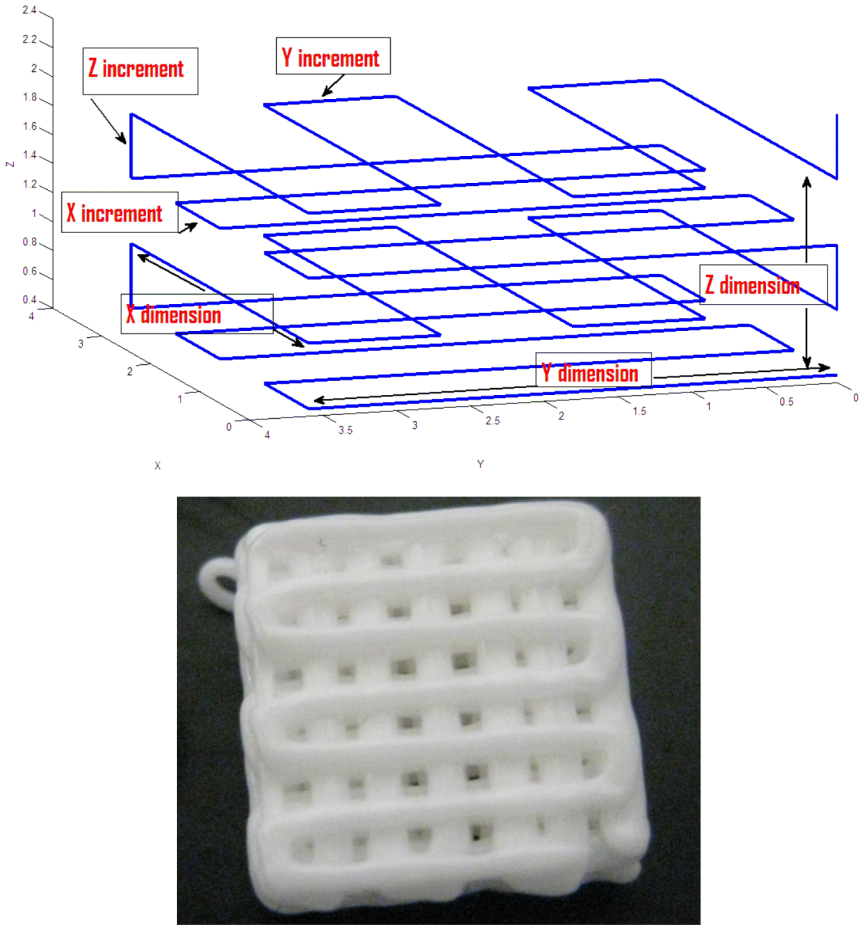


Figure 3.5: Silicon scaffold before and after fabrication

material was silicone, since silicone is very elastic material, and if the produced part is wanted to be rigid, it can be covered Polijel F-213 gelcoat. Also there is no any pre- or post-process needed. The material is filled into the syringe and can be used directly. After the production process, the produced part vulcanizes in a time period between 15 and 20 minutes depending on the size of the part, and it is fully cured in one day. The resultant part is elastic. Since the curing does not occur immediately, during the production, some supporting materials, such as wires, can be embedded into the part. Thus first fabricated parts were manufactured using silicone.

Finally it was decided to use polycaprolactone (PCL) as a synthetic polymer for scaffold fabrication. PCL was chosen because it is one of the most commonly used synthetic polymers in tissue engineering. The material is frequently used as a secondary material for implants and orthotics to increase their rigidity and has been used in several US FDA-approved devices. While it is degradable, and the products of its

breakdown are not toxic, this occurs on the time scale of years, greater than desirable for most wound-healing applications. The breakdown products are either eliminated through the body by the renal system or broken down further by the tricarboxylic acid cycle [49]. The material is an easily workable biologically compatible polymer with significant stiffness and exceptional durability.

3.7. Biocompatible Polymeric Scaffolds

After scaffolds were fabricated using silicone it was decided to fabricate them using other polymeric materials, such as polycaprolactone (PCL). Firstly PCL ($M_n \approx$ 100,000 Dalton) was prepared by Iskender Yilgor in Polymer Research Laboratory at Koc University. Then polymer powder was dissolved in different organic solvents, such as carbon tetrachloride, dichloromethane (DCM). Finally by using appropriate parameters scaffolds were fabricated with required mechanical properties and dimensions. The process is explained in detail in next section.

3.7.1. Preparing of polymer solution

Firstly PCL polymer in powder form was prepared in the polymer laboratory. Then polymer powder was dissolved in DCM and Carbon Tetrachloride. Different solutions containing various weight ratios of solvent and polymer were prepared at room temperature. It was experienced that weight ratio 60/40 of DCM and PCL showed the best result. In fact DCM was chosen because of its low boiling point at 40 $^\circ$C. After that they were put in the oven at temperature 35 $^\circ$C for 12 hours to dissolve the polymer and obtain a homogenous solution.

3.7.2. Fabrication of scaffolds

Produced PCL solution was used in a system for scaffold fabrication after it was prepared. Thereafter gear ratios of servo drivers and frequency of step motor for 27 Gauge needle and 20 mL syringe were tuned up. It was found that gear ratios of X, Y, Z motors and frequency of step motor are 30, 30, 3 and 200 mHz consequently. It is possible to fabricate only 10 layers, because it starts to detach from the stage. Some of the

fabricated scaffolds can be seen on Figure 3.6 and Figure 3.7. Average diameter of a scaffold strut was around 500 microns.

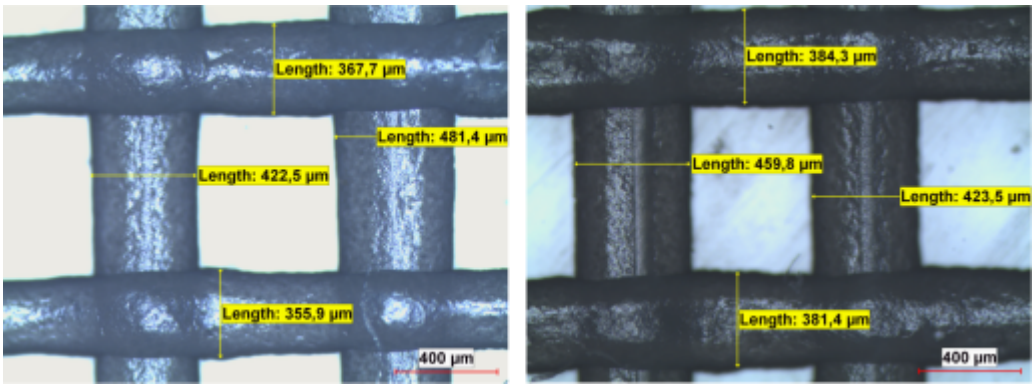


Figure 3.6: Microscope images of manufactured scaffolds.

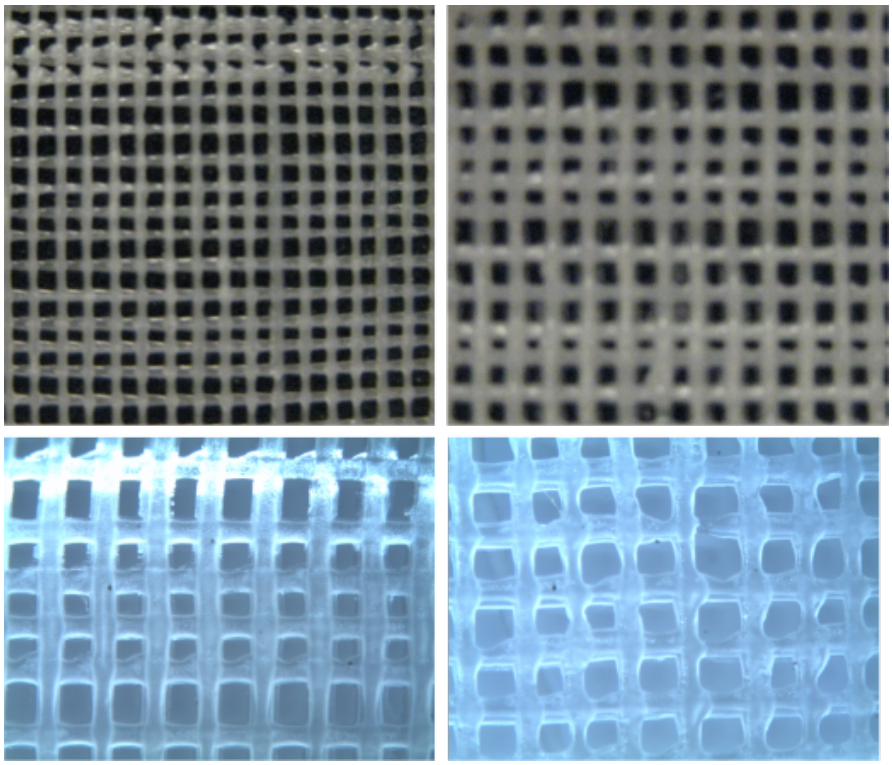


Figure 3.7: Microscope pictures of some produced polymeric scaffolds.

Chapter 4

Development of a Second Generation OARP System

4.1. Introduction

While operating our previous open architecture rapid prototyping (OARP) system we experienced a few drawbacks, such as, the system is heavy, almost immobile, and the deposition system is not modular and does not have back suction. In order to solve these problems we decided to build a new OARP system as it was more reasonable to build a new one than modifying the old one. We call the new system "Si-Dau 10" after the authors of the project, abbreviations for Sinan and Daulet (Figure 4.1). During the designing of our system we took into consideration existing OARP models such as RepRap, Fab@Home and our previous system [50],[47],[51]. By designing a new OARP system we intended to bring a native solid free form rapid prototyping system kit to universities and individuals who are interested in rapid prototyping especially in Turkey. This research developed a compact, lightweight and mobile biofabrication system which has modular deposition system and back suction. Also few algorithms were developed to manufacture some parts on the system. Algorithms allows user to manufacture some simple geometrical parts and grid like structures called as a scaffold. Moreover as system is light weight, biofabrication can be done in different places which have sterile conditions. Furthermore it is practical to have deposition system modular, because depending on applications different size syringes can be used. Finally back suction lets system freeform fabrication of parts having multiple contours.

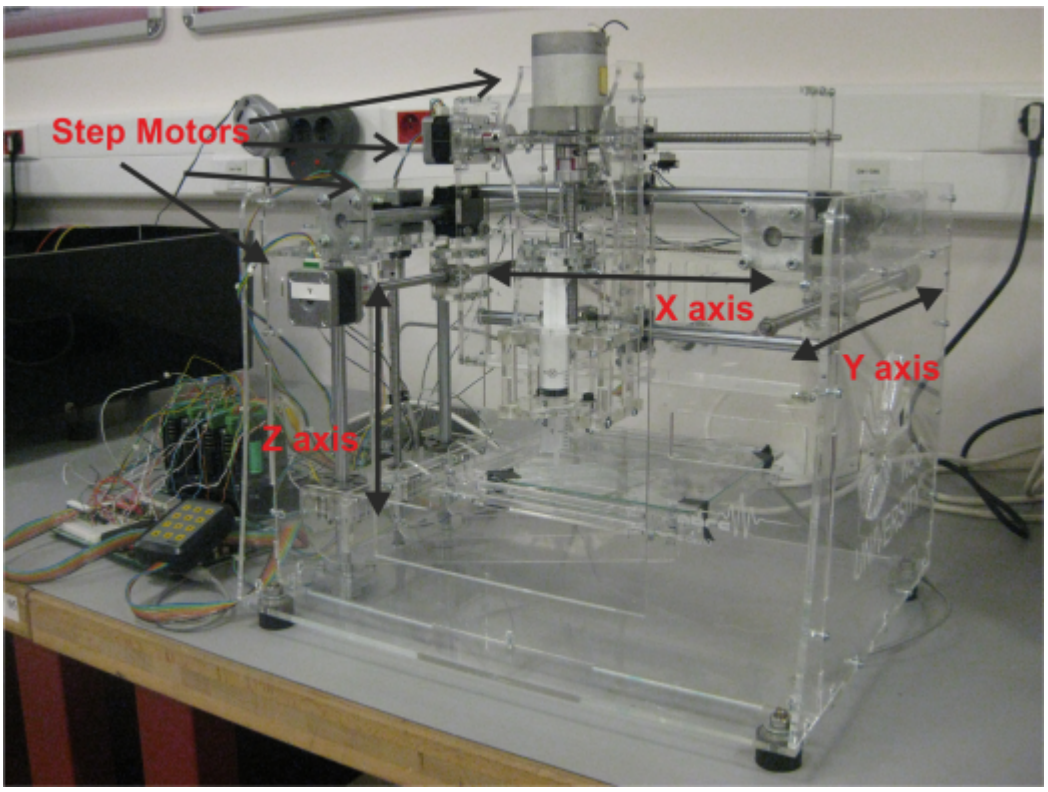


Figure 4.1: Si-Dau 10 OARP System

4.2. Hardware

4.2.1. Design

In the Koc University at Manufacturing and Automation Research Center, two different additive manufacturing (AM) machines were designed. One was designed several years ago and was used to produce silicon based soft materials by using primitive geometries. This system was explained in the previous chapter. While the first designed system was used, some drawbacks appeared such as unportability and weak versatility. Therefore, "Si-Dau 10" (Figure 4.1) was designed. The new system has a 3 axis gantry type structure and is actuated by a step motor-ball screw mechanism. Different feeding systems can be assembled into the system which increases the machine modularity. Algorithms for microcontroller were written in MARC. The controllable numbers of motors are limited by the microcontroller limit. They can be also increased via slave-master microcontroller usage. The communication between microcontroller and computer is fulfilled with a USB. By USB communication, any type of computer can be used to run the software. The designed algorithm takes data from the touchpad

or by directly changing parameters in the software then it calculates positions for the AM machine and sends them to the microcontroller.

Various types of other design studies are investigated. The literature investigation revealed that there are similar structural systems such as RepRap, Fab@home, Cupcake CNC [50],[47],[52]. The disadvantages of these systems are piece breakage during shipping, high resolution of the system which relates to a semi-open architecture software limitation and structure of the machines. For example, by using the software of Fab@home or RepRap the minimum resolution is 10 microns. In fact, this minimum resolution is theoretical due to structural flexibility and the vibration of actuated step motors. Several precautions are taken to avoid these negative effects on resolution.

Other factors to be considered in the design phase of the machine are the availability of machine equipments in the market, production of the parts and easy assembly of the components. In addition, through assembling components, the minimum number of tools and skills are required. This means that the designer should not care about the assembly of the machine, he/she should only take care of the production of his/her design. In order to assemble and use the AM machine, USB connected PC, wrench, soldering machine, some cables, connectors, pliers and side cutters are required. The laser cut acrylic parts are snapped to each other by their geometrical design. Just after snapping the parts to each other, fasteners are used to complete the assembly. The machine is ready to use after connecting the microcontrollers, motor drivers and motors. While preparing the electronic connection and drawing, the system is built based on plug and play logic.

4.2.2. Architecture of a System

Fundamental acrylic components of our system were cut with Epilog Helix 60 W laser engraver (Epilog Inc.). In fact it was easy to find such engraving equipment as this system is frequently used in the sign making, and trophy and gift engraving industries in Turkey. Though there were other options for cutting them, such as manufacture using CNC machine and water jet cutting. CNC manufacturing was not favored regardless of the fact that we have 3 axis and 5 axis CNC machines in our laboratory, for the reasons that it takes a long time and a lot of effort while designing CAM and man-

ufacturing. As a matter of fact most laser-cutting setups unlike CNC machines does not use CAM software instead they use almost any vector based graphics software and image editor such as Corel Draw to create or customize laser cutting and engraving. Moreover as water jet cutting is not very appropriate for cutting detailed brittle materials like acrylic, it was not preferred. Firstly whole system was designed in Solid Works (Figure 4.2). Afterward 63 acrylic items were cut by using drawing files from Solid Works. Eventually after all parts were built and all other items were bought we started to assemble the system. Acrylic parts were fastened by using snap-fit mounting and basic T-nut style screw/nut fastening. In T-nut style there is opening in one part for hex nut and threaded or normal hole for the screw in the part that is at right angle to previous part. T-nut style can be seen on Figure 4.3. Actually tolerances accomplished by laser cutting facilitated assembly of the system and increased accuracy of the machine. Some basic technical specifications of the rapid prototyping system are given in Appendix-3.

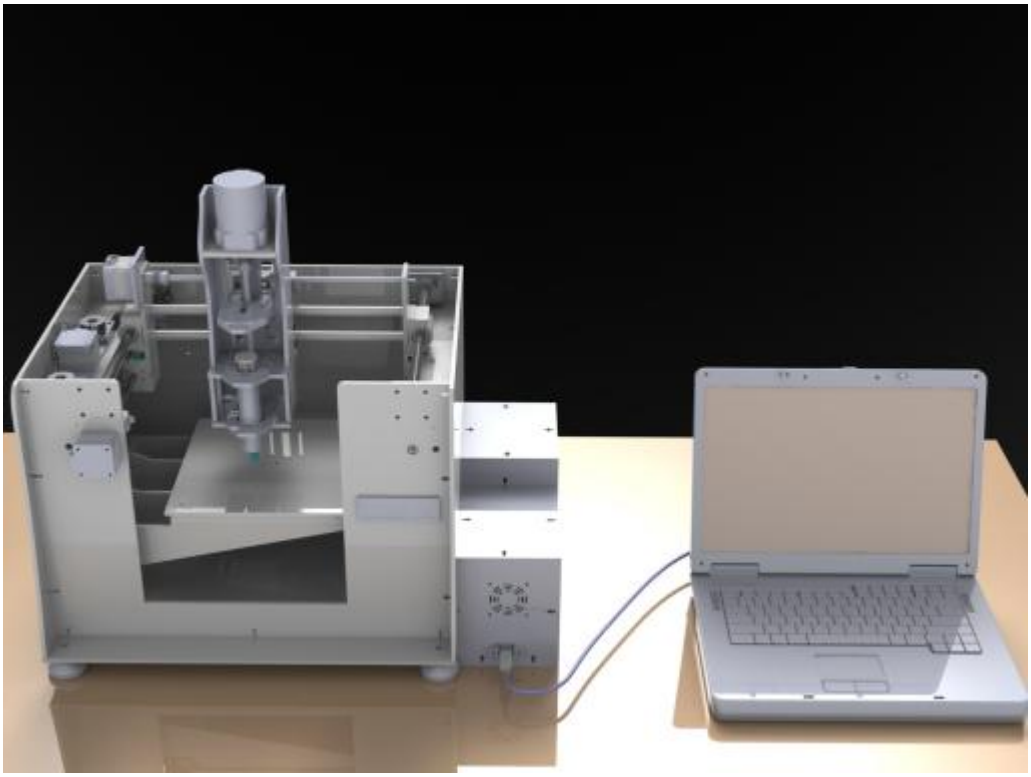


Figure 4.2: Designed “SiDau” 10 System.

Figure 4.3: T-nut style.

4.2.3. Positioning

In positioning system commercial flanged ball bearings and linear bearing pillow blocks were used in combinations with shafts (GTEN Inc.). Two types of shafts were used, one is threaded with 8 mm diameter and other is not with 12 mm diameter. Two separately moving stage like structures were designed, where one is travelling on both X and Y axes and remaining along Z axis (Figure 4.6). X axis has two ball screws to move on while Y, Z, axes have only one. Along X axis to achieve symmetrical motion slave ball screw was connected to the motor screw by using timing belt and pulleys (Figure 4.4). On XY stage modular deposition system was bolted on Y axis according to design (Figure 4.5). Decidedly two separated stages were chosen instead of one three dimensional stage to reduce acceleration of parts during manufacturing. For driving mechanism of X-, Y-, and Z- axes we selected NEMA 17 unipolar E-Minebea hybrid step motors because of their high performance in small sizes at low speeds. As a matter of fact their high resolution angular positioning makes them optimal for open loop control. In case if accuracy would be insufficient stepper can be operated as a closed loop by putting them together with an encoder. Positioning resolution of our system travels minimum 1.56 μm/pulse (in microstep mode, 1600 steps/rev) and

maximum 12.5 μm/step (in half step mode, 200 steps/rev) per full step, a nominal top speed 18 mm/s.

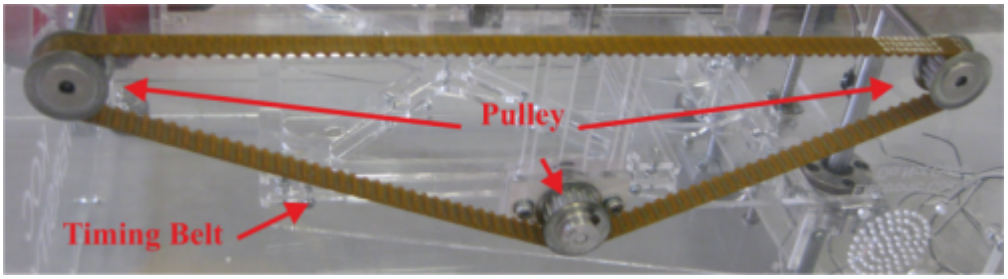


Figure 4.4: Timing belt and pulley

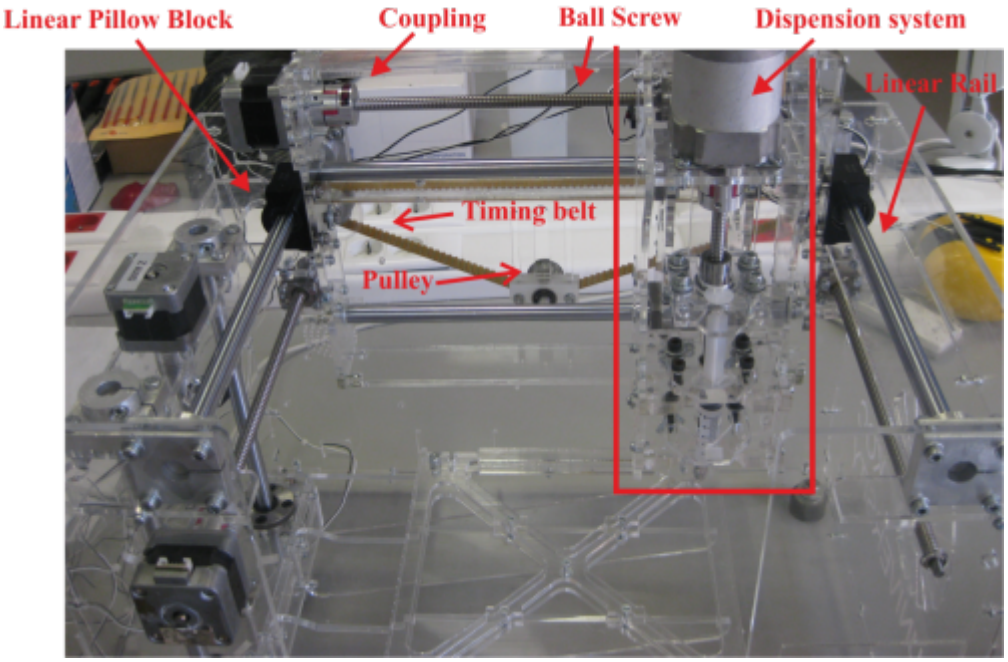


Figure 4.5: X and Y carriage.

4.2.4. Material Deposition System

The deposition system of the machine is modifiable (Figure 4.7). The whole system can be changed from single or multi syringe into fused deposition mechanism according to applied material and deposition strategy. Before using any type of head mechanism, an additional calibration must be considered. In this study, the developed material deposition system is available for different size of syringes, whose injection and withdraw is also possible to better control the flow of the solution (Figure 4.8).

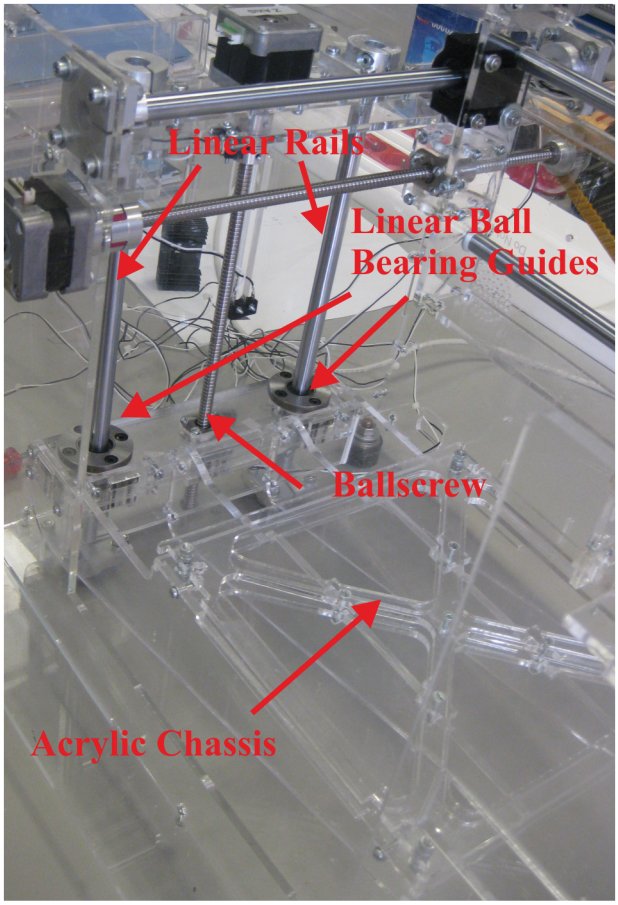


Figure 4.6: Z stage.

Whereby the powerful selected NEMA 23 type actuator provide to imprint almost all types of fluids without thinking about the viscosity of fluid. The flow rate is controllable via the sent signal frequency from microcontroller to step motor driver.

Normally, the deposition systems use commercial barrel in similar system. The barrels increase the operational cost of the machine compared to disposable syringes. It is possible to use various size syringes with different size needles. Needles with 100 μm, 250 μm, and 550 μm inner diameter (EFD Inc. and Hayat Inc.) have been used so far with disposable syringes. It is hard to find less than 100 μm sized needles, which limit the resolution of the designed machine. These aforementioned subjects dramatically reduces the operational costs and provide an advantage when compared to similar systems in literature.

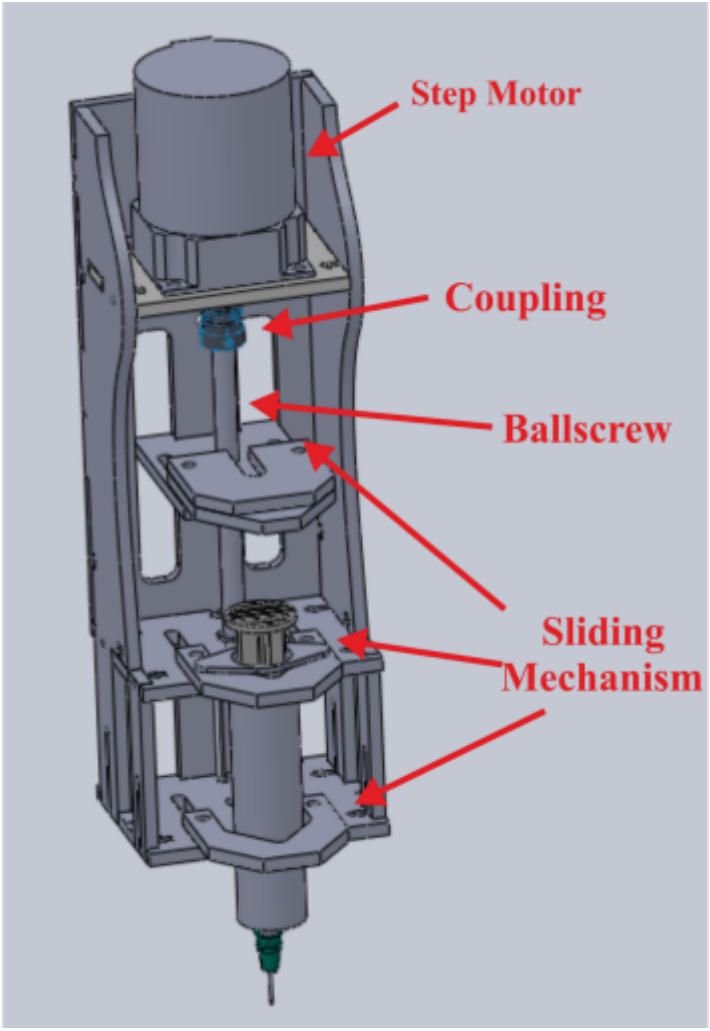


Figure 4.7: Syringe Mechanism.

4.2.5. Electronic Hardware

Nowadays, most of desktop and laptop computers do not have serial and parallel ports. Instead the personal computers which are used in community have standard USB interfaces. Serial and parallel ports are usually used in hobby robotics and microcontroller applications because of ease of applications. As a result of these points, the USB connection was selected in this study.

By taking into consideration availability of electronic equipment in Turkey it was decided to select PIC16F877. As a matter of fact, PIC16F877A is useful as a reference device because it has a minimal instruction set but full range of features such as high number of I/O pins, internal Analog to Digital Converter, PWM, Counter/ Timer, USART, SPI, I2C connections, resemblance of pin diagram with 18 series controller. Therefore, if it becomes clear that more power or special feature is needed, higher

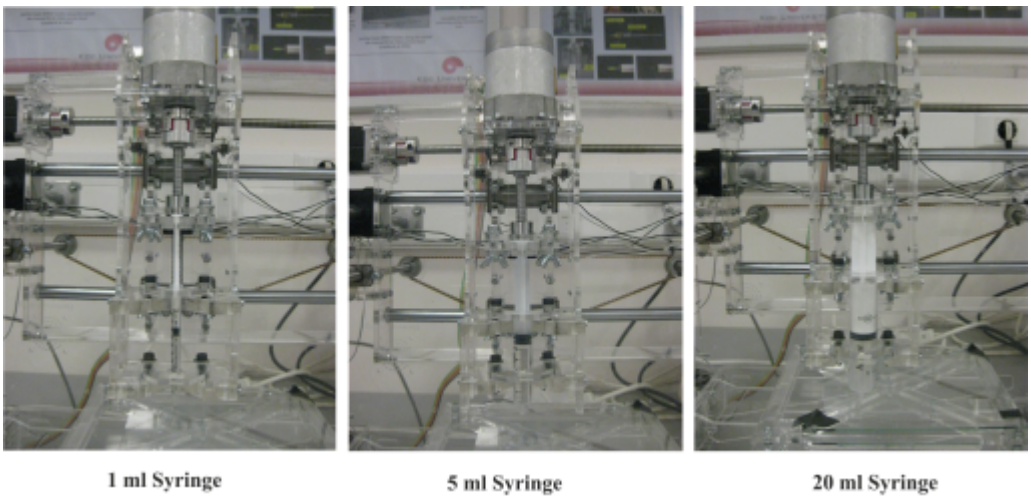


Figure 4.8: Deposition System.

specified 18/24 series can be used. Due to sequential running of the microcontroller code, in one instruction 4 step motors are controllable by position and velocity.

The cabling of the system was made by using an interface card which was installed on AM machine. It was decided to use multi-purpose Tekopic2 board as an interface card. Cabling of the system can be seen on Appendix-2. Finally all electronic components were purchased from Turkey.

4.3. Software

As claimed in the title of this work, the additive manufacturing machine is controlled by using open-architecture based software. Open architecture means that the user has full control on software source code and there is no limitation in any part of code even the code has interaction with different electronic hardware such as microcontroller. This interaction between microcontroller and computer or a keypad actualizes in programming, which controls the electronic hardware directly by using microcontrollers, digital signal processors or FPGAs. In this new system a keypad was used to directly interact with microcontroller. The keypad has inputs from the client such as the requested different production parameters.

In this study, 16 series PIC microcontroller was used. To program these types of microcontrollers several options are available. In practice, high level language based compilers such as CCS C, PIC C, PIC Basic Pro, JAL are used. Even though these

compilers create wide range availability, each of them has also some advantages and disadvantages. In any case, C programming language is chosen for the first part because of its robustness, easy to program and modular functions. In written program, the following operations may be possible;

- Parsing the incoming commands from the keypad,
- Storing the necessary actuation commands,
- Activating the actuators and sending the step and direction signals to motor driver,
- Fabricate multilayer quadrilaterals and grid like structures called scaffolds,

If the system needs more powerful controller, 16F8xx will be changed into 18F4xx without changing any electronic hardware. As it was stated before, the 16 series microcontroller could be changed into 18 series without changing any hardware. The 18 series microcontroller can handle 40 million line of assembly instructions in one second by using 40 MHz crystal. This property of the electronic system proves the upgradeability of the system.

There are also different computer software such as Java based RepRap software. The program written by RepRap is multiplatform (Microsoft Windows, Linux, Mac OS, and Solaris) based due to usage of Java language but has limits because of having semi open architecture. Semi open architecture means, even though the client has control on the second part of program, he/she does not have control on the first part thus the controllability of the system decrease.

Two algorithms were designed and implemented in the system. One algorithm enables fabricating equilateral quadrilaterals and other is for fabricating grid like structures called scaffold. In the first algorithm before manufacturing quadrilaterals different parameters should be adjusted first. To adjust them no external hardware was used, so parameters are adjusted directly in the code. So speed of the motors and dimensions of the parts are required to be defined in the code itself. Manufactured parts are different size equilateral quadrilaterals. Thus operator needs to enter x and y Cartesian

coordinates of one side of quadrilateral, increment in Z direction and number of layers (Figure 4.9). Actually this algorithm can be developed and then used in fabricating free form surfaces in Si-Dau 10'system, as it has ability to fabricate lines with different angles. In the second algorithm it was decided to use keypad for interacting with microcontroller. Basically algorithm is the same with algorithm developed for previous system, which was mentioned in previous chapter. Before fabricating scaffolds main parameters are entered by keypad, while other should be changed in the program. Main parameters such as height, width and length of a scaffold, also a distance between the struts are entered through a keypad. Mainly algorithm has two types of layers as shown on Figure 4.10. Before fabrication operator can have different designs of scaffolds by choosing different amounts of each layer. Thus it is possible to fabricate a scaffold with different amount of each type of layer stuck on each other. The speed of the motors is changed in the code. Actually firstly speed of the motors is should be adjusted for required application. Then it remains same in application with same material, thus there is no need to enter it every time you fabricate parts.
The necessary steps to run the AM machine is,

1. Connecting the computer to microcontroller board via USB-RS232 converter,
2. Adjusting the parameters according to the selected needle and used materials,
3. Starting the computer applications and entering basic parameters into program,
4. Injecting selected material into syringe and arranging the z level position of syringe with respect to the platform
5. Entering parameters from the keypad or changing parameters within the program,
6. Setting the zero X-Y position of the syringe,
7. Starting the production sequence,

Before manufacturing parts, simulations were run on Proteus ISIS software (Figure 4.11). Simulations allow seeing whole manufacturing process for each algorithm implemented in it, so it is possible to see frequency and travelled distance of each motor.

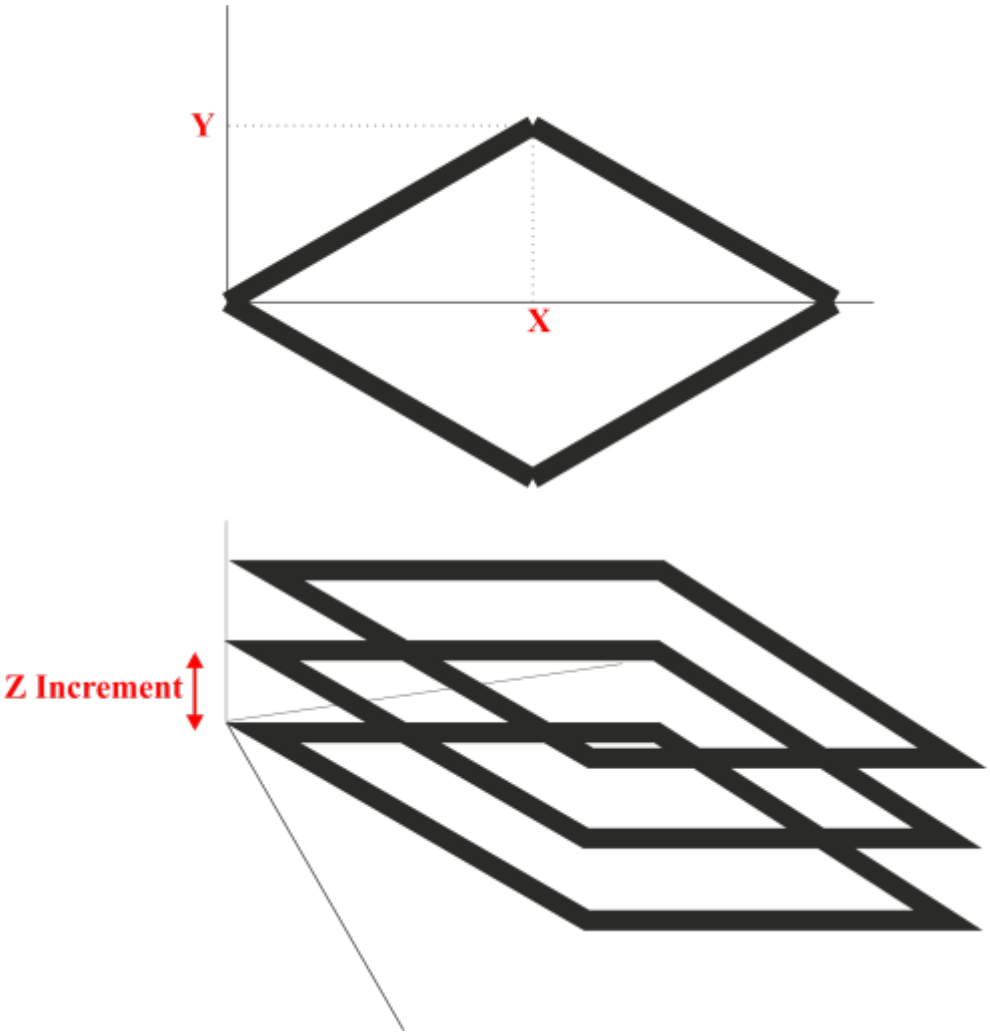


Figure 4.9: Designed quadrilaterals by first algorithm.

4.4. Accuracy Test of a Developed System

Mechanical part of developed system consists of two subsystems: a 3-axis machine and deposition system. Each axis of the machine is driven by microcontroller controlled step motors. The system is able to be controlled with a kinematic resolution of 1.56 μm in each axis. However, because acrylic parts are not rigid and there is no feedback system in step motors it is difficult to achieve this resolution. To estimate dimensional errors of produced parts 3 axis CNC Mazak FJV-200 UHS machine was used (Figure 4.12). Mechanical comparator (Mahr GmbH) was used in accuracy test of a system. Parts were not actually manufactured during the test. Since the production material, silicone, does not solidify immediately and is viscous enough to spread on the surface after it is deposited, some deformation occurs causing a produced line to lose its circular form tolerably. As a result, dimensional errors occur. Thus system was

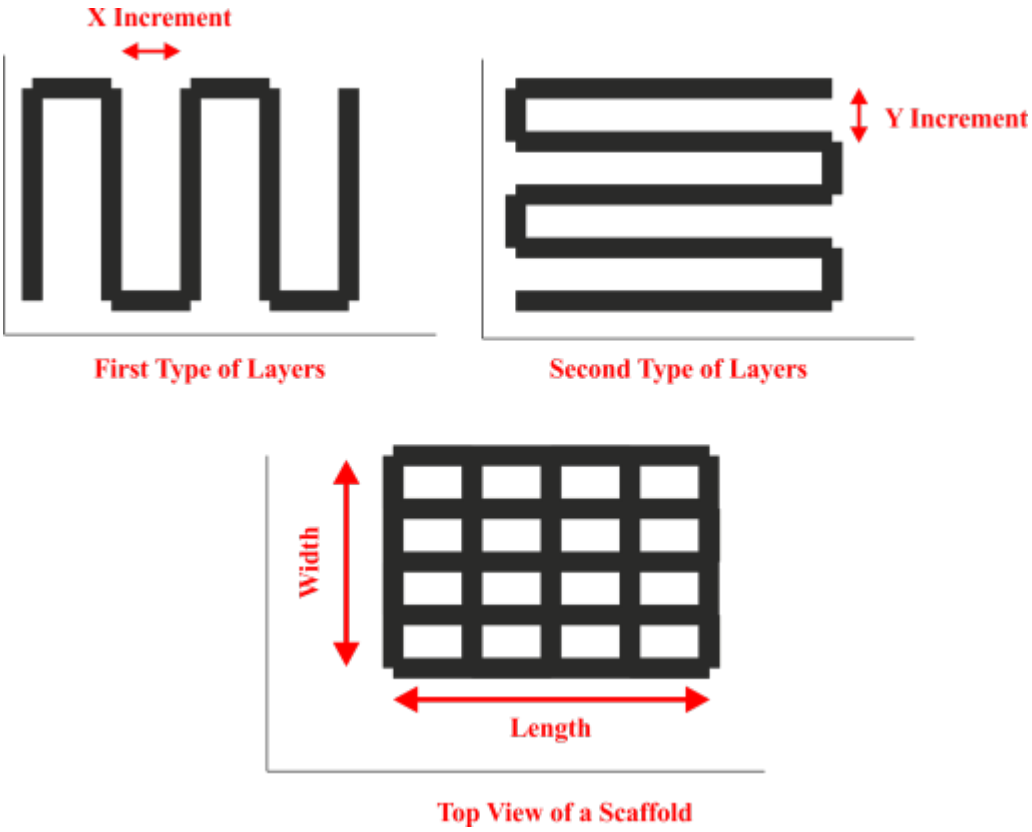


Figure 4.10: Designed scaffolds by second algorithm.

actuated repeatedly different distances in X, Y and Z directions and then was checked by 3 axis machine. Error was measured by moving comparator the same distance as the system move. With repeated measurements for 40, 20 mm translation in X,Y and Z directions, kinematic accuracies of +X,-X,+Y, -Y, -Z and +Z direction are found as 0.04 mm, 0.05 mm, 0.6 mm, 0.5 mm and 0.3/0.3 mm respectively. Also average errors in X, Y and Z directions are 0.162%, 1.84% and 0.97% respectively (Table 4.1). Average percentage errors in three directions are different, thus it can be concluded that the errors in the produced parts are directional with highest error in Y direction. The reasons of the errors can be lack of feedback mechanism in driving system and low precision of assembly of a system.

Dimensional errors were also investigated during Proteus simulations. In fact it is possible to estimate travelled distance from total send pulses. Repeated simulations were run to observe total send pulse number for different entered values. In Table 4.2 results are shown for simulations which were run to observe pulses for 40 mm (25600 pulses), 20 mm (12800 pulses), 10 mm (6400 pulses) and 2 mm (1280 pulses) simulated lines in +X,-X, +Y, -Y, +Z and -Z directions. Simulations showed that in +X,-X,

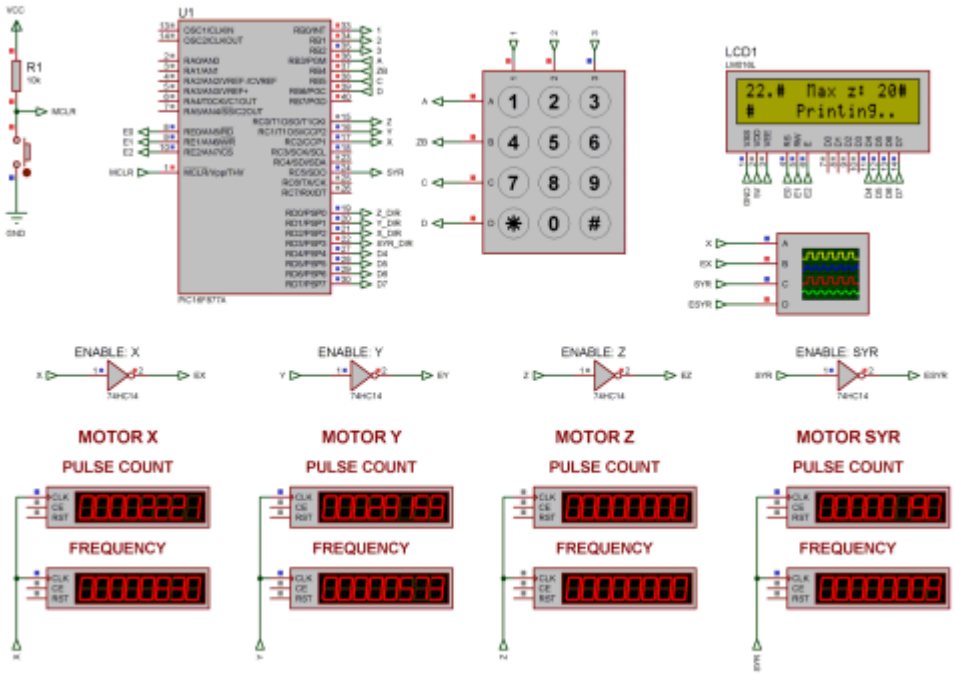


Figure 4.11: Simulation run using Proteus ISIS software.

Table 4.1: Measured errors by CNC machine in X, Y, and Z direction.

Distance Expected (mm)	X axis Measured (mm)	Error %	Y axis Measured (mm)	Error %	Z axis Measured (mm)	Error %
-40	-40.07	0.175	-40.7	1.75	-40.35	0.875
-20	-20.035	0.175	-20.35	1.75	-20.2	1
20	20.035	0.175	20.35	1.75	20.2	1
40	40.05	0.125	40.85	2.125	40.4	1
Average Error		0.162		1.84		0.97

+Y, -Y, -Z and +Z directions there are 0/0, 1.56/1.56 μm, 1.56/1.56 μm errors accordingly. However those errors are very small comparing to the errors found during measurements. Travelled distance is calculated as shown below:

Pitch Distance = 2.5 mm
Number of pulses per rotation = 1600
Travelled distance= No of Sent Pulses * Pitch Distance
/ No of Pulses per Rotation

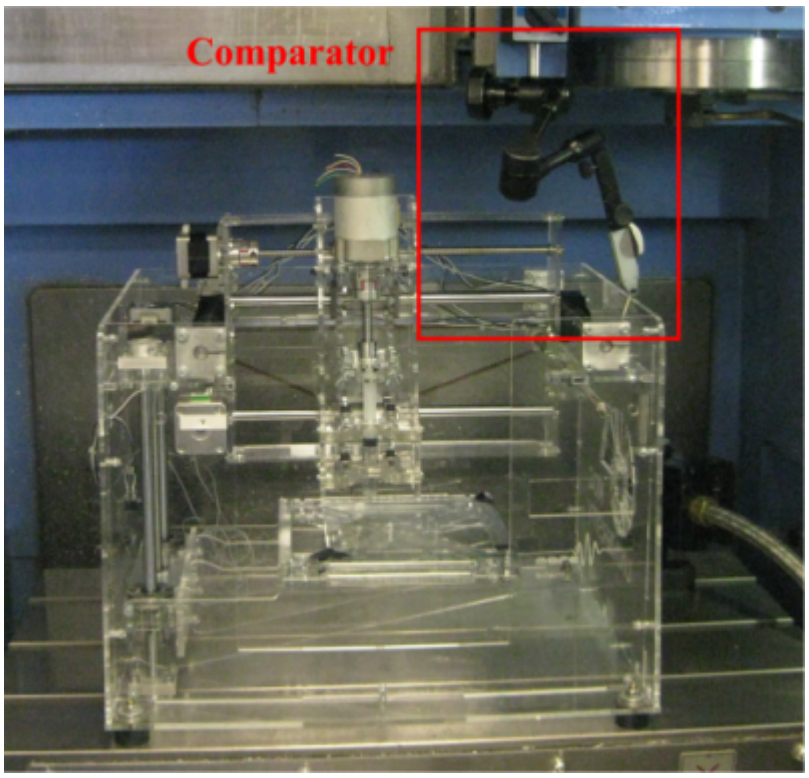


Figure 4.12: Si-Dau 10 System.

Table 4.2: Measured errors during simulations in X, Y, and Z direction.

Pulses Expected	X motor counted pulses	Error Pulses	Y motor counted pulses	Error Pulses	Z motor counted pulses	Error Pulses
25600	25600	0	25599	-1	25599	-1
12800	12800	0	12799	-1	12799	-1
6400	6400	0	6399	-1	6399	-1
1280	1280	0	1279	-1	1279	-1

4.5. Some of the Manufactured Parts

In this section, some of the parts which were produced by developed system are presented. It is verified that the system is able to manufacture parts designed by the algorithms. On Figure 4.13 and Figure 4.15 it can be seen that it is possible to manufacture multilayer quadrilaterals by using first algorithm. Also manufactured 3D silicon scaffold is given on Figure 4.14 and Figure 4.15. Only silicone was used as a production material because of the lack of time to tune the system to use other materials such as polymer solution used by first generation system. Thus it is clear that algorithm works

properly and it is possible to manufacture polymeric scaffolds in future work. Finally it is also possible to implement first algorithm into the second, to manufacture scaffolds with fibers having angles different than 90 degrees.

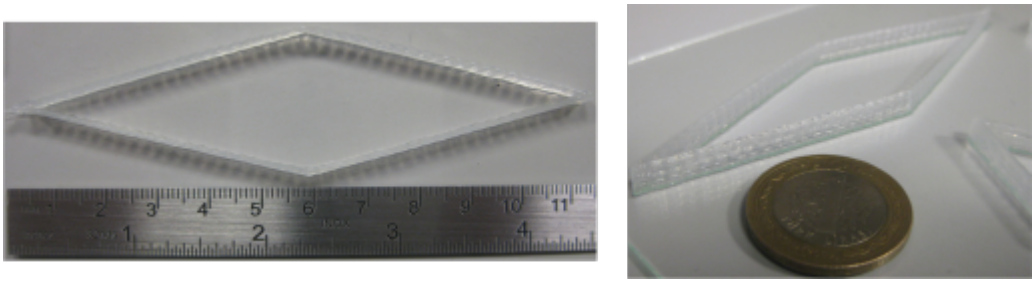


Figure 4.13: One of the manufactured parts (19 layers).

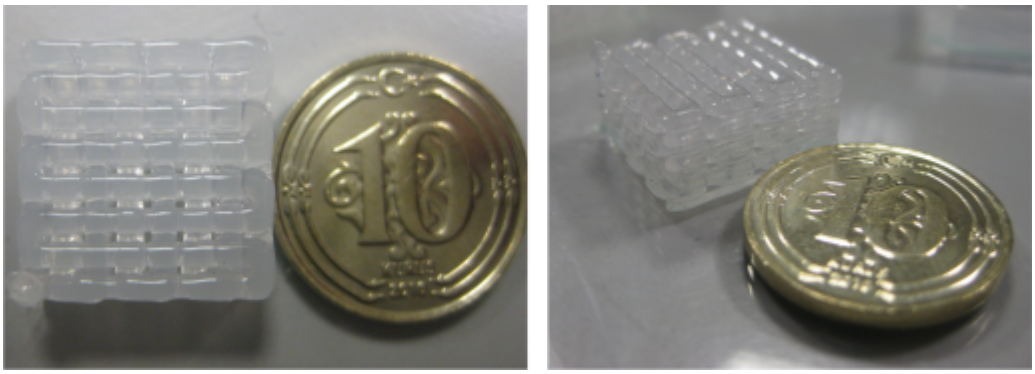


Figure 4.14: Manufactured Silicon Scaffold (20 layers).

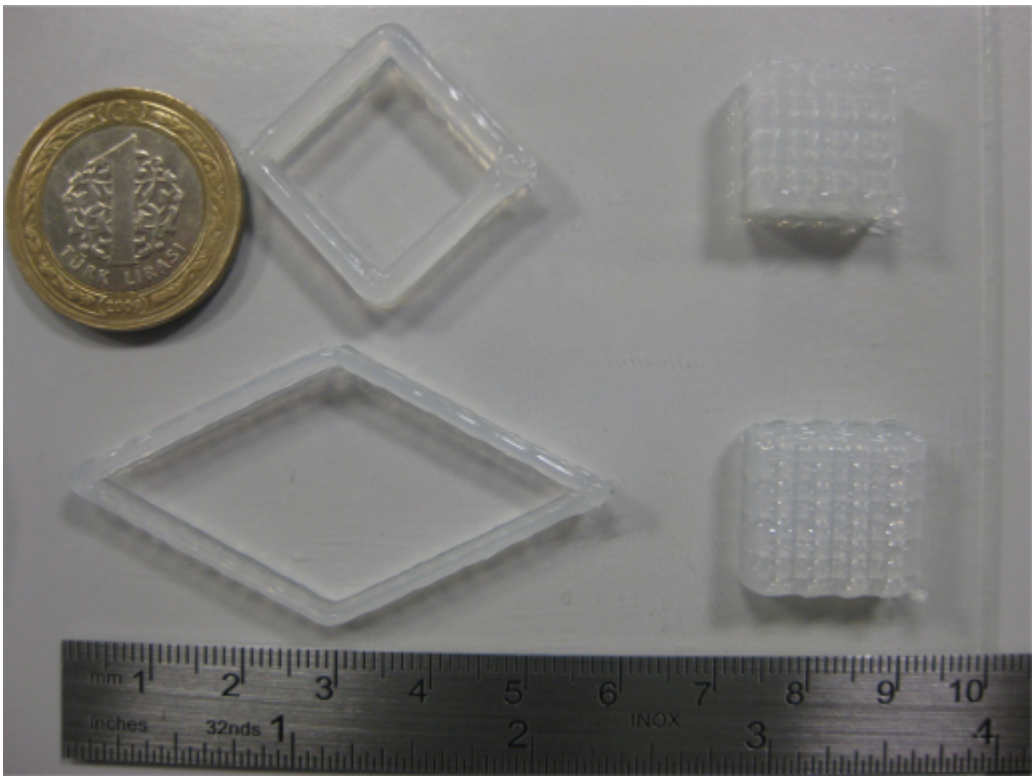


Figure 4.15: Picture of some of the produced parts.

Chapter 5

Biological Analysis

5.1. Introduction

Two open architecture rapid prototyping systems were developed at Koc University Manufacturing and Automation Research Center for the fabrication of polycaprolactone (PCL) scaffolds for tissue engineering. The systems utilized dichloromethane (DCM), a carcinogenic and highly volatile organic chemical (boiling point is 40 $^{\circ}$C), as a solvent in chemical solution deposition fabrication of PCL scaffolds. After the PCL solution is extruded from the rapid prototyping machine, DCM rapidly vaporizes from the solution, causing the PCL to solidify into scaffolds. Trace amounts of DCM can remain in the scaffolds post fabrication and render the scaffolds unsuitable for growing artificial tissue or in vivo implantation. However, DCM can be completely removed in a vacuum oven.

Biological tests were performed on the scaffolds to assess if the material could be used for tissue engineering applications. New scaffolds were fabricated on the first generation system, vacuum dried at room temperature for 48 hours to remove residual DCM, and UV sterilized for 1 hour in preparation for cell culture. In cell culture experiments, the scaffolds were assessed for cell attachment, cytotoxicity, and proliferation. These biological tests assess if the prototyping system and post processing steps can produce scaffolds suitable for growing artificial tissue.

In similar rapid prototyping systems, PCL scaffolds were used for bone tissue engineering [53], [37]. The cell lines used are typically mesenchymal stem cells or bone marrow cells. Since our university does not have access to stem cell and bone marrow cultures, we instead performed our biological assessments with NIH3T3 and HeLa

cells. HeLa cells are oldest immortalized permanent human cell line. The line was derived from cervical cancer cells. Moreover it is very common to use this cell line in various tissue culture techniques because of their aggressive growth characteristics [54].

NIH3T3 cells are immortalized mouse fibroblasts cells. NIH3T3 cells exhibit fibroblast like morphology and grow like mesenchymal stem cells and bone marrow cells [7]. Although the species and cell type of NIH3T3 are completely different from human mesenchymal stem cells and bone marrow cells, NIH3T3 cells are still useful only for obtaining preliminary biological assessments on biocompatibility of PCL scaffolds produced by our rapid prototyping system.

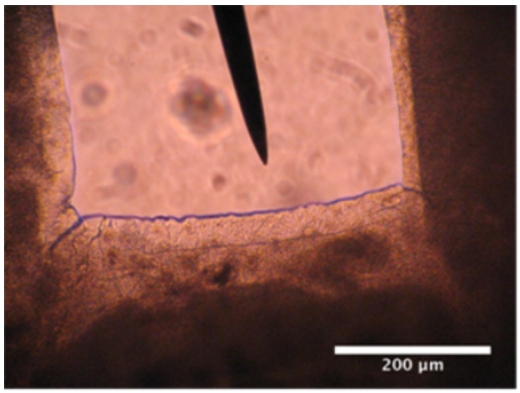
5.2. Cell Attachment

Cell attachment is the strength to which cells binds to a material and is a crucial design parameter in engineering of tissue substitutes. Almost all mammalian cell lines are attachment dependent. If cells cannot attach to a surface, they cannot grow and perform normal functions. The degree to which each cell lines have affinity for a material depends on the chemical composition surface, geometry, and mechanical properties of the material. Assessing cell attachment is important in determining which materials are suitable for which biological applications and how a material can be modified to better support the growth of a particular cell line. The goal of this biological test was to demonstrate that fibroblasts can grow on PCL scaffolds.

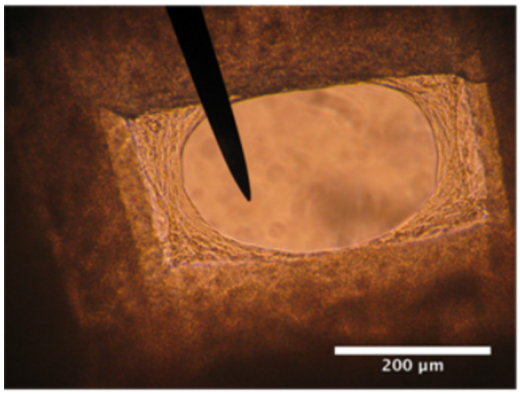
5.2.1. Culture Conditions

PCL scaffolds were fabricated by rapid prototyping system. Scaffolds were vacuum dried for 48 hours to remove residual DCM. After vacuum drying, scaffolds were cut into 7 x 7 mm square pieces and sterilized under UV lamp for 1 hr. After UV sterilization, scaffolds were transferred into complete medium (low glucose DMEM, 10% Fetal Bovine Serum, 1% Penicilin-Streptomycin-L-Glutamine) and prewetted for 24 hour. Prewetting allows serum protein to soak into and coat the scaffolds, equilibrating the scaffold with the culture medium and hypothetically makes the scaffolds more

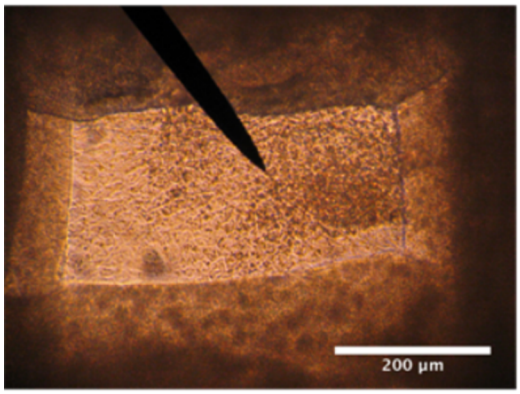
conducive to cell attachment. After prewetting, 250000 NIH3T3 cells were seeded into the scaffolds and cultured over the course of 9 days. Microscope pictures of the scaffold cultures were used to track the progression of cell growth and attachment on the scaffolds (Figure 5.1).



Seeded Scaffolds Day 1



Seeded Scaffolds Day 5



Seeded Scaffolds Day 9

Figure 5.1: Microscope Images of NIH3T3 Fibroblast Cells.

5.2.2. Hematoxylin and Eosin Staining

Hematoxylin and Eosin (H & E) staining was performed because light microscopy is insufficient to reveal the cell morphology. In fact H & E staining reveals cell morphology via staining of nuclei and cytoplasm. H & E staining was performed according to manufacturer instructions [55]. Scaffolds were first washed with PBS to remove medium and fixed with 1 mL paraformaldehyde (4%) for 10 minutes. Paraformaldehyde was washed away with PBS. Scaffolds were incubated with 500 μL hematoxylin staining solution at ambient temperature for 3 minutes. Hematoxylin staining solution was aspirated away. Scaffolds were washed with 1 mL PBS three times. Scaffolds were then incubated in eosin staining solution for 3 minutes. After eosin staining, scaffolds were washed with 1 mL of PBS three times and mounted on slides for light microscopy. Microscope pictures were taken of scaffolds stained on day 9 of culture, to observe NIH3T3 cell attachment on fabricated scaffolds (Figure 5.2). It can be seen that cells are attached on the surface of the scaffold and proliferated towards the macro pores. Thus it is proven that NIH3T3 cells are attached and proliferated on fabricated PCL scaffolds.

5.3. Cytotoxicity

Cytotoxicity is the degree to which a chemical or material adversely affects cell growth. When cells are cultured in the presence of a cytotoxic material, cellular metabolism may be inhibited and cells may stop dividing or undergo apoptosis.

Although previous works have shown that PCL is a biodegradable and biocompatible material, we cannot be sure if our PCL scaffolds are suitable for in vitro and in vivo testing. Our PCL stock was synthesized by the chemistry laboratory, and the purity of the PCL stock was never assessed. Additionally, our rapid prototyping system used DCM, a carcinogenic chemical, as a solvent in fabrication. Although vacuum drying was used to remove DCM and other volatile chemicals in the scaffold, chemical analysis was never performed to assess the purity of the PCL scaffolds. Residual amounts of DCM, byproducts from PCL synthesis, and solvents used in synthesis of PCL can remain in the scaffolds and may adversely affect cell growth on our PCL scaffolds.

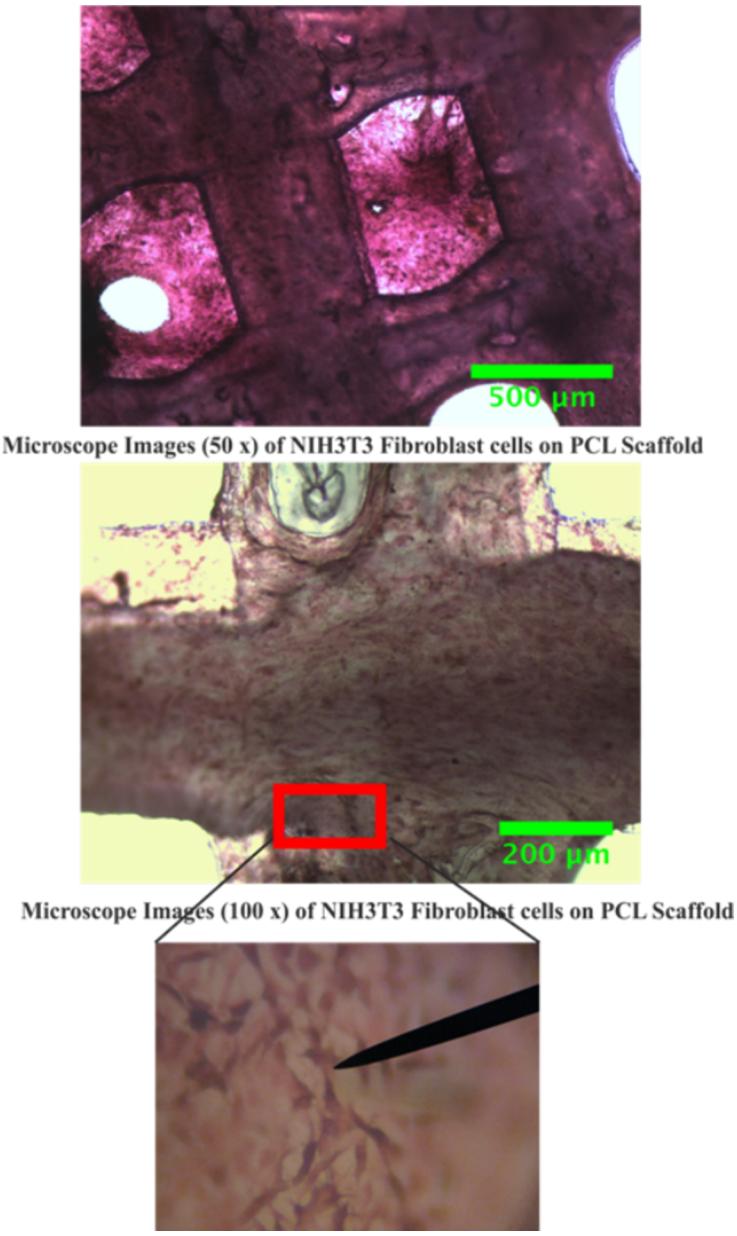


Figure 5.2: Microscope Images of H&E Stained Scaffolds (Day 9).

5.3.1. Preparation of PCL Scaffold Powder

PCL scaffolds were sonicated into fine powder in PBS. The sonication machine was set at 25% power and 4 cycles. Scaffolds were sonicated for 15 seconds in 1 minute intervals until scaffolds were broken into fine powder. Sonication was performed on ice to prevent melting of the PCL material. After sonication, scaffold powder in PBS solution was sterilized under a UV lamp for 1 hour.

To determine if the PCL scaffolds are cytotoxic, cell monolayers were cultured in the presence of PCL scaffold material and MTT assay was used to measure changes in

metabolic rate of the monolayers. Different amount of scaffold materials were tested two times. The treatments groups were 2 μL, 5 μL, 10 μL of scaffold material, PBS positive control, and DMSO (Dimethyl sulfoxide) negative control. Samples were replicated twice. MTT assay was performed in 24 hours after addition of material and 72 hours after addition of material. MTT assay protocol is given in appendix D.

5.4. Cell Proliferation

Cell proliferation is the propagation of cells on a scaffold or matrix. For scaffolds to be useful for tissue engineering, cells must be able to proliferate and populate the scaffold. Over time, the number of cells on the scaffold should increase until the material reaches maximum capacity.

5.4.1. Culture Conditions

Assess cell proliferation, 25000 NIH3T3 were seeded into 7 x 7 mm PCL scaffolds and cultured over the course of 6 days in complete medium. The scaffolds were prepared for cell culture by the method described in Section 5.2. of the thesis. The scaffolds were not prewet with medium prior to the addition of the cell suspension. Scaffolds were cultured in agarose coated well plates to prevent unwanted cell proliferation on the tissue culture plate.

5.4.2. Quantification of cell number

Quantification of cell number on the scaffolds was performed on day 3, 4, and 5. On the day of cell counting, scaffolds were transferred to microcentrifuge tubes and washed with PBS to remove culture medium. Afterwards, scaffolds were incubated in 500 μL trypsin at 37 $^\circ$C for 10 min to enzymatically detach NIH3T3 cells from the scaffolds. 500 μL complete medium was added to halt trypsinization of cells. Cells were centrifuged down at 600 g for 5 minutes to concentrate the cells. The cell suspension volume was decreased to 250 μL. Cells were resuspended and 10 μL samples were taken from the cell suspension for cell counting via a hemocytometer. Cells from each scaffold samples were counted four times.

5.5. Results

Microscopy images show that NIH3T3 cells can attach and grow on the PCL scaffolds (Figure 5.1). The selected images demonstrate that fibroblasts attached to the PCL scaffolds and over time grew to fill in the spaces between the scaffolds. Moreover H&E staining shows that NIH3T3 cells exhibit fibroblast like morphology on the PCL scaffolds (Figure 5.2). NIH3T3 cells are not just present in the spaces between the scaffolds, but are also covering the whole material. Finally these preliminary results indicate mouse fibroblasts can grow on PCL scaffolds fabricated by our machine.

MTT assay results are shown on Figure 5.3. Higher absorbance corresponds to higher metabolic rate due to more metabolism of the formazan salt used in the assay. As expected, cells cultured in DMSO exhibit low metabolic rate. Cells cultured with increasing amounts of scaffold material show increasing metabolic rate. This data shows that our scaffold material is not significantly cytotoxic to HELA cells.

Cell proliferation was observed by counting cells on the scaffold. The averages of cell counts from each scaffold on each day are shown in Figure 5.4, Table 5.1 and Table 5.2. The cell counts on the scaffolds generally increase over time. High standard deviation errors in Table 5.1 and Table 5.2 are due to low resolution of hemocytometer. The cell count from day 5 of culture is higher than the number of cells initially seeded into the scaffold. The increase in cell number can only be attributed to cell proliferation on the PCL scaffolds.

Table 5.1: Cell Count of Scaffold 1

Day	Count 1	Count 2	Count 3	Count 4	Average	Standard Deviation
3	2500	17500	2500	7500	7500	6124
4	15000	7500	12500	17500	13125	3698
5	65000	52500	62500	92500	68125	14830

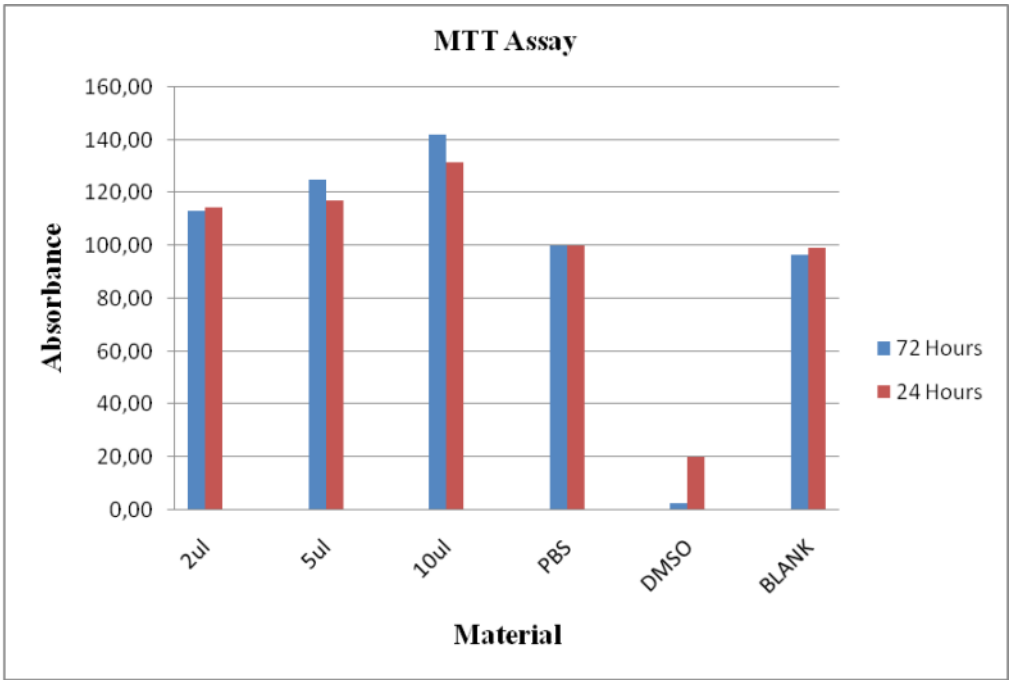


Figure 5.3: MTT Assay test results.

Table 5.2: Cell Count of Scaffold 2

Day	Count 1	Count 2	Count 3	Count 4	Average	Standard Deviation
3	20000	27500	15000	17500	20000	4677
4	50000	45500	62500	60000	54375	7153
5	105000	112500	110000	110000	109375	2724

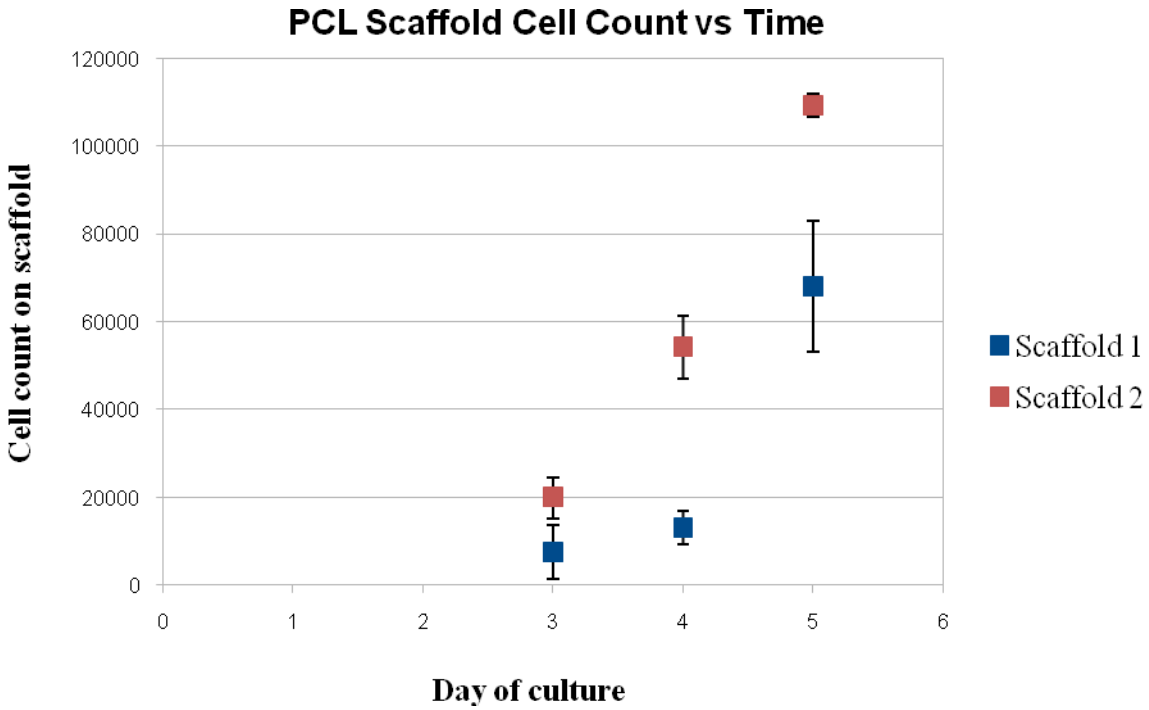


Figure 5.4: Cell count over time on PCL scaffolds.

Chapter 6

Conclusions

Main aim of this work was to develop a system which can design and manufacture bioactive scaffolds for tissue engineering approaches. It was decided to use rapid prototyping systems in scaffold fabrication. Two open architecture rapid prototyping systems were designed and developed in Manufacturing and Automation Research Center at Koc University. First generation open architecture rapid prototyping (OARP) system was developed in previous work (48). Few modifications in the electronic wiring were done in the first generation system. Isolated BNC connection box was changed with homemade designed electronic card. Second generation OARP system called Si-Dau 10 was developed in this work.

Si-Dau 10 apparatus is built as the base of the rapid prototyping (RP) machine. Motions in all directions are provided by step motors. RP machine works by extruding a material onto a table through a syringe which is mounted on a motorised X and Y axis. The table can move down in the Z direction to allow for each layer that is created. The designed modular deposition system is very suitable for RP research in medicine, because different size standard sterile medical syringes are used in this system as reservoir.

Two different algorithms were developed for each system. Main aim of the developed algorithms was to design and fabricate different size scaffolds and simple geometries without using any CAD/ CAM software. The algorithms were implemented in both system and different parts were produced to prove that algorithms work properly. For first generation OARP system designed algorithms are able to fabricate 3D grid like structures called scaffolds and hollow spheres. Algorithms for Si-Dau 10 system are similar to previous one and can produce scaffolds and quadrilaterals. To

fabricate parts using designed algorithms operator required to enter dimensions of the manufactured manually.

Initially, liquid silicone which is technically known as polysiloxane is used as a production material for both systems. Then mixture of polycaprolactone (PCL) solution in dichloromethane (DCM) was used in the fabrication process. This solution was used on both systems. However, it was only possible to manufacture polymeric scaffolds on the first generation system. After polymeric scaffolds were manufactured they were tested for cytotoxicity, cell attachment and proliferation. Tests showed that scaffold material is not toxic and NIH3T3 fibroblast mouse cells were attached and proliferated on the scaffolds.

As a result, the objective of the study was achieved: two open architecture rapid prototyping systems were developed by using only domestic resources, both hardware and software, and it also was proven that these systems are capable of producing basic 3D scaffolds. Even though polymeric scaffolds were only manufactured on the first generation system, it is clear that it will be also possible to manufacture them on the Si-Dau 10 system after main fabrication parameters will be tuned for polymeric scaffolds production.

Appendix A

Electrical Connection Diagram

In the previous work outputs of the microcontroller corresponds to X, Y and Z signals were transferred through BNC cables to servo drivers. BNC cables were removed and new electronic board was implemented. Schematic view o a board is shown in Figure A.1 and Figure A.2. Signals come from the micro controller are sent via board to the servo drivers. The numbers seen in Figure A.1 correspond to pin numbers of CN cables of servo drivers.

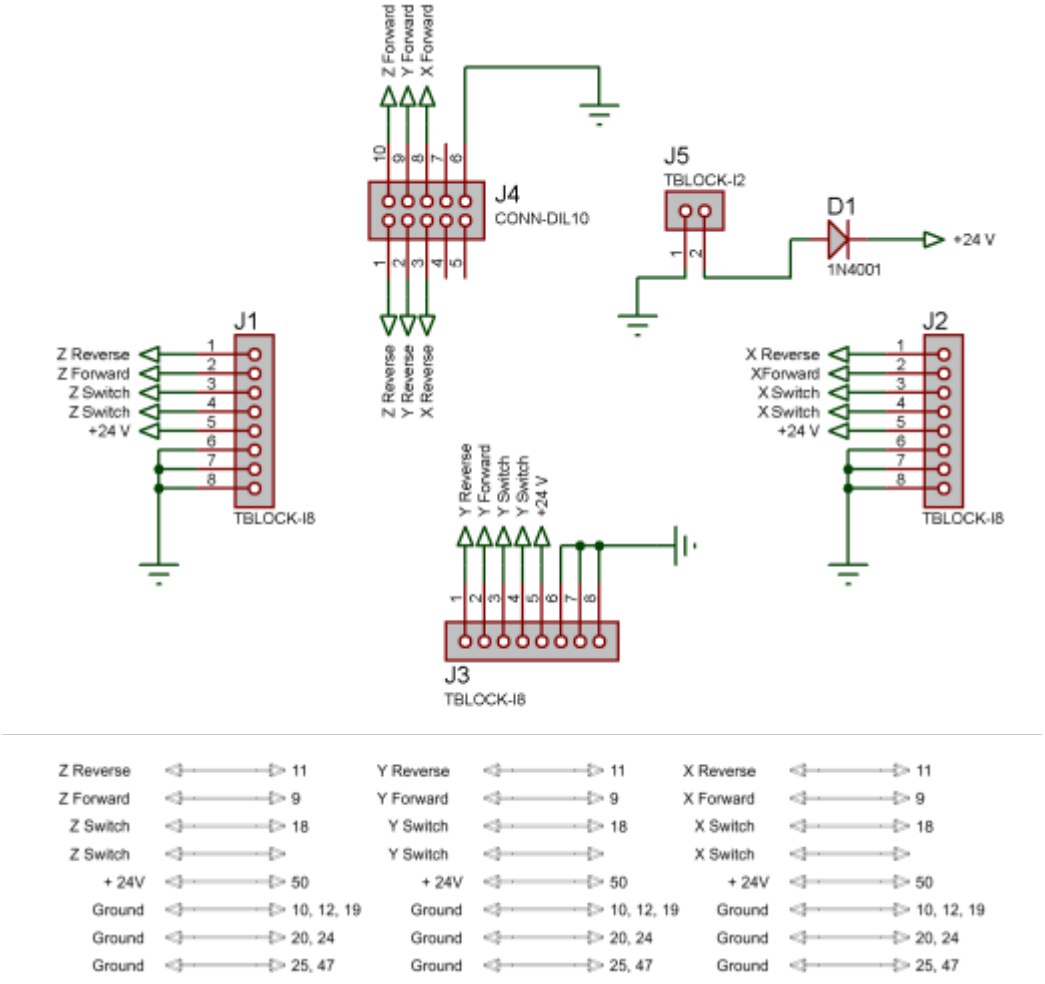


Figure A.1: Wiring of a PCB board.

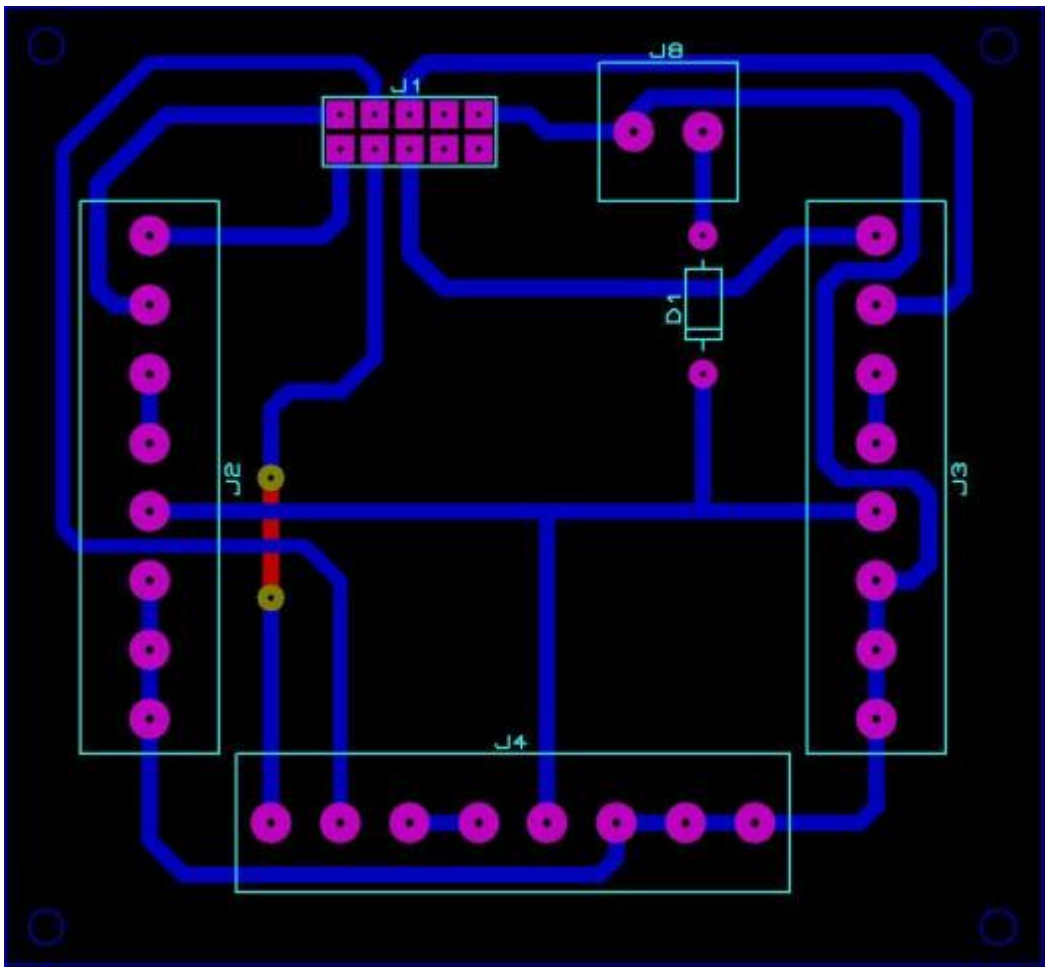


Figure A.2: Schematic view of a PCB board.

Appendix B

Electrical Connection Diagram for Si-Dau ‘10

Electrical connections diagram which was developed for second generation rapid prototyping system is given below.

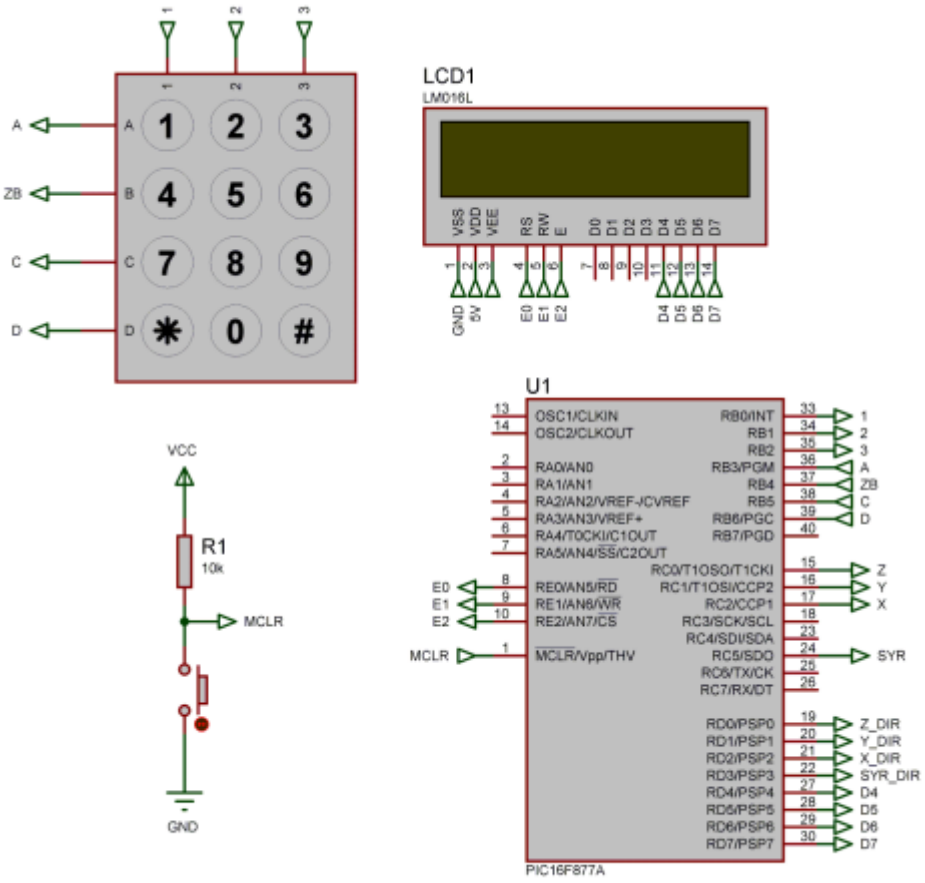


Figure B.1: Electrical Connection Diagram for Si-Dau ‘10.

Appendix C

Technical Specifications of Second Generation OARP System

Some of the main specifications of a developed Si- Dau 10 system is given below. More details on the developed system was given in Chapter 4.

Table C.1: Technical specifications of Si-Dau 10

			X	Y	Z
Dimensions of a Machine	Height Length Width	356 mm 370 mm 470 mm			
Z stage Carrier Platform	Length Width	224 mm 224 mm			
Workspace Dimensions			209 mm	126 mm	110 mm
Kinematic Resolution		1.56 μm			
Electrical Requirements		DC Power Supply 24 V & 5 V			
Nominal Speed			18 mm/s	18 mm/s	18 mm/s
Ball Screws			2.5 mm	2.5 mm	2.5 mm

Appendix D

Protocol for MTT Assay

1) Split the cell into 96-well plate (25000 cell), 10-15 μL cell and 85 μL medium

2) Growth overnight

3) Add appropriate amount of chemicals, DMSO, Tris into each respective well.

4) Growth overnight

5) Prepare fresh MTT(stored keep at + 4 ˚C)

6) Prepare solution of 5mg /mL in 1x PBS buffer, for each well 50 μL solution used

7) Suck the old medium from each well

8) Add 200 μL fresh growth medium(Dulbeccos Modification of Eagles Medium)

9) Add 50 μL MTT solution and wait 3.5 hour at 37 C incubator(cell culture)

10) Suck the medium carefully (do not disturb precipitate)

11) Prepare DMSO/EtOH solution (1:1) and add 200 μL DMSO/EtOH solution

12) Wait 5 min. and measure absorbance in Elisa Reader.

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