NOVEL TECHNIQUES OF FABRICATING POROUS, BIODEGRADABLE POLYMER SCAFFOLDS FOR SUSTAINED DRUG RELEASE

by

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During my stay here in UT Arlington, I found research as a challenging and motivating task. In research one can not expect positive results always and that was one of the lessons I learned. In this vast ocean of science and engineering, my contribution seems to be insignificant, just like a drop of water, but I see ocean as an integration of drop of water having no limits. With a deep sense of gratitude I thank Dr. Pranesh Aswath, my advisor, who not just taught but also motivated me. Without his support and valuable guidance this work was impossible. As a researcher, he asked questions but also taught how to answer.

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ABSTRACT

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RELEASE

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Porous scaffolds made from biodegradable polymers can be effectively used to mimic the nature of tissue, if bioactive chemicals (growth factors) are embedded in scaffolds and are released with a constant, determined rate. Two types of porous scaffolds were made from biodegradable polymers and their drug release trends and rates were studied. In first method, the emulsion of PLLA (Poly L-Lactic Acid) in chloroform solution and PVA (Poly Vinyl Alcohol) and acetaminophen (drug) in de-ionized water solution was made by using homogenizer. The emulsion was blended with PLLA solution by using blender. The resultant emulsion was freeze vacuum dried for 12 hours. Various combinations were obtained by varying Acetaminophen concentration, Homogenizer speed and Blender speed. The drug release study was conducted *in-vitro* by following the release of the drug over one week using a UV spectrophotometer. The

influence of these variables on drug release rate was studied by using Design of Experiments software. It was observed that, these scaffolds had high drug release rate and hence could release the drug for a short time. In the second method, scaffolds had three layers. The top and bottom layers were made by blending PLLA solution with PVA solution by using blender. The middle layer was made by blending PLLA solution with a mixture of PVA and Acetaminophen in de-ionized water solution using a blender. The emulsion layers were freeze vacuum dried for 12 hours. Various combinations were obtained by varying Acetaminophen and PVA concentrations and Blender speed. The drug release study was conducted over a period of 25 days. The pore size and porosities were obtained for scaffolds made by this method. It was seen that, these scaffolds had low drug release rate and hence could release the drug for a longer time. To study the viability of scaffolds made by second method, four different types of scaffolds were fabricated. In all the cases 10% w/v PLLA: PGA (85:15) copolymer solution was used. Different combinations of PVA concentration and Blender speed were obtained. There was no drug embedded into the scaffolds, initially. The cells were cultured and their presence into the scaffolds after 10 days was observed.

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CHAPTER 1

INTRODUCTION

Tissue loss is often devastating and a costly health issue all over the world. Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ [6].

Biologic tissues consists of cells, extracellular matrix and the signaling system, which are brought into play through differential activation of genes or cascade of genes whose secreted or transcriptional products are responsible for cueing tissue building and differentiation [6].

Extra cellular matrix is made up of cell secretions immobilized in spaces continuous with cells and mainly consist of collagen, glycoproteins, hyaluronic acid, proteoglycans, glycosaminoglycan and elastins. It harbors molecules such as growth factors, cytokines, matrix degrading enzymes and their inhibitors. Tissue regeneration is an attempt to imitate nature of biological tissue. Biodegradable polymers can be used to make scaffolds which will imitate the nature of extra cellular matrix. These scaffolds can be enriched with signaling molecules, which may be bound to them or infused into them [6]. The cells of the required tissue type can be seeded onto these scaffolds and kept in a

suitable environment so that the cells can proliferate, differentiate provided the required growth factors supplied at a desired rate.

Biodegradable polymers are the polymers which degrade *in vivo* without producing any harmful sideproducts and are of great interest in tissue engineering. An attempt in this study has been made to regenerate tissue on drug releasing, porous and biodegradable polymer scaffolds.

The properties of scaffold like pore size, porosity, biocompatibility, drug release rate, mechanical strength, viability for cells growth are essential in the study of tissue engineering. It's a challenge to design a scaffold which will satify all the requirements and should not have any short coming. In this study, an attempt is made to address the maximum number of "ideal scaffold " requirements.

The scaffolds were made by two different methods. In the first method, scaffolds with pore sizes upto 250 μm were made for a short term drug release study with high release rates. In this method polymer microspheres were embedded into the scaffold during fabrication, the emulsion freeze dry method was followed to fabricate the scaffolds. In second method the scaffolds were made in such a way that they have prolonged drug release period with a low release rate. The pore size and porosity was almost the same as obtained in the first method. In this method, three layers ware made from the emulsion freeze dry method, the drug was embedded in the middle layer.

The influence of pore size, porosity, drug concentration and relative proportions of biopolymers are studied in the context of drug release rate for both types of scaffolds. An attempt was made to study the viability of scaffolds made by second method for cells proliferation.

The objective of this work was to fabricate scaffolds with a sustained drug release rate with possible elimination of burst release, it was also desired to have scaffolds with pores in the range of 50 – 250 µm, which was assumed to be suitable for cells growth . It was hypothesized that, if the drug is associated with a water soluble polymer like Poly vinyl Alcohol (PVA), then by controlling the pore size and porosity, the access of water to the PVA and drug can be controlled and hence the drug release.

CHAPTER 2

BACKGROUND

The cell is the structural and functional unit of all living organisms and hence sometimes called as the "building block of life." Humans are multicellular organisms. Cells that have similar functions are grouped into a categories called as tissues. Grouping of the tissues into anatomical and functional units is called organ. Organs, in turn, may be grouped together by common functions into a system. The body is made up of numerous systems which act in a coordinated fashion to maintain an entire organism**.**

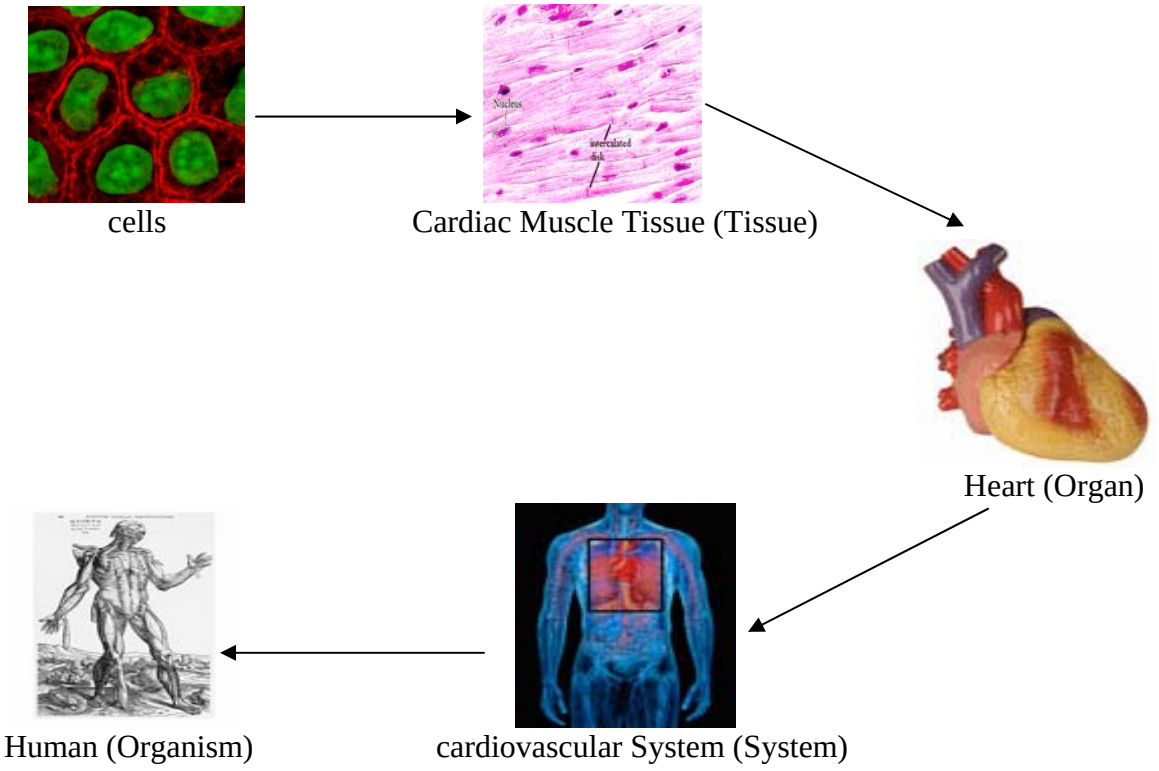


Fig 2.1 Bilogical constituents of an organism, an integration from cells to organism. [8]

The loss or failure of an organ or tissue is a frequently devastating and costly problem in health care, occuring in millions of patients every year [7].

2.1 Therapeutic Approaches for Lost Tissue or Organ Function

2.1.1 Transplantation

Organs or parts of organ are transplanted from a cadaveric or living related donor into the patient suffering from lost organ function. Although organ transplantation has been established as a curative treatment for end stage diseases of liver, kidney, heart, lung and pancreas, it is substantially limited by a critical donor shortage [7]. Also, problems with the immune system produce chronic rejection and distruction over time[6]. The other major problem of organ transplantation remains the necessity of lifelong immunosuppression therapy [7] which creat an imbalance of immune surveilance and can creat new tumor formation[6].

2.1.2 Surgical Reconstruction

Organs or tissues are moved from their original location to replace lost organ function in a different location. There are a number of problems associated with this treatment as replacing tissues consisting of different tissue type can not replace all of the functions of the original tissue[7]. Biological changes are seen because of abnormal interaction of tissues at its new location [6]. Diverting urine into the colon can produce fatal colon cancer 20-30 years later, making esophageal tubes from the skin can result in skin tumors 30 years later, using intestine for urinary tract replacment can result in scarring and obstruction over time[6]. There is also the risk of complications and surgical morbidity at the donor site.

2.1.3 Artificial Prosthesis

The use of artificial, non biological materials in mechanical heart valves, blood vessels, joint replacement prostheses, eye lenses or extracorporeal devices such as dialysis or plasmapheresis machines can cause infection, limited durability of the prostheses materials, lack of mechanism of biological repair and remodeling, chronic irritation, occlusion of vascular grafts and necessity of anticoagulant therapy and its side effects. Regarding the pediatric patient population, not all artificial implants can provide a significant growth or remodeling potential, which offer results in repeated operations associated with substantial morbidity and mortality [7].

2.1.4 Supplement of Metabolic Products of Diseased Tissue or Organs

The metabolic products of the diseased tissue or organ can be supplemented by oral or intravenous medication. e.g. in the case of loss of endocrine tissue function, hormonal products such as insuline or thyroid, adrenal or gonadal hormones can be supplemented. But supplemental therapy can not replace natural feedback mechanism, frequently resulting in dysregulation of hormone levels [7].

Organ transplantation has become the only established method of treatment for many end stage organ diseases and has enjoyed tremendous success in improving and saving patient lives. The following chart describes the current status of organ requirement and their availability in the United States.

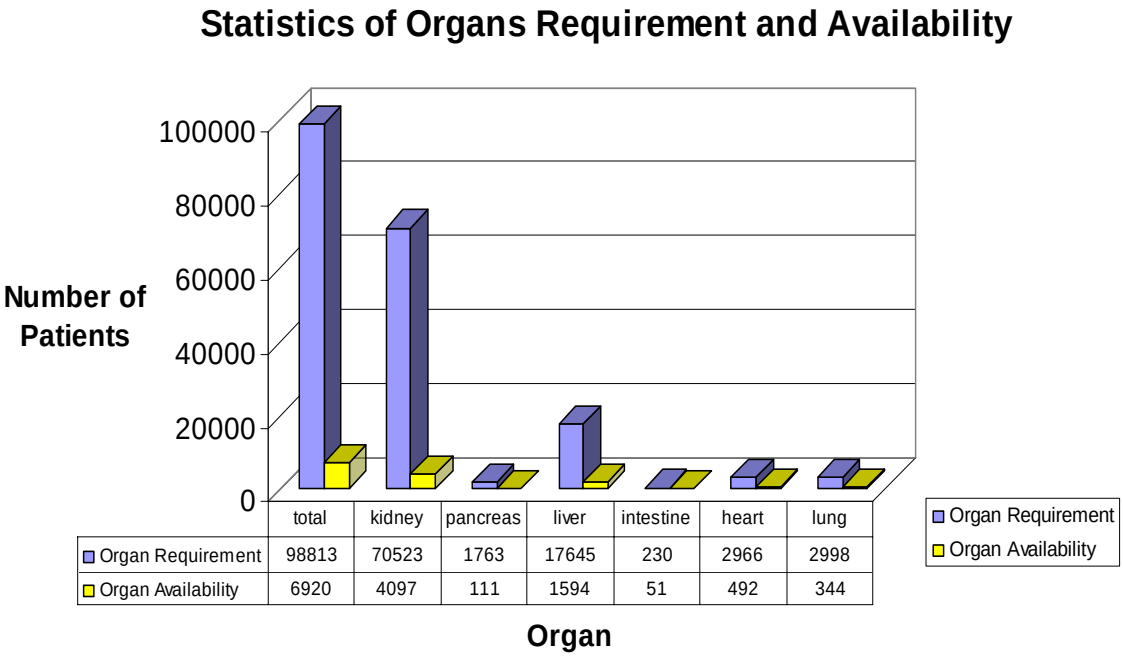


Fig 2.2 Statistics of Organs Requirement and Availability [9]

The following chart gives the statistics of organ requirement, their availability and percentage of organs available.

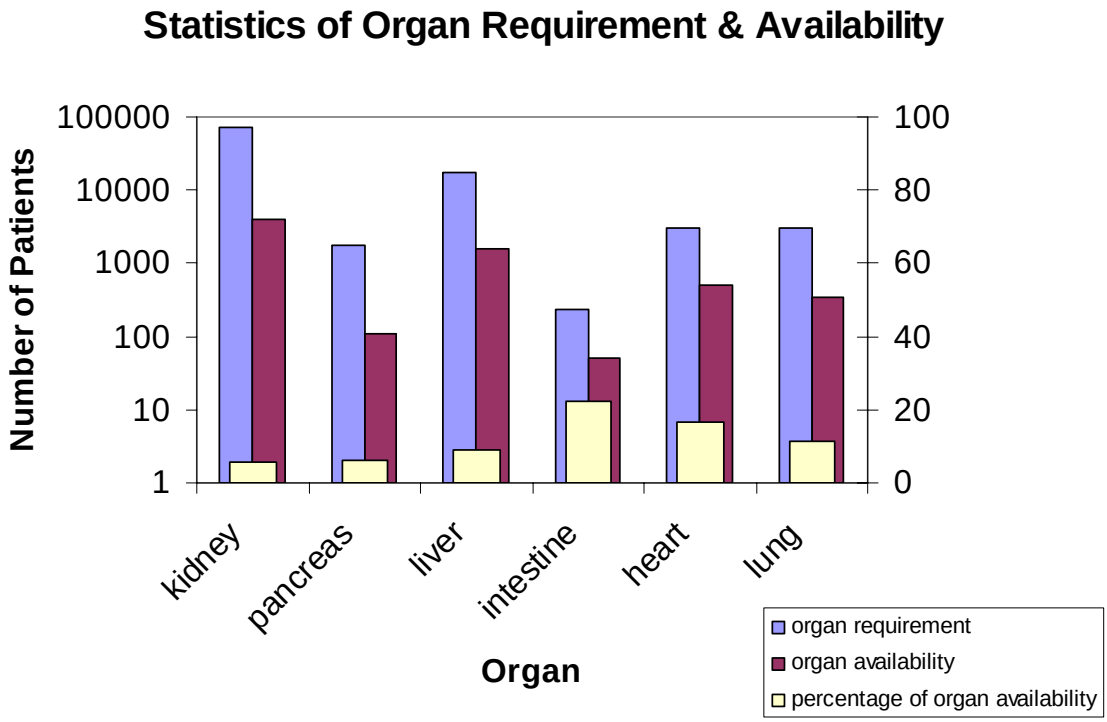


Fig 2.3 Statistics of Percentage of Organs available. [9]

It's clear from the chart that the percentage of total number of organs available is 7% of the actual number of organs required. The severe scarcity of donor organs, especially in the pediatric population, has become a major limitation and stimulated investigation into selective cell transplantation and the emergence of tissue engineering as an alternative approach to treatment of end stage organ diseases [5].

The transplantation of only essential tissue elements has many potential advantages, which include: [6]

1. Alleviation of the donor shortage by utilizing cells from a small tissue source, expanding them in culture and implanting them into multiple recipients.
2. Reduction in the risk and expense associated with major surgical procedures and protracted hospitalizations.
3. Potential for use of autologous cells eliminating the need for immunosuppresion.
4. Capacity for organ directed gene therapy and
5. Capacity for tissue growth.

Biological tissues consist of cells, extracellular matrix and the signaling system. Extracellular matrix is made up of a complex of secretions immobilized in spaces continuous with the cells and signaling system is brought into play through differential activation of genes or cascades of genes whose secreted or transcriptional products are responsible for cueing tissue building and differentiation.

2.2 Tissue Engineering as an approach for Tissue Regeneration

Tissue engineering is an endeavor in which nature is imitated or at least attempted to imitate. Meeting the challenge reconstituting tissues is in a way dependent on how the nature is imitated. There are three objectives to imitate nature: [6]

1. It can mean making an exact or closely approximate biologic replica that exhibits at least of the biologic properties of the original tissue at the time it is implanted.

2. It can mean providing a much less well developed precursor for a biologic substitute, with the expectation that it will be built into a faithful replica.

3. It can mean using a nonbiologic replacement.

2.2.1 Basic Components of Tissue Engineering

Tissue engineering is based on three basic components of biologic tissues and can be given as follows:

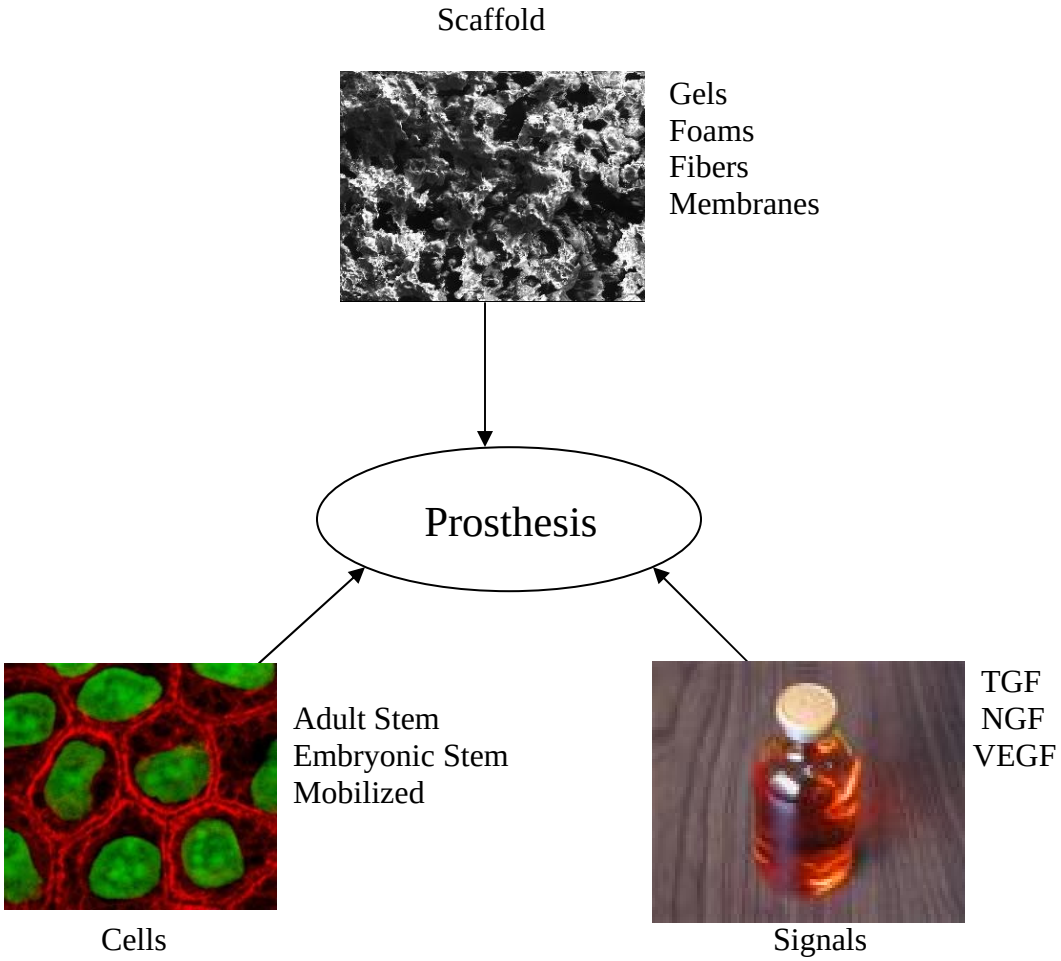


Fig 2.4 Triad of Tissue Engineering. TGF- Transforming Growth Factor, NGF- Nerve Growth Factor, VEGF- Vascular Endothelial Growth Factor. [6]

The strategy of tissue engineering generally involves following steps: [4]

1. An appropriate cell source is identified, isolated and produced in sufficient numbers.

2. An appropriate biocompatible material that can be used as a cell substrate or cell encapsulation material is isolated or synthesized and manufactured into the desired shape and dimensions.

Biocompatibility is the ability of a material to perform with an appropriate host response in a specific application [7]. A biocompatible materials have several key roles, which include facilitating cellular migration or invasion into the implanted material, guiding wound healing and tissue regeneration and providing specific cues through cell/matrix interactions and tissue responses to the material.[3].

3. The cells are uniformly seeded onto or into the material and grown in a bioreactor.

4. The engineered structure is placed into the appropriate *in vivo* site. Depending on the site and the structure, vascularization may be necessary.

2.2.1.1 Cells

There are three types of sources from where cells can be obtained and are named as Autologous, Allogeneic and Xenogeneic. Each source has its own benefits and limitations.

2.2.1.1.1 Autologous

If the cells are obtained from the patient's own body, then these cells are called as autologous cells. Because the cells are obtained from the patient's body, these cells are immune acceptable but are not available on the shelf and must be obtained from the patient when needed.

2.2.1.1.2 Allogeneic

If the cells are obtained from the other human sources, then the source is called as Allogeneic cells source and the cells as Allogeneic cells. These cells can be obtained off the shelf but may require immune engineering to avoid possible immune rejection by the host immune system.

2.2.1.1.3 Xenogeneic

If the cells are obtained from different species (e.g. animals) then the cells are called as Xenogeneic cells. These cells require immune engineering as these cells are rejected by human immune system. Extra precaution needs to be taken about viral transmission from animals, as the cells might be infected by viruses.

2.2.1.1.4 Stem cells as a source

Stem cell is a cell which can replicate and produce cells that take on more specialized functions [6]. Stem cells that give rise to only one type of differentiated cell are termed unipotent [6], oligopotent, multipotent and pluripotent represent an increase in the number of differentiated cell types from few to many or most [6]. A totipotent cell is

one that can generate the totality of cell types that comprise the organism. So far four types of stem cells have been identified and are named as Embryonic Stem Cells, Hematopoietic Stem Cells, Neuronal Stem Cells and Mesenchymal Stem Cells.

Table 2.1 Stem cells and their daughter cells [6]

Stem Cell Types	**Daughter Cells**
Embryonic stem cells	All type
Hematopoietic Stem Cells	Blood cells
Neuronal Stem cells	Neurons, glia
Mesenchymal Stem Cells	Muscle, bone, cartilage, tendon

2.2.1.2 Scaffolds

Extra Cellular Matrix (ECM) is a basal lamina of all tissues. The tissue organization is maintained by cell-cell as well cell matrix adhesive interactions, as well as ECM in which cells are embedded [6]. ECM is a molecular complex that has as basic components collagens and other glycoproteins, hyaluronic acid, proteoglycans and elastin [6]. The ECM also harbors molecules such as growth factors, cytokines, matrix degrading enzymes and their inhibitors. Cells- ECM interactions participate directly in promoting cell adhesion, migration, proliferation, differentiation and programmed death (apoptosis)[6].

Synthetic and naturally occurring polymers are an important element in new strategies for producing engineered tissue. Several classes of polymers have proved to be most useful in biomedical applications, including situations in which polymers remain in

intimate contact with cells and tissues for prolonged periods. These polymers can be appropriate for tissue engineering applications. But to select appropriate polymers for tissue engineering, it is necessary to understand the influence of polymer on cells viability, growth and functions.

How synthetic and naturally occurring polymers can help cells adhere, migrate, proliferate, differentiate into a specific cell with a particular function can be explained as follows:

2.2.1.2.1 Adhesion

Interactions between cells and the extra cellular matrix are mediated by cell surface glycoprotein and proteoglycan receptors interaction with proteins bound within the extra cellular matrix. Most tissue derived cells are anchorage dependent and require attachment to a solid surface for viability and growth. In tissue engineering, cell adhesion to a surface is critical because adhesion precedes other events such as spreading, migration and often differentiated cell function.

For cells attached to a solid substrate, cell behavior and function depend on the characteristics of the substrate [6]. There is a relationship between chemical or physical characteristics of the substrate and behavior or the function of attached cells. Polymers can frequently be made more suitable for cell attachment and growth by surface modification. So far no general principle has been identified which will allow prediction of the extent of attachment, spreading or growth of cultured cells on different polymer surfaces.

2.2.1.2.2 Migration

The migration of individual cells within a tissue is critical element in the formation of the architecture of organs and organisms. Hence cell migration is an important phenomenon in tissue engineering, because the ability of cells to move will be an essential part of new tissue formation or regeneration. Cell migration is promoted when fibronectin binds simultaneously to integrins through its cell binding domain and to proteoglycan receptors through its haparin binding domain [6]. Polymer scaffolds can be surface modified to express these molecules.

2.2.1.2.3 Proliferation

Most normal cells grow only when attached and spread on a solid substrate (Folkman and Moscona, 1978). Cells attach and spread in vitro either by depositing new ECM components or by binding to Exogenous ECM [6]. Polymer scaffolds can work as an exogenous ECM, which ultimately help cells attach and spread.

2.2.1.2.4 Differentiation

Interaction of cells with ECM molecules, hormones and growth factors is required to activate genes that are specific for differentiation [6]. Polymer scaffolds which can be harbored with hormones and growth factors can help cells differentiate.

2.2.1.2.5 Scaffolds Fabrication Methods [6]

The major requirement of any polymer processing technique is not only the utilization of biocompatible materials, but also the process should no way affect the biocompatibility of the polymer.

The ideal scaffold should have suitable pore size, so that the cells can ingrow and the internal surface area will be available for cells attachment. It should have a large surface area so that a high number of cells, sufficient to replace or restore organ function, can be cultured. It should also be suitable to incorporate bioactive molecules. Some of the common scaffolds fabrication methods are discussed below.

2.2.1.2.5.1 Fiber Bonding [6]

In this procedure, two biocompatible polymers are used. The fibers are made from the first polymer. Second polymer is dissolved into a solvent in which the first polymer one is insoluble. The resultant solution is cast over a nonwoven mesh of first polymer's fibers. The solvent is allowed to evaporate and residual amount is removed by vacuum drying. The resultant composite of two polymers is heated to a temperature above the melting point of first polymer for a particular time period. During heating the fibers of first polymer join at their cross points, but two polymers don't interact. The composite is quenched to prevent further melting of first polymer. Second polymer is selectively dissolved by a solvent of second polymer but not first one. The resultant structure is a bonded fibers of the first polymer. The second polymer is required to maintain a fiber like structure of the first polymer. It also prevents the fibers from collapsing at elevated temperatures.

The scaffolds made by this method have high area: volume ratios and high porosity, but this method lacks complex three dimensional structure, which is often required for tissue regeneration.

2.2.1.2.5.2 Solvent Casting and Particulate Leaching [6]

A biocompatible polymer is dissolved into a solvent. Sieved particulate particles (usually salt) are dispersed in a polymer solution. The resultant solution is casted into a container. The particulate particles are insoluble in the solvent in which the polymer is dissolved. The solvent is allowed to evaporate and residual amount is removed by vacuum drying. Thus the composite is a matrix of polymer with particulate particles dispersed. Such composite is kept into a medium in which the particulate is soluble but not polymer (usually water). As the time passes, the particulate particles dissolve leaving behind the pores of the same size.

Highly porous and interconnected pores scaffolds can be obtained by this method, but the scaffolds are usually brittle and hence can not be used for soft tissue applications.

2.2.1.2.5.3 Melt Molding [6]

In this method, the polymer is mixed with gelatin microspheres. The mixture is then poured into a mold of required shape. The mold is heated to a temperature above the glass transition temperature of the polymer. The composite is subsequently removed from the mold and placed into distilled or de ionized water. The gelatin which is soluble in water leaches out leaving behind the porous structure. The pore size and porosity can be changed by using gelatin spheres of different diameters and number of spheres.

This method is suitable to incorporate bioactive materials into the scaffold, but this capability of this method is limited by the polymer which is used to make scaffold, as some polymers may require heating above their melting points depending on their structure, which can be non compatible to bioactive materials.

2.2.1.2.5.4 Gas Foaming [6]

In this method polymer pellets are compression molded into solid disks. After compression, the disks are exposed to high pressure CO_2 to saturate the polymer. The subsequent reduction in pressure to ambient levels caused the nucleation and formation of pores in the polymer matrix from the CO_2 gas.

The scaffolds made by this method can have a closed pore morphology which is undesirable for tissue engineering applications. Again there is uncertainty of incorporation of bioactive materials, as exposure to high pressure and CO_2 may hamper their activity.

2.2.1.2.5.5 Freeze Drying [6]

In this method a desired polymer is dissolved in a solvent. Water is added so the solution. As water is immiscible with the polymer solution, the mixture is homogenized to form an emulsion. The emulsion is poured into a metal container or mold and quenched in liquid nitrogen. After quenching, the polymer scaffold is freeze dried to remove water and solvent.

By this method highly porous, interconnected porous scaffold structure can be obtained. This method also offers higher specific pore surface area as well as the ability to make thick scaffolds. This method is also suitable to incorporate bioactive material,

provided it is soluble in water. This method is also useful to fabricate drug delivery devices.

Hence in this study, freeze dry method is extensively used to make scaffolds. The scaffolds are incorporated with a drug. The viability of the scaffolds is also studied for cells culture.

2.2.1.3 Signals

Signals are mechanical and biochemical entities which regulate tissue development and maintenance in vivo. Mechanical signals include physical stimuli, hydrodynamic conditions, gravitational forces etc. The signals that are responsible for dictating tissue pattern are often mechanical in nature. The pattern generating effects of compression on bone, shear on blood vessels and tension on muscles are examples of mechanical signals dictating tissue pattern.

Biochemical signals include oxygen concentration, pH, cytokines, growth factors etc. Regulated growth factors/ hormone release from matrices or transplanted cells can create refined and controlled approach for tissue regeneration. Immobilized bioactive ligands on or within biomaterials control single and multiple cellular morphologies and functions via receptor mediated processes [3]. Inclusion of growth factors within tissue engineered therapies mimics the natural tissue environment and will presumably improve healing [3]. Several classes of polymers have proved to be most useful in biomedical applications, including situations in which the polymers remain in intimate contact with tissues for prolonged periods [6].

Table 2.2 Polymers and their potential Medical Applications. [6]

Polymer	Typical Application
Polydimethylsiloxane, silicone elastomers (PDMS)	Breast, Penile and testicular prostheses, catheters, drug delivery devices, heart valves, hydrocephalus shunts, membrane oxigenetors.
Polyurethanes (PEUs)	Artificial heart and ventricular assist devices, catheters, pacemaker leads.
Poly (Tetrafluoroethylene) (PTFE)	Heart valves, vascular grafts, facial prostheses, hydrocephalus shunts, membrane oxigenators, catheters, sutures.
Polyethylene (PE)	Hip prostheses, catheters.
Polysulfone (PSu)	Heart valves, penile prostheses.
Poly (methyl methacrylate) (pMMa)	Fracture fixation, intraocular lenses, dentures.
Poly (2-hydroxyethylmethacrylate) (pHEMA)	Contact lenses, catheters.
Polyacrylonitrile (PAN)	Dialysis membrane.
Polyamides	Dialysis membranes, sutures.
Polypropylene (PP)	Plasmapheresis membranes, sutures.
Poly (Vinyl Chloride) (PVC)	Plasmapheresis membranes, blood bags.
Poly (ethylene-covinyl acetate)	Drug delivery devices.
Poly (L-Lactic Acid), Poly (Glycolic Acid) and Poly (Lactide-co-glycolide) (PLA, PGA and PLGA)	Drug delivery devices, sutures.
Polystyrene (PS)	Tissue culture.
Poly (Vinyl pyrolidone) (PVP)	Blood substitutes.

2.2.2 Polymers

2.2.2.1 Synthetic Polymers

Cell behavior and function depend on the characteristics of the substrate. Cell adhesion appears to be maximized on surfaces with intermediate wettability [6]. Cells viability may also be related to interactions with the surface [6]. Polymers can frequently be made more suitable for cell attachment and growth by surface modification. The surface chemistry of polymers influences cell interactions *in vivo.*

2.2.2.2 Biodegradable Polymers

These polymers slowly degrade and then dissolve following implantation. This feature is important for many tissue engineering applications, because the polymer will disappear as functional tissue regenerates. Biodegradable polymers may provide an additional control over cell interactions: during polymer degradation, the surface of the polymer is constantly renewed, providing a dynamic substrate for cell attachment and growth [6].

Homopolymers and copolymers of poly (L-Lactic Acid), poly (Glycolic Acid), and poly (Lactide-co-Glycolide) (PLA, PGA and PLGA) are frequently used as cells culture substrate, because they have been successfully used as implantable sutures for several decades.

Polymers like PLA, PGA and PLGA undergo hydrolytic degradation. Hydrolysis is the scission of susceptible molecular functional groups by reaction with water. It may be catalyzed by acids, bases, salts or enzymes. It is a single step process in which the rate of scission is directly proportional to the rate of initiation of the reaction. A polymer's

susceptibility to hydrolysis is the result of its chemical structure, morphology, dimensions and the body's environment. The rate of hydrolysis tends to increase with a high proportion of hydrolysable groups in the main or side chain, other polar groups which enhance hydrophilicity, low crystallinity, low or negligible cross link density, a high ratio of exposed surface area to volume and very likely mechanical stress [6].

PGA is the simplest linear aliphatic polyester. It is highly crystalline. Because of its high crystallinity, it has a high melting point and low solubility in organic solvents.

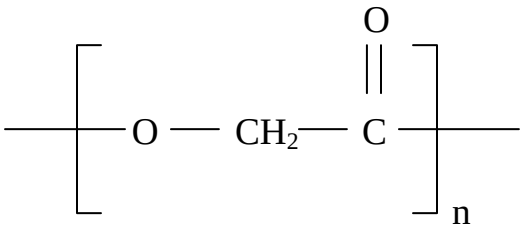


Fig 2.5 Poly Glycolic Acid (PGA)

Poly Lactic Acid (PLA) is linear polyester which is similar to Poly Glycolic Acid (PGA) with an extra methyl, which confers hydrophobicity to the polymer. Relatively PLA is more hydrophobic than PGA. The structural form of PLA can be given as follows.

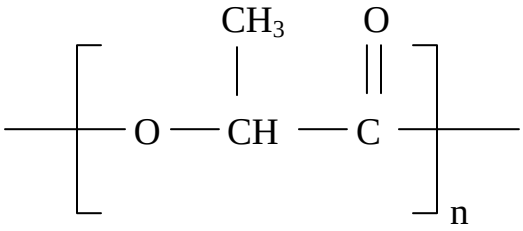


Fig 2.6 Poly Lactic Acid (PLA)

PLA is a chiral molecule and exists in two stereo isomeric forms that give rise to four morphologically distinct polymers. *d*-PLA and *l*-PLA are two stereo regular polymers, *d,l*- PLA is a racemic polymer obtained from a mixture of *d* and *l*-lactic acid and meso-PLA can be obtained from *d,l*-lactide.

The copolymers of PGA and PLA have a wide range of applications. The hydrophobicity of PLA limits water uptake. The crystallinity of PGA is lost in PLA-PGA copolymer. This morphologic change leads to an increase in the rates of hydration and hydrolysis. Thus copolymers of PLA-PGA tend to degrade faster than either PLA or PGA [6]. There is no linear relationship between the ratio of glycolic acid and lactic acid and the physicomechanical properties of their copolymers.

CHAPTER 3

MATERIALS AND METHODS

Two types of scaffolds were prepared by emulsion freeze dry method. Both types of scaffolds were studied for drug release by using Acetaminophen as a model drug.

3.1 Polymer Microspheres Dispersed Scaffold

In this type of scaffolds, polymer micro spheres embedded with drug were dispersed into another polymer solution and the freeze dry method was followed to make the scaffold.

3.1.1 Materials

Poly (L-Lactic Acid) (PLLA), was obtained from Dow Cargill with molecular weight ~110,000 D. 100 % hydrolyzed Poly Vinyl Alcohol (PVA) with average Molecular Weight of 14000 D was obtained from Aldrich Chemicals. Acetaminophen obtained from Sigma-Aldrich was used as a model drug, it's a hydrophilic drug commonly used to relive pain and aches associated with many conditions. Dulbecco's Phosphate Buffered Saline (10X DPBS) was obtained from Cambrex Bio Science. Chloroform was obtained from EM science.

A homogenizer with speed from 11000 RPM to 30000 RPM and a blender with speeds from 560 RPM to 1160 RPM were purchased from CAT and Toastmaster respectively.

3.1.2 Method

10 % (w/v) PLLA solution was prepared by dissolving 1 gm of PLLA in 10 ml chloroform. PVA solution was prepared by dissolving PVA in De ionized water at temp slightly greater than 65ºC. Acetaminophen solution was prepared by dissolving known

amount of Acetaminophen in already prepared PVA solution at room temp. An emulsion of PLLA solution and PVA + Acetaminophen solution with a ratio 1:1 (2ml of each) was homogenized using homogenizer at various speeds. The resultant emulsion was blended with remaining amount of PLLA solution by using blender, again at various speeds. The blended mixture of PLLA, PVA and Acetaminophen was poured into ~4.5 cm diameter aluminum Petri dish and was immediately frozen by using liquid Nitrogen. The frozen emulsion was freeze vacuum dried for approx. 12 hours and vacuum dried for another 12 hours. Various combinations were obtained for scaffolds fabrication by varying PVA and Acetaminophen concentration, homogenizer speed and blender speed and are described in the following table.

Table 3.1 Different parameters for scaffolds fabrication

S.N.	Acetaminophen Solution Ingredients		Amount of Acetaminophen solution used (ml)	Homogenizer Speed (RPM)	Blender Speed (RPM)
	PVA Conc. (% w/v)	Acetaminophen Conc. (% w/v)			
1	0.5	0.5	2	11000	560
2	0.5	0.5	2	11000	1120
3	0.5	0.5	2	22000	1120
4	1.5	1.0	2	22000	1120
5	1.5	1.0	2	11000	1120
6	1.5	1.0	2	22000	560
7	1.5	1.0	2	11000	560
8	0.5	0.5	2	22000	560

3.1.3 SEM Analysis of the Scaffolds

To analyze the scaffolds under SEM, small pieces were cut from the scaffolds. Using sputtering machine, the samples were coated by Carbon at 8 mTorr of gas pressure for 8 minutes. The samples were mounted on sample holder and observed under SEM at 15 kV.

3.1.4 Drug Release Study

3.1.4.1 Derivation of Acetaminophen Concentration Equation

Acetaminophen was dissolved in 10X DPBS. Absorbance spectra for Acetaminophen solutions at concentrations 1.5, 3, 7.5, 10, 12, 15, 30 and 50 µg/ml was obtained by using UV/ Vis Spectrophotometer in Absorbance mode.

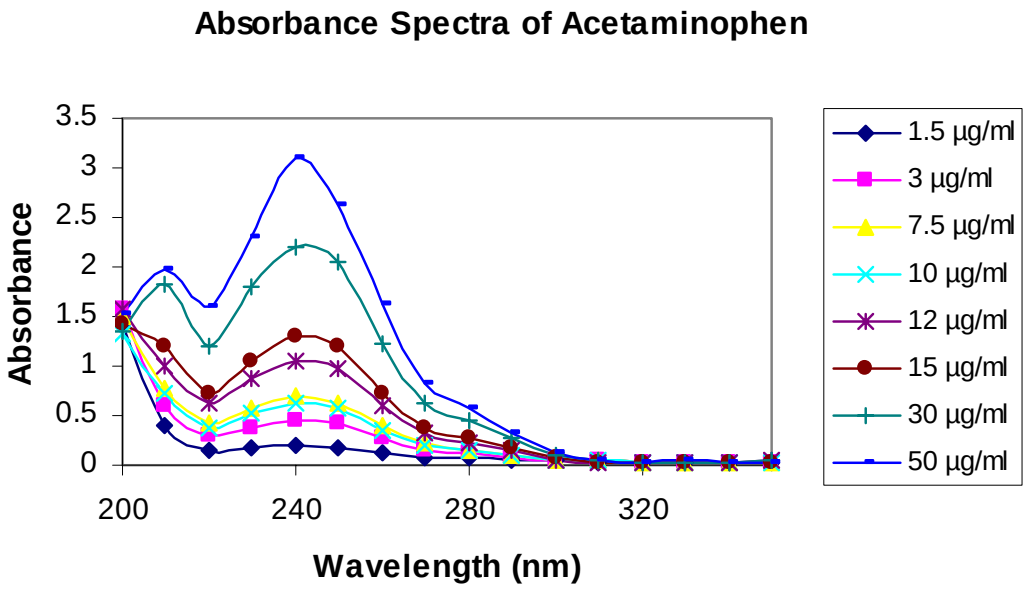


Fig 3.1 Absorbance spectra of Acetaminophen at different concentrations

It was observed that Acetaminophen has a maximum absorbance at a wavelength of 240 nm. Hence absorbance at this wavelength was used to calibrate a curve.

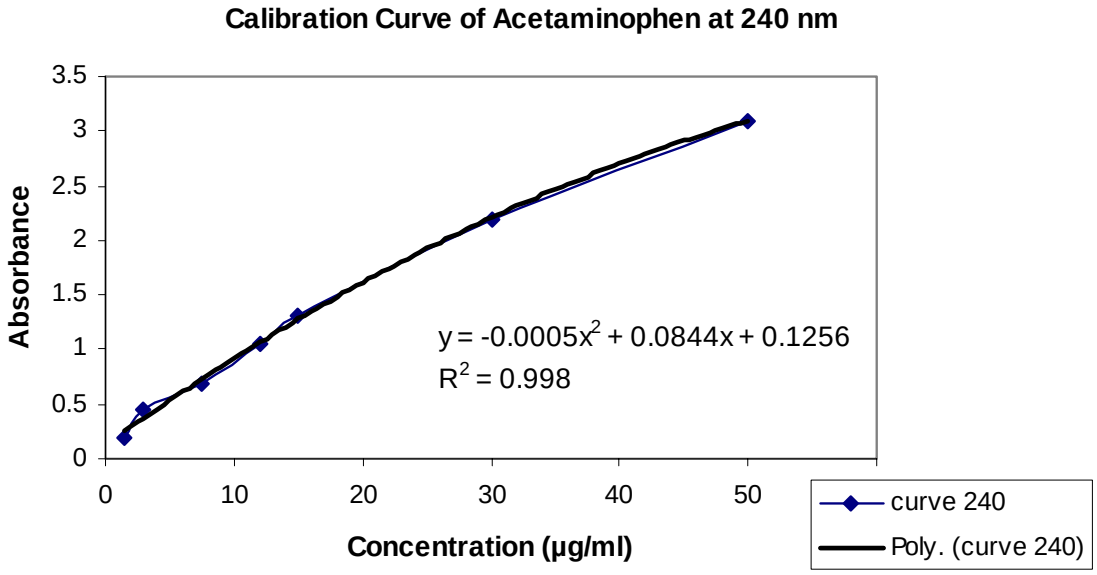


Fig 3.2 Calibration curve of Acetaminophen at a wavelength of 240 nm

A polynomial equation was obtained from the trend line of the original curve. The equation for the concentration of Acetaminophen can be given as:

$$\boldsymbol{Y = -0.0005 \times x2 + 0.0844 \times x + 0.1256}$$

Where $\boldsymbol{x}$ = Absorbance at 240 nm,

And $\boldsymbol{Y}$ = Acetaminophen Concentration in µg/ml.

3.1.4.2 Acetaminophen Release Study

Small pieces of scaffolds were cut and weighed. Each piece was placed into clean 22ml capacity vials filled with 10X DPBS (Dulbecco's Phosphate Buffered Saline). The vials were kept on a rotating frame inclined at ~35 º and at a speed of 9 RPM. Due to the high porosity and low density of the polymers, it was observed that the scaffolds floated on DPBS. To submerge the scaffolds into the DPBS, a spiral made from stainless steel wire was fixed to each piece, which submerged the scaffolds into DPBS although not completely sunk to the bottom and hence the scaffolds were completely surrounded by DPBS. The whole setup was kept into a thermal chamber maintained at 37º C (±1 º C).

To minimize the effect of surface area of scaffolds exposed to DPBS, a ratio of 0.0999 (gm of Scaffold): 5 (ml of DPBS) was maintained for all scaffolds. The weight of each piece of scaffold and amount of DPBS is given as follows:

Table 3.2 Samples specifications for drug release study

S.N.	Weight of Scaffold (gm)	Amount of DPBS (ml)
1	0.0942	4.71
2	0.0964	4.82
3	0.0973	4.87
4	0.1068	5.34
5	0.0999	5
6	0.1062	5.31
7	0.0955	4.78
8	0.1029	5.11

To measure the concentration of Acetaminophen, 3 ml of DPBS was taken out of each vial and poured into a clean cuvette. The cuvette was placed into a UV/Vis Spectrophotometer and analyzed under Absorbance mode. The absorbance for each sample was noted. By using MATLAB software, the concentration of Acetaminophen in each vial was calculated from Acetaminophen concentration equation. The solution in the cuvette was returned back to the vials after UV/ Vis Spectrophotometer study.

The drug release study of each sample of scaffold was done 1, 2.5, 5, 11, 24, 48, 72, 96, 120 and 144 hours after the samples were kept into DPBS. The amount of acetaminophen in each scaffold was calculated.

3.2 Layered Scaffolds

In this type of Scaffolds, Drug solution emulsified with a polymer solution was sandwiched between two layers of emulsions of the same polymer but without drug.

3.2.1 Materials

The material used for this type of scaffolds was the same as that was used for the Polymer Microsphere Dispersed Scaffolds.

3.2.2 Method

10 % (w/v) PLLA solution was obtained by dissolving 1 gm of PLLA in 10 ml chloroform. Two PVA solutions were prepared by dissolving known amount of PVA in 50 ml De ionized water at temp slightly greater than 65ºC. 1 % (w/v) Acetaminophen solution was prepared by dissolving 0.5 gm of Acetaminophen in one of the PVA solutions already prepared at room temp.

3.2.2.1 Preparation of Bottom layer

A known amount of PLLA solution was blended with PVA solution by using a blender at a specific speed. The emulsion was poured into an aluminum Petri dish of diameter ~4.5 cm. This emulsion was frozen by using liquid nitrogen in such a way that approximately half bottom part of the emulsion was frozen and remaining upper half still emulsion. An emulsion made for middle layer was poured on the top of this half frozen emulsion.

3.2.2.2 Preparation of Middle Layer

A known amount of PLLA solution was blended with a known amount of PVA + Acetaminophen solution and blended by using a blender at a particular speed. This emulsion was poured on top of the bottom layer which was almost half frozen as described in bottom layer preparation method. The middle layer emulsion was frozen by using liquid nitrogen in such a way that half bottom of the middle layer was frozen and

upper half still an emulsion, and this was visually determined. Later the top layer emulsion was poured on top of the middle layer emulsion.

3.2.2.3 Preparation of Top Layer

A known amount of PLLA solution was blended with a known amount of PVA solution by using a blender at specific speed. The emulsion was poured on top of half frozen middle layer emulsion and was completely frozen using liquid nitrogen.

Pouring an emulsion of subsequent layer on top of half frozen previous layer emulsion made sure that there was no boundary between layers and two emulsions mixed homogenously to form a scaffold with uniform pore size and porosity.

The resultant frozen emulsion was Freeze vacuum dried for 12 hours and vacuum dried for another 12 hours. Chloroform and water escaped from the scaffold by sublimation.

3.2.3 Drug Release study

Three different combinations were obtained in two different studies of drug release from layered scaffolds by varying PVA concentration, blender speed and amount of PLLA solution and PVA solutions (PVA with Acetaminophen and without Acetaminophen) in different layers of the scaffold.

3.2.3.1 Drug Release Study 1

Table 3.3 Constituents of the scaffold for drug release study 1 of Layered scaffold

Layer	**PVA Concentration (% w/v)**	**Amount of PLLA solution (ml)**	**Amount of PVA solution (ml)**	**Amount of PVA + Drug solution (ml)**	**Blender speed (RPM)**
Bottom	1.5	3	1	-	1120
Middle	1.5	3	-	1	1120
Top	1.5	3	1	-	1120

3.2.3.1.1 Sample Preparation

A piece of scaffold weighing 0.0990 gm was cut. Four surfaces (except top and bottom) were coated by a PLLA solution to prevent direct release of Acetaminophen from exposed middle layer of the scaffold which had embedded drug into it. To prevent the scaffold from floating into DPBS, a spiral of stainless steel wire was fixed to the bottom of piece of scaffold. The scaffold with a stainless steel wire spiral was placed into a 22ml capacity clean vial. 10 ml of DPBS was poured into the vial. The vial was kept on a rotating frame inclined at ~35º and rotating at a speed of 9 RPM. The whole setup was kept into a thermal chamber maintained at 37º C (±1 º C).

3.2.3.1.2 Acetaminophen Release Study

3 ml of DPBS solution was taken out of the vial and poured into a clean cuvette. The cuvette was placed into UV/ Vis Spectrophotometer and analyzed under Absorbance mode. The concentration of Acetaminophen was calculated from the equation derived earlier by using MATLAB software. The drug release study was performed every after a period of 24 hours and for 25 days. The amount of Acetaminophen present into the sample was calculated and percentage of drug release from the sample was analyzed.

3.2.3.2 Drug Release Study 2

Two different scaffolds were prepared. The scaffolds are numbered as **1** and **2**. Acetaminophen concentration in both the scaffolds was 1% (w/v). The constituents of the scaffolds are given as follows:

Table 3.4 Constituents of the scaffold for drug release study 2 of Layered scaffold

Layer	**PVA Concentration (% w/v)**		**Amount of PLLA Solution (ml)**		**Amount of PVA Solution (ml)**		**Amount of PVA + Drug Solution (ml)**		**Blender Speed (RPM)**	
	1	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
Bottom	1	1.5	3	3	1	1	-	-	560	560
Middle	1	1.5	4	4	-	-	1	1	560	560
Top	1	1.5	3	3	1	1	-	-	560	560

3.2.3.2.1 Sample Preparation

The pieces weighing 0.0894gm and 0.1202 gm were cut from scaffolds 1 and 2 respectively. Four surfaces (except top and bottom) of the scaffolds were coated by a PLLA solution to prevent direct release of Acetaminophen from exposed middle layer of the scaffold which had embedded drug into it.

To prevent the scaffold from floating into DPBS, a spiral of stainless steel was fixed to the bottom of each piece of scaffold. The scaffold pieces with a stainless steel wire spirals were placed into two 22ml capacity clean vials. To minimize the effect of variations in weights of the scaffolds a ratio of 0.1202 gm (scaffold):10 ml (DPBS) was used. Hence in case of scaffold 1, 7.5 ml of DPBS and for scaffold 2, 10 ml of DPBS was poured into the vial. The vials were kept on a rotating frame inclined at ~35º and rotating at a speed of 9 RPM. The whole setup was kept into a thermal chamber maintained at 37º C (±1 º C).

3.2.3.2.2 Acetaminophen Release Study

3 ml of DPBS solution was taken out from each vial and poured into a clean cuvette. The cuvette was placed into UV/ Vis Spectrophotometer and analyzed under Absorbance mode. The concentration of Acetaminophen was calculated from the equation derived earlier by using MATLAB software. The drug release study was performed every after a period of 24 hours and for 21 days.

3.2.3.2.3 Cross Section Analysis of Scaffolds

Approximately 6mm × 4mm pieces were cut from each scaffold. The pieces were fixed into wax by pouring molten wax at ~ 47 ºC into a plastic container. The assembly of samples in wax was let to cool for 1 day. The sections of the wax were prepared by using microtome. The thickness of the sections was in the rage of 35 µm to 60 µm. the sections were fixed on glass slides and soaked in Xylene for 1-2 hours. Xylene de waxed the samples. The glass slides were observed under optical microscope.

3.2.3.2.4 Calculation of pore size and porosity

Pore size was calculated from SEM pictures and Cross Section pictures of the scaffolds. Porosity is a ratio of volume of pores to the total volume of scaffold. Density of a material relates it's mass to volume. If any two entities of this relation are known then, third entity can be determined. The same principle was followed to calculate the porosity. PLLA was dissolved in chloroform. The solution was poured into flat container. The chloroform evaporated and a thin sheet of PLLA was formed. A piece was cut from the sheet. The dimensions of the piece were measured by using a micrometer screw gauge and found to be 0.0479" × 0.2238" × 0.5455". The piece weighed 0.0439 gm. The density of PLLA was obtained from weight and volume of the piece of PLLA sheet and was found to be 0.4581 gm/ml. The density of Acetaminophen is 1.2083 gm/ml [10]. The density of PVA was 1.25 gm/ml [11].

3.3 Cells Culture on Scaffolds

3.3.1 Scaffold fabrication

3.3.1.1 Materials

85:15 PLLA: PGA copolymer was purchased from Birmingham Division of DURECT Corporation, Pelham, AL. All other materials were same as described in earlier Materials section of scaffolds.

3.3.1.2 Method

10 % (w/v) solution of polymer was prepared by dissolving 1gm of PLLA: PGA copolymer in 10ml of chloroform. A PVA solution was prepared by dissolving known amount of PVA in De Ionized water at temp ~65º C. The PVA solution was let to cool down at room temperature. The polymer solution was blended with 3 ml of PVA solution by using a blender at various speeds. The emulsion was poured into a Petri dish of ~3.5 cm diameter and was immediately frozen using liquid nitrogen. The frozen emulsion was freeze vacuum dried for 12 hours and vacuum dried for another 12 hours. Following combinations were obtained by varying PVA concentration and speed of the blender.

Table 3.5 Parameters for scaffolds fabrication for cells culture

S.N.	PVA Conc. (% w/v)	Blender Speed (RPM)
1	0.5	560
2	0.5	1120
3	1.5	560
4	1.5	1120

3.3.2 Calculation of Pore size and Porosity

The pore size was measured from SEM pictures of the scaffolds. Here the same principle of density, weight and volume was followed to calculate the porosity of the scaffolds. The density of PLLA: PGA (85:15) Copolymer was obtained from the company from which the polymer was purchased and was 1.27 gm/ml. Other data was similar as obtained in earlier study of measurement of Pore size and Porosity of scaffolds.

3.3.3 Cells Culture

3.3.3.1 Materials

3T3 cells were used as model cells for culturing**.** Dulbecco's Modified Eagle Medium (DMEM) was used a culture medium**.** Four types of scaffolds prepared by the method described in section 3.3.1 were used to culture cells.

3.3.3.2 Method

Approximately 7mm × 7mm pieces were cut from each four types of scaffolds. Each piece was dipped into 70% for 3min and later in 100% ethanol for 3min. The scaffolds were allowed to dry at room temperature for ~10 min. The pieces were kept in different wells of 12 well plate. 5ml of DMEM and 0.5 ml of calf serum was added to wells containing scaffolds. The well plate was kept into incubator at 37º C and 5% CO_2. The scaffolds were observed for any color change in the DMEM medium for 3 days. The medium was changed and 3T3 cells with a concentration of 72,500 cells/ml was obtained from cells at a concentration of ~5,12,727 cells/ml. ~ 5000 cells were seeded on each scaffold by dropping 70µl of cells medium on top of each scaffold. 5 ml of DMEM with

serum was added to each well and the well plate was kept into incubator. The medium was changed every after a period of 3 days. The scaffolds were kept for cells culture for 2 weeks.

3.3.3.3 Histological study of Scaffolds

3.3.3.3.1 Scaffolds fixing and embedding in wax

One of the four scaffolds was taken out of the culture medium and placed into formalin for 1 hour. The scaffold was then placed into a series of ethanol with concentration varying from 75 % to 90 % to 100 % for 15 minutes each. Later the sample was kept into a solution of 50 % Xylene / 50 % ethanol for 5 minutes. Later the sample was kept into 100 % Xylene for another 5 minutes. Lastly the scaffold was kept to dry for ~ 15 minutes. The air dried scaffold was placed into a small plastic container. A paraffin wax was melted and poured into the container at ~ 47 º C. The wax was allowed to cool for one day.

3.3.3.3.2 H & E staining

The sections of 35 µm were prepared by using a microtome. The sections were dropped in hot water at ~ 48 º C. The sections were then placed on glass slide. The H & E staining protocol used in histological study of the scaffolds is given as follows:

1. Samples were dipped into 100 % Xylene for 15-20 minutes.
2. Samples were dipped into 50 % Xylene / 50 % ethanol for 3 minutes, 1 time.
3. Samples were kept into 100 % ethanol for 2 minutes, 2 times.
4. Samples were washed by Distilled water for 2 minutes, 2 times.
5. Samples were air dried for 10 minutes.
6. Samples were kept in 33 % Formalin / 33 % ethanol / 33 % water for 3 minutes at room temp.
7. Samples were washed by distilled water, 10 dips, 3 times.

8. Samples were stained by SIGMA Harris Hematoxylene for 2 minutes at room temperature.
9. Samples were washed by distilled water, 10 dips, 3 times.
10. Samples were kept in 0.1 % HCl for 1 minute.
11. Samples were dipped in Scott's solution. (A mixture of 15 gm $MgSO_4$ + 1.5 gm Na_2CO_3 in 1 liter of distilled water).
12. Samples were washed by distilled water, 10 dips, 2 times.
13. Samples were stained by SIGMA Eosin Y for 2 minutes at room temperature.
14. Samples were dipped in 95 % ethanol for 1 minute.
15. Samples were dipped in 100 % ethanol for 1 minute, 2 times.
16. Samples were dipped in 50 % Xylene / 50 % ethanol for 1 minute.
17. Samples were dipped in 100 % Xylene for 1 minute, 2 times.

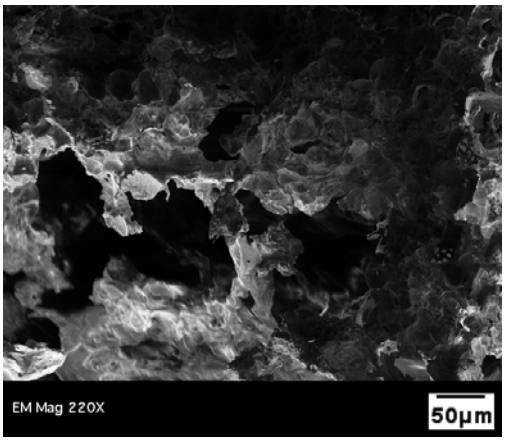
The samples were observed under optical microscope.

CHAPTER 4

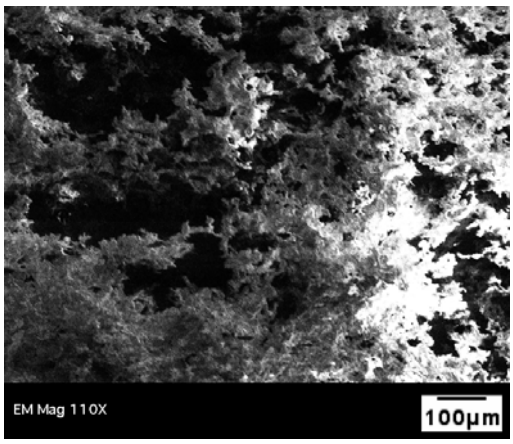
RESULTS

4.1 Polymer Microspheres Dispersed Scaffold

4.1.1 SEM Analysis of Scaffolds

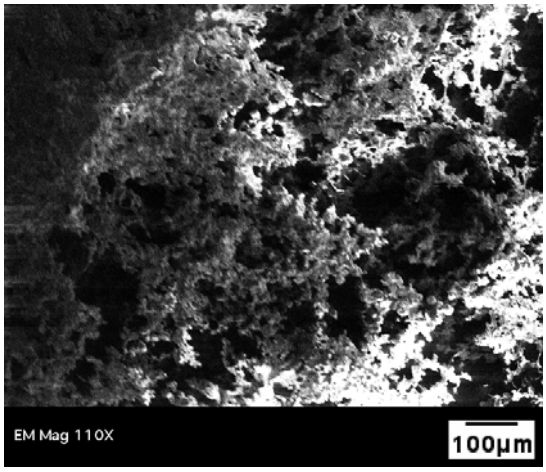


(a)

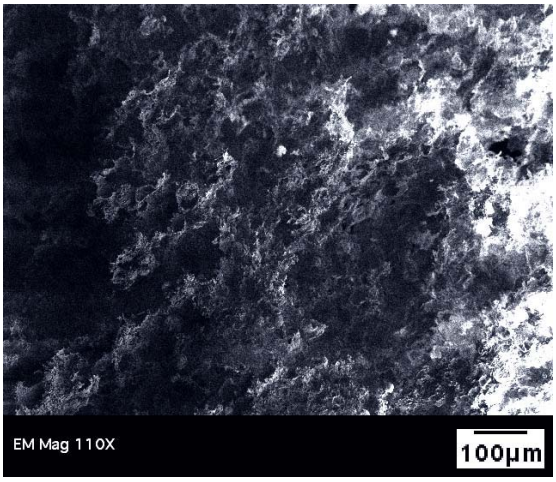


(b)

Fig 4.1 SEM pictures of scaffolds, prepared with (a) 0.5 % w/v concentration of PVA and Acetaminophen, Homogenizer speed of 11000 RPM and Blender speed of 560 RPM. (Scaffold 1) (b) 0.5 % w/v concentration of PVA and Acetaminophen, Homogenizer speed of 11000 RPM and Blender speed of 1120 RPM. (Scaffold 2)

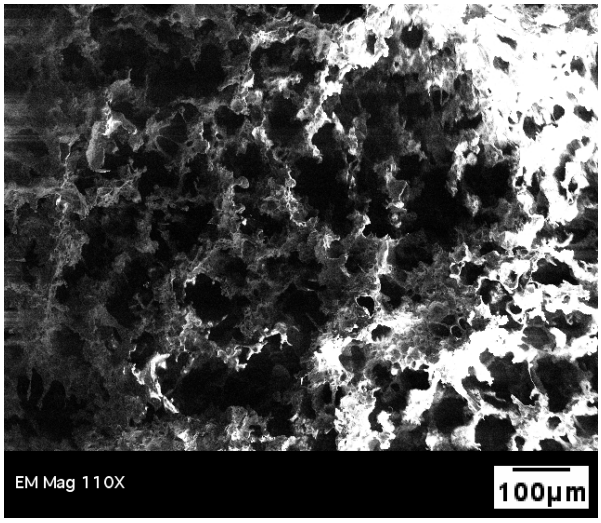


(a)

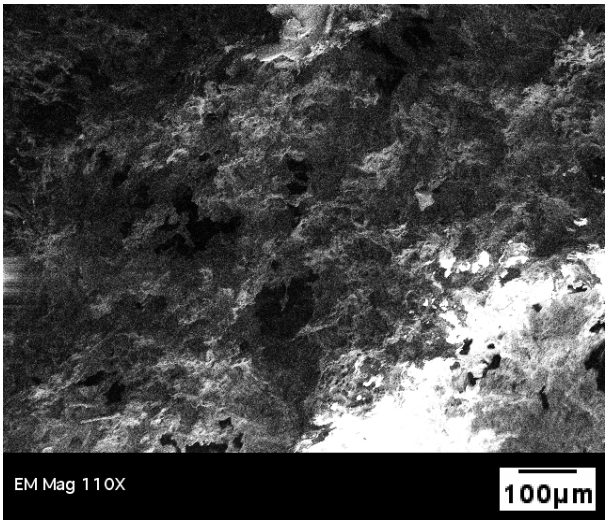


(b)

Fig 4.2 SEM pictures of scaffolds, prepared with (a) 1.5 % w/v concentration of PVA and 1.0 % w/v concentration of Acetaminophen, Homogenizer speed of 22000 RPM and Blender speed of 1120 RPM. (Scaffold 4) (b) 1.5 % w/v concentration of PVA and 1.0 % w/v con concentration of Acetaminophen, Homogenizer speed of 22000 RPM and Blender speed of 560 RPM. (Scaffold 6)



(a)



(b)

Fig 4.3 SEM pictures of scaffolds, prepared with (a) 1.5 % w/v concentration of PVA and 1.0 % w/v concentration of Acetaminophen, Homogenizer speed of 11000 RPM and Blender speed of 560 RPM. (Scaffold 7) (b) 0.5 % w/v concentration of PVA and Acetaminophen, Homogenizer speed of 22000 RPM and Blender speed of 560 RPM. (Scaffold 8)

From fig 4.1 (a) and (b), it can be seen that the scaffolds 1 and 2 are highly porous. These scaffolds had same constituent values like, Acetaminophen concentration (0.5 % w/v), homogenizer speed (11000) and PVA concentration (0.5 % w/v), only the difference was in blender speed. Scaffold 1 was made at 560 RPM speed of blender and scaffold 2 at 1120 RPM. It can be observed that, these scaffolds have pores having size in the range of 25 µm to 250 µm, though scaffold 2 has pores uniformly distributed though out its body as compared to scaffold 1. This might have caused because of higher blender speed. At high speed of blender, the emulsion is more uniformly blended than at lower speed.

From fig 4.2 (a) and (b), it is observed that, scaffolds 4 and 6 have same constituent values like, Acetaminophen concentration (1.0 % w/v), homogenizer speed (22000) and PVA concentration (1.5 % w/v), only the difference is in blender speed. Scaffold 4 is made at 1120 RPM speed of blender and scaffold 6 at 560 RPM. As compared to scaffold 6, scaffold 4 is more porous and has higher proportion of large pores, though pore size in both scaffolds is in the range of 25 µm to 250 µm. Scaffold 6 has very less pores and hence the resultant pores are not interconnected. Although it was made at 560 RPM speed of blender, the blending time was not monitored, and as a result the emulsion might not have blended thoroughly.

From fig 4.3 (a), it can be seen that, scaffold 7 is highly porous. It has pores in the range of 25 µm to 250 µm and pores are uniformly spread. It has larger pores and they are interconnected. This scaffold is made at 11000 RPM speed of homogenizer, which resulted into the bigger microspheres and blender speed of 560 resulted into larger

pores. As the proportion of larger pores into the scaffold increases, the pores get interconnected.

From fig 4.3 (b), it cab be seen that, the scaffold 8 is not very porous, though it has larger pores, the proportion of pores to remaining mass of scaffold is very less. The larger pores are caused because of the low speed of blender (560 RPM) but high speed of homogenizer (22000 RPM) resulted into smaller microspheres. Having less proportion of large pores, it lacks connectivity of pores; hence there might be some pores which are not connected at all.

Overall it can be seen that, majority of the scaffolds made at 560 RPM speed of blender have larger pores than scaffolds made at 1120 RPM. Because of higher proportions of larger pores, the pores are interconnected. Scaffolds 1, 2, 4 and 7 are highly porous and have large connectivity of pores. It can be observed that, scaffold 6 is the least porous scaffold and scaffold 8 is intermediate to other scaffolds morphologies.

4.1.2 Drug Release Study

A polynomial equation for Acetaminophen concentration was derived from the absorbance spectra of Acetaminophen at various concentrations and is described in section 3.1.4.1. The equation is;

$$\boldsymbol{Y = -0.0005 \times x2 + 0.0844 \times x + 0.1256}$$

Where $\boldsymbol{x}$ = Absorbance at 240 nm,

And $\boldsymbol{Y}$ = Acetaminophen Concentration in µg/ml.

The amount of drug in a system can be given as;

Accumulation = Drug Incoming - Drug Outgoing + Drug Generation – Drug Consumption.

In the present study, it was clear that, there was only Drug Incoming from scaffold and no drug outgoing, generation or consumption, in the system. Hence the concentration of Acetaminophen into the vial was equivalent to amount of Acetaminophen released from the scaffolds. The concentrations of Acetaminophen were calculated by using the equation given above.

It was observed that, there was a structural irregularity in scaffolds numbered as 3 and 5; the reason could be human error or technical error. These scaffolds showed little or no drug release after 24 hours, indicating that, no pores were connected in the interior of the scaffolds or got collapsed in the absence of liquid nitrogen while freeze drying and , hence were not considered for drug release study. During freeze drying, if the emulsion is not kept frozen for entire procedure, the chloroform melts, resulting in collapse of an emulsion.

The scaffold 3 was prepared with 0.5 % w/v concentration of PVA solution having 0.5 % w/v concentration of Acetaminophen, 22000 RPM homogenizer speed and 1120 RPM blender speed. The scaffold 5 was prepared with 1.5 % w/v concentration of PVA solution having 1.0 % w/v concentration of Acetaminophen, 11000 RPM speed of homogenizer and 1120 RPM speed of blender.

For convenience the drug release curves are named after the variables with which the scaffolds are made. The numbers are prefixed by letter P, A, H and B representing **P**VA concentration, **A**cetaminophen concentration in PVA solution, **H**omogenizer speed and **B**lender speed respectively.

Table 4.1 Scaffolds numbers and their corresponding names of curves

# of Scaffold	**Name of corresponding Curve**
1	P0.5, A0.5, H11K, B560
2	P0.5, A0.5, H11K, B1120
4	P1.5, A1, H22K, B1120
6	P1.5, A1, H22K, B560
7	P1.5, A1, H11K, B560
8	P0.5, A0.5, H22K, B560

The cumulative release of Acetaminophen can be given as follows:

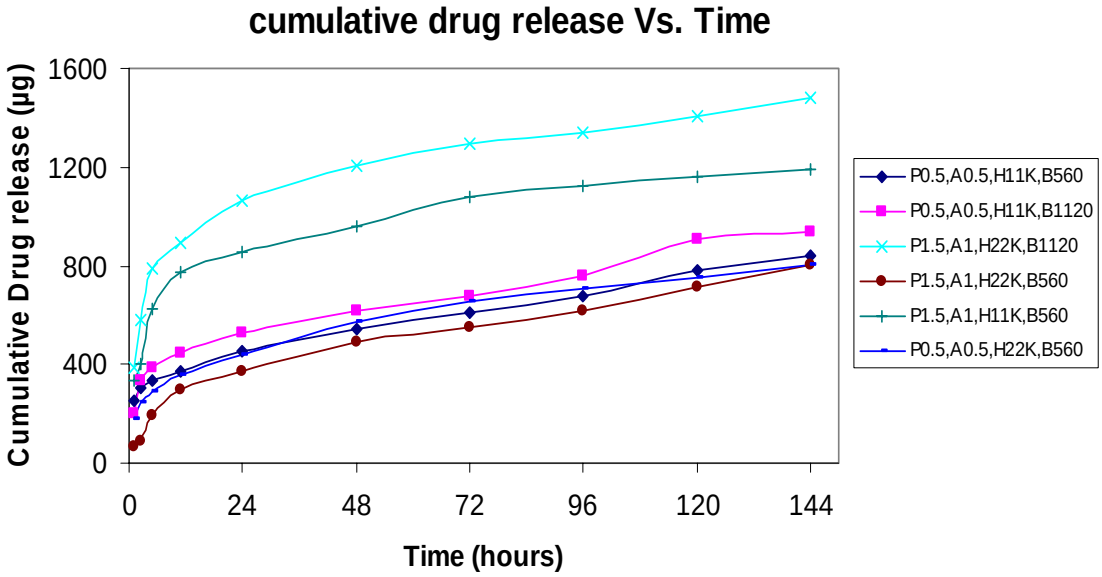


Fig 4.4 Cumulative Drug Release from the scaffolds plotted against time

From the graph it can be seen that, the scaffolds with lower drug concentration have smaller cumulative drug release, and the scaffolds having higher drug concentration have higher cumulative drug release, which is quite natural. Only the exception is seen with scaffold 6 (P1.5,A1,H22K,B560). In spite of higher concentration of drug, this scaffold has low cumulative drug release. From fig 4.2 (d), it can be seen that, this scaffold is not very porous; as a result the pores are not connected to large extent. For a good drug release, pore size as well as porosity is highly important. The release of drug does not depend on just drug concentration, there are other factors which have a great influence on drug release like blender speed and homogenizer speed, which are designing the structure of the scaffolds.

The total amount of Acetaminophen present in the scaffolds was calculated for each piece of scaffold.

Table 4.2 Weights and amount of Acetaminophen present in scaffolds

S.N.	Weight of Sample (g)	Amount of Acetaminophen (µg)
1	0.0942	923.52941
2	0.0964	945.09804
4	0.1068	2034.2857
6	0.1062	2022.8571
7	0.0955	1819.0476
8	0.1029	1008.8235

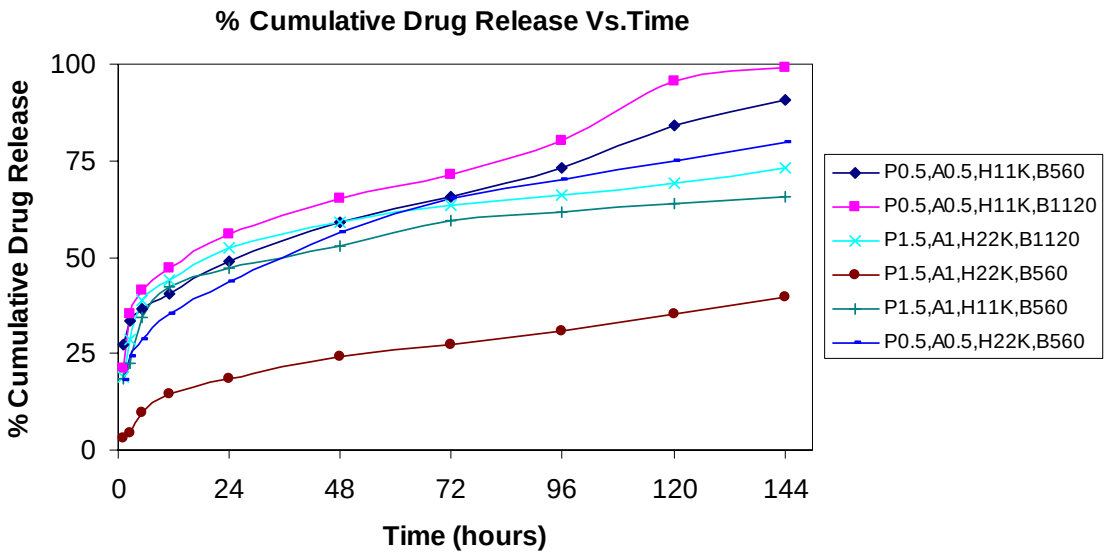


Fig 4.5 Percentage Cumulative Drug Release from the scaffolds plotted against time

Because the scaffolds had different drug concentrations, the relative comparison of drug release is possible only when the percentage of total drug released by the scaffold is studied. From fig. 4.5, it can be seen that, the scaffolds with low drug concentration

have higher percentage of drug released at a particular time. For scaffolds having same drug concentration, there are other factors associated with the drug release such as blender speed and homogenizer speed. For example, in case of scaffold 1 and 2, scaffold 2 has a higher percentage of drug release than scaffold 1. There are three factors, like PVA, drug concentration and homogenizer speed, same for both of these scaffolds; only the difference is in blender speed. Because the scaffolds have different combinations of factors, it is important to determine the effect of each factor on drug release. The influence of these factors on drug release is studied in details in Design of Experiments Drug release study.

Since the drug release is a curve with second order polynomial equation, the drug release rate varies with time. The drug release rate was calculated from the drug release data and given as follows:

$$R = \frac{D_2 - D_1}{T_2 - T_1}$$

Where:

R Drug Release Rate (µg/ hr)

D_2 Amount of Drug Released after Time T_2 (µg)

D_1 Amount of Drug Released after Time T_1 (µg)

T_2, T_1 Time in hours at which the Drug Release was measured.

By using this formula various time periods are considered to calculate the rate of drug release and are given as follows.

4.1.2.1 Drug Release Rate R (µg/hr) with $T_2 - T_1 = 1$ (hour) for each scaffold

Table 4.3 Drug Concentrations and Release Rates with T2 =1 hr and T1 = 0 hr

S.N.	Drug Concentration at Time T1= 0 hr (% w/w)	Actual Amount of Drug Released During this Interval	Drug Release Rate (µg/hr)
1	0.98	252.6435	252.64
2	0.98	197.8735	197.87
4	1.90	384.667	384.67
6	1.90	64.03595	64.04
7	1.90	332.7783	332.78
8	0.98	181.1326	181.13

4.1.2.2 Drug Release Rate R (µg/hr) with $T_2 - T_1 = 4$ (hours) for each scaffold

Table 4.4 Drug Concentrations and Release Rates with T2 = 5 hrs and T1 = 1 hr

S.N.	Drug Concentration at Time T1= 1 hr (% w/w)	Actual Amount of Drug Released During this Interval	Drug Release Rate (µg/hr)
1	0.71	85.223211	21.31
2	0.78	191.98783	48.00
4	1.55	403.174806	100.79
6	1.85	128.645901	32.16
7	1.56	292.96859	73.24
8	0.81	109.259465	27.31

4.1.2.3 Drug Release Rate R (µg/hr) with T_2 - T_1 = 19 (hours) for each scaffold

Table 4.5 Drug Concentrations and Release Rates with T2 = 24 hrs and T1 = 5 hours

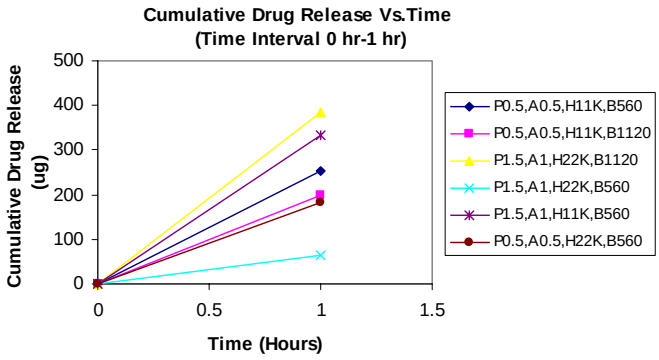
S.N.	Drug Concentration at Time T1 = 5 hrs (% w/w)	Actual Amount of Drug Released During this Interval	Drug Release Rate (µg/hr)
1	0.62	114.438399	6.02
2	0.58	138.241456	7.28
4	1.18	275.486862	14.50
6	1.73	179.389854	9.44
7	1.26	232.860568	12.26
8	0.70	150.134866	7.90

4.1.2.4 Drug Release Rate R (µg/hr) with T_2 - T_1 = 120 (hours) for each scaffold

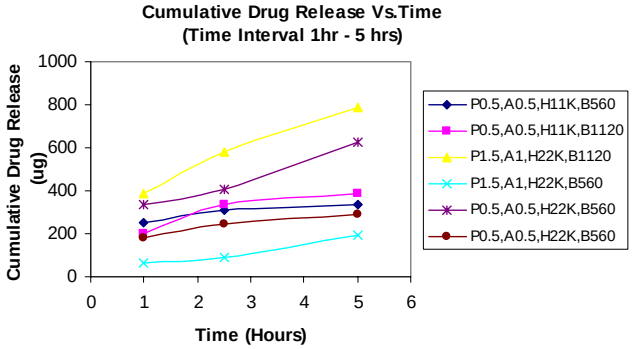
Table 4.6 Drug Concentrations and Release Rates with T2 = 144 hrs and T1 = 24 hours

S.N.	Drug Concentration at Time T1 = 24 hrs (% w/w)	Actual Amount of Drug Released During this Interval	Drug Release Rate (µg/hr)
1	0.50	385.844323	3.22
2	0.43	409.29358	3.41
4	0.92	420.227772	3.50
6	1.56	428.166595	3.57
7	1.01	334.28198	2.79
8	0.55	361.841421	3.02

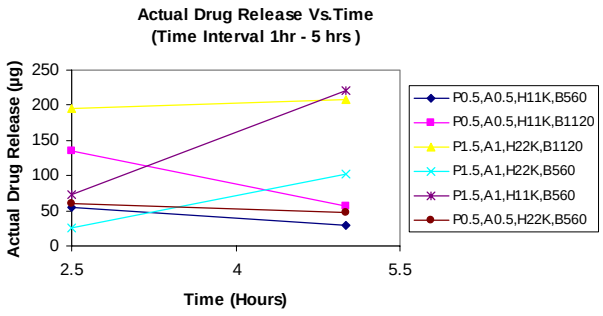
The cumulative and actual drug release from the scaffolds during these four time intervals can be given as follows.



(a)

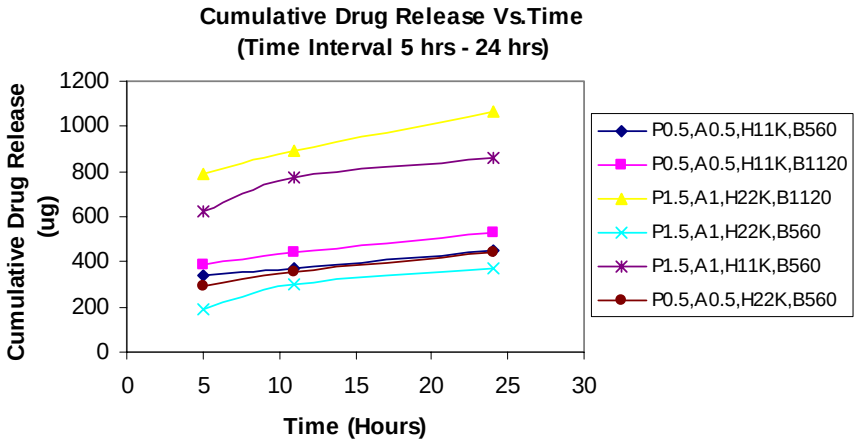


(b)

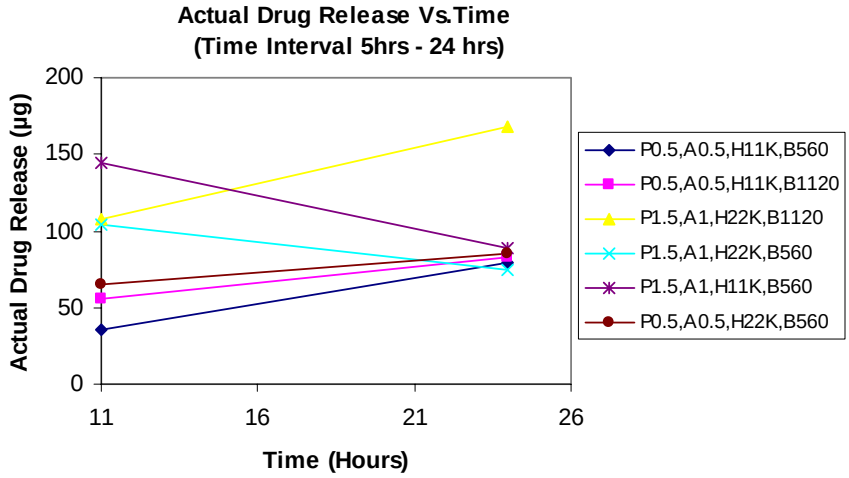


(c)

Fig 4.6 (a) cumulative drug release for time interval 0 hr – 1 hr, (b) cumulative drug release during time interval 1 hr – 5 hrs, (c) actual drug release during time interval 1 hr – 5 hrs

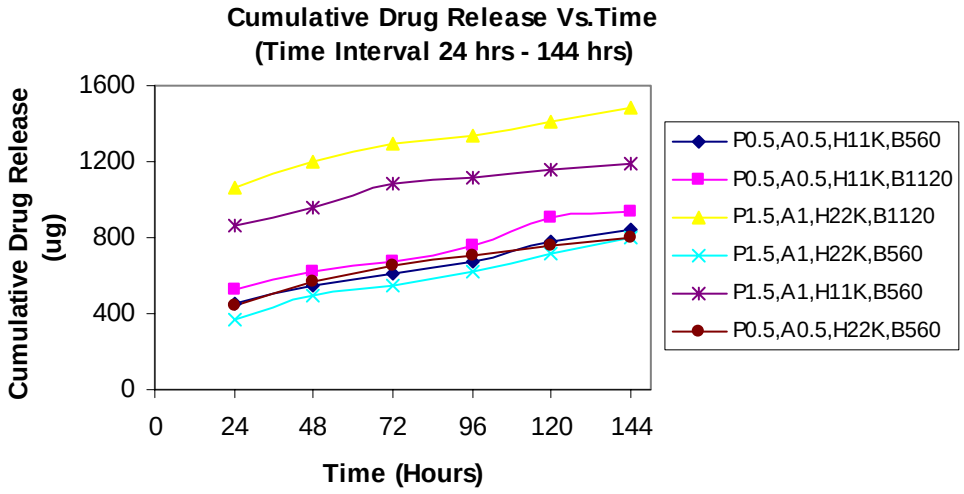


(a)

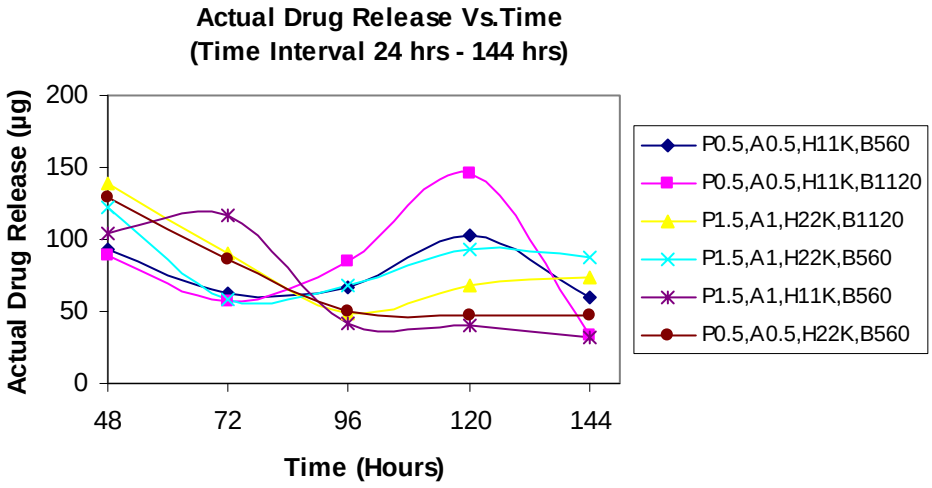


(b)

Fig 4.7 (a) cumulative drug release during time interval 5 hrs – 24 hrs (b) actual drug release during time interval 5 hrs – 24 hrs.



(a)



(b)

Fig 4.8 (a) cumulative drug release during time interval 24 hrs – 144 hrs (b) actual drug release during time interval 24 hrs – 144 hrs

4.1.3 Drug Release Study Using Design of Experiments Software

Outcome of an experiment is based on the input variables. If an experiment is dealing with numerous variables, it's a difficult task to analyze the influence of particular variable on the experiment. Design of Experiment is software, which predicts the design of experiment on the basis of input variables and their values. In this software, the user enters the number of input variables and their boundary values, corresponding outputs are also entered.

In the present study, there are three input variables; blender speed, homogenizer speed and drug concentration. The output of the experiment is drug release rate. As the concentration of drug changes with time, two boundary values were obtained from the cumulative drug release data. The drug release rates were obtained from the formula given in section 4.1.2. Four time periods were considered to calculate the drug release rates. The input variables values are given as follows.

Table 4.7 Input Variables and their values for Design of Experiments Drug Release study

S.N.	Time Interval for Drug Release Rate (Hrs)	Blender Speed (RPM)		Homogenizer Speed (RPM)		Drug Concentration (% w/w)	
		Min.	Max.	Min.	Max.	Min.	Max.
1	0 – 1	560	1120	11000	22000	0.98	1.9
2	1 – 5	560	1120	11000	22000	0.71	1.85
3	5 – 24	560	1120	11000	22000	0.58	1.73
4	24 – 144	560	1120	11000	22000	0.43	1.56

In this table, drug concentration values are obtained from table 4.3 to 4.6 for corresponding time intervals.

4.1.3.1 Influence of variable" Blender Speed" on Drug Release Rate at speed 560 RPM

4.1.3.1.1 Time Interval 0 hr – 1 hr

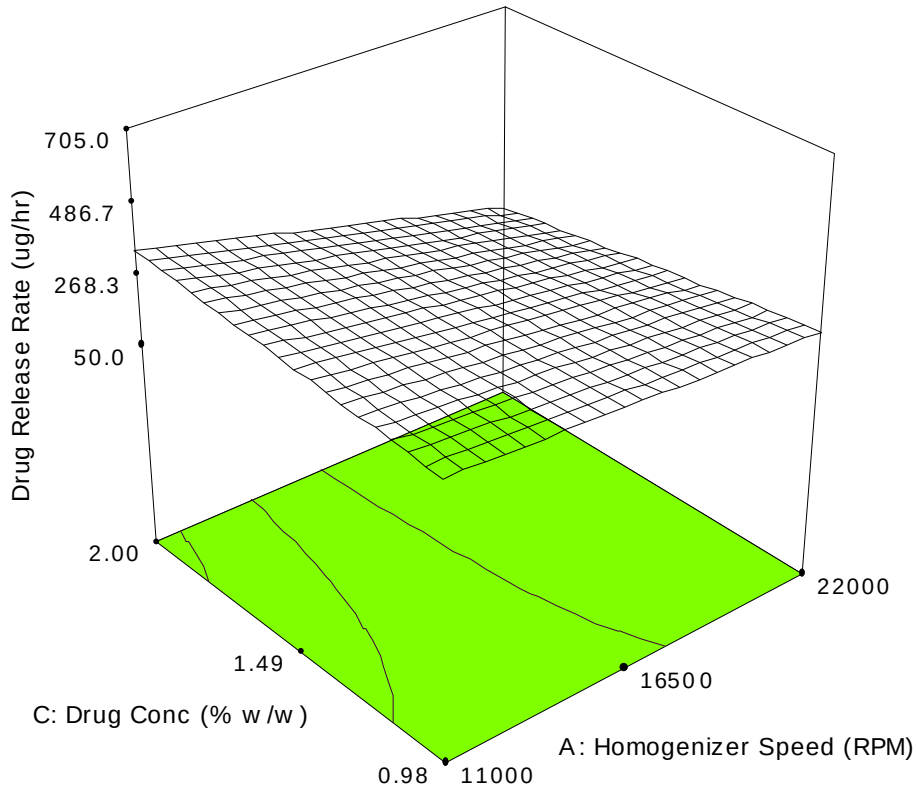


Fig 4.9 Design of Experiments study of Drug Release Rate at Blender speed of 560 RPM for a time interval 0 hr -1 hr.

From the 3 D graph, it is observed that, the drug release rate decreases slightly with increase in homogenizer speed at high concentration of drug (2.0% w/w). It's clear from the fig. 4.6 (a), that drug release from scaffold 4 is greater than scaffold 7. It is also seen that, at high speed of homogenizer (22000 RPM), the drug release rate decreases with increase in drug concentration. This is clear from fig 4.6 (a). Scaffold 8 has a higher drug release than scaffold 6. It is also clear that, the drug release rate for this interval is

very high. Owing to higher pore size and porosity, it can be estimated that, the surfaces of the scaffolds exposed to medium will easily release the drug in a short time.

4.1.3.1.2 Time Interval 1 hr – 5 hrs

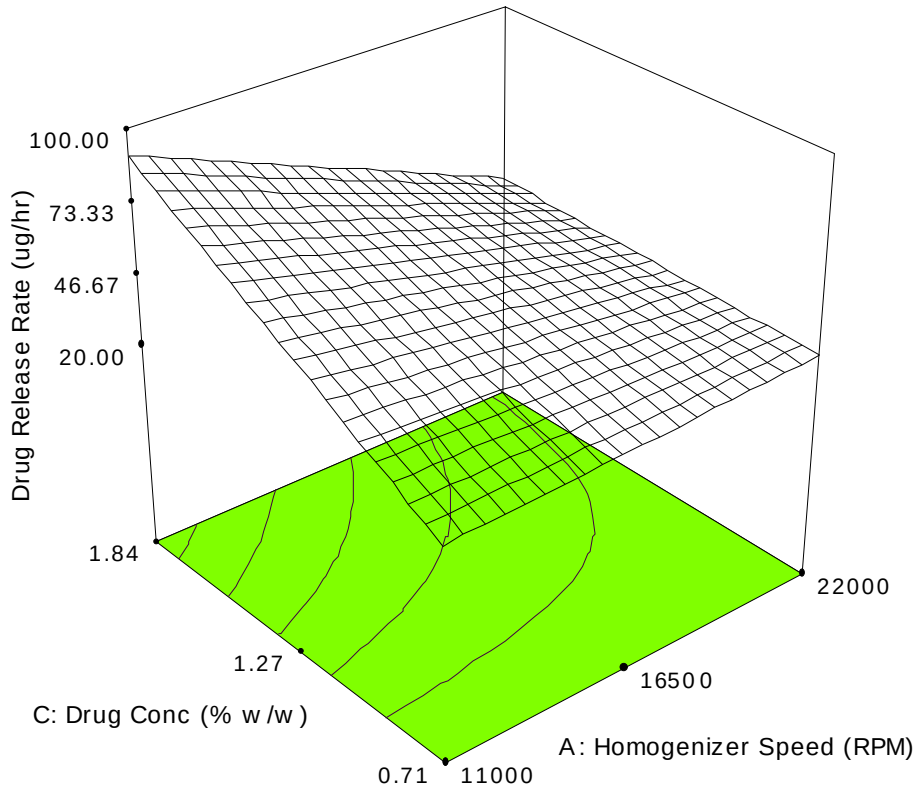


Fig 4.10 Design of Experiments study of Drug Release Rate at blender speed of 560 RPM for a time interval 1 hr -5 hrs

For time interval 1hr- 5 hrs, it can be seen that, the drug release rate increases significantly with increase in the concentration of drug, at low homogenizer speed (11000 RPM). From fig 4.6 (b), it can be seen that, scaffold 7 has a higher cumulative drug release than scaffold 1, and hence the release rates. At low concentration of drug (0.71 % w/w), the drug release rate is invariable for homogenizer speed, though at high concentration (1.84 % w/w), the release rate decreases as the homogenizer speed

increases. There is no significant effect of drug concentration on release rate at high homogenizer speed (22000 RPM).

4.1.3.1.3 Time Interval 5 hrs – 24 hrs

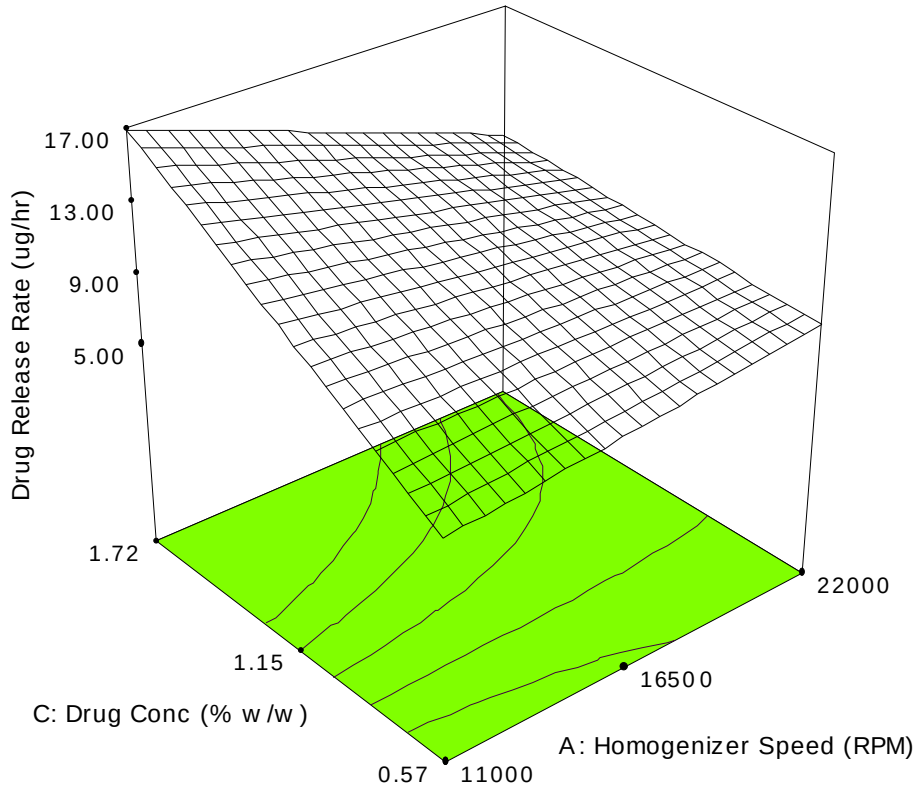


Fig 4.11 Design of Experiments study of Drug Release Rate at blender speed of 560 RPM for a time interval 5 hrs -24 hrs

For a time interval 5hrs – 24 hrs, a similar trend of drug release as that for 1 hr- 5 hrs is observed, however, the drug release rate is lower. As the drug is released from the scaffolds, their concentration decreases and hence drug release rate decreases.

4.1.3.1.4 Time Interval 24 hrs – 144 hrs

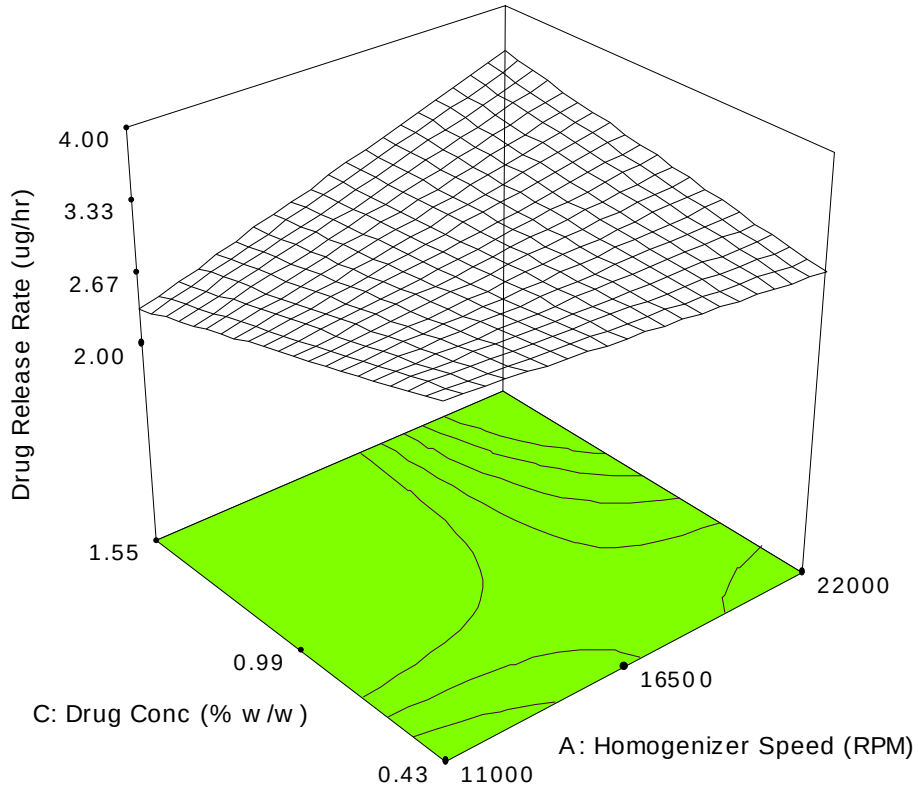


Fig 4.12 Design of Experiments study of Drug Release Rate at blender speed of 560 RPM for a time interval 24 hrs -144 hrs

For interval 24hrs – 144 hrs, it can be seen that, the drug release rate decreases as the concentration of drug increases, though not that significantly, at low speed of homogenizer (11000 RPM). The highest release rate is obtained at high drug concentration and high homogenizer speed (22000 RPM). The release rate is almost indifferent for all speeds of homogenizer at low concentration of drug (0.43 % w/w), though at high concentration of Drug (1.55 % w/w), it increases with homogenizer speed.

4.1.3.2 Influence of variable" Blender Speed" on Drug Release Rate at speed 1120 RPM

4.1.3.2.1 Time Interval 0 hr – 1 hr

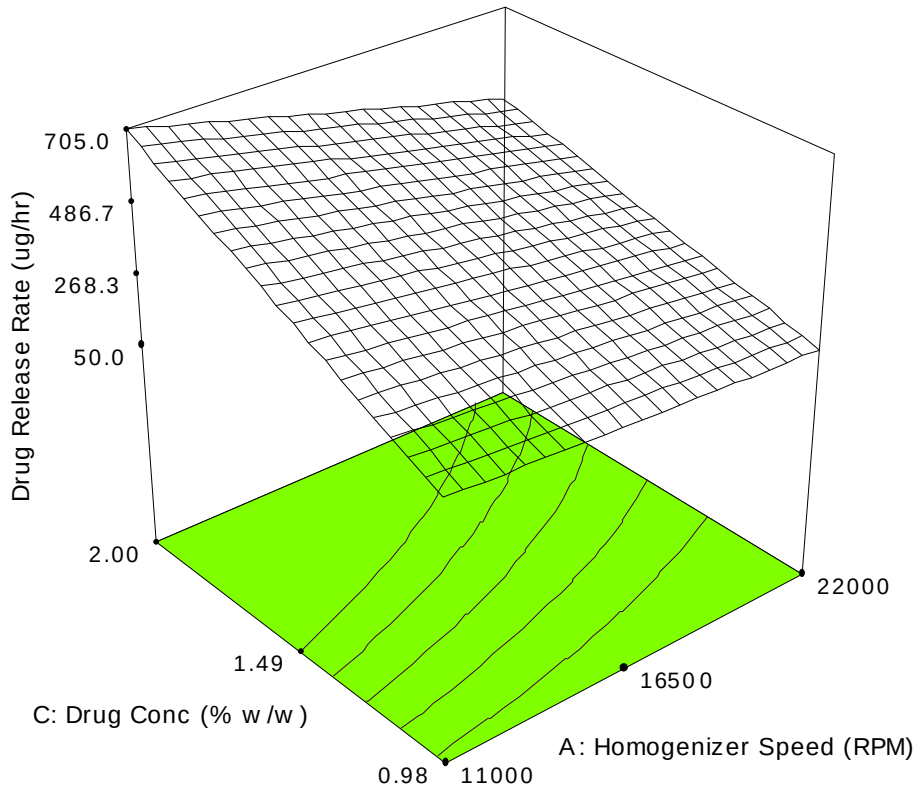


Fig 4.13 Design of Experiments study of Drug Release Rate at blender speed of 1120 RPM for a time interval 0 hr -1 hr

For high speed of blender, it can be seen that, the drug release rate increases with concentration of drug, both at low and high speeds of homogenizer (11000 & 22000 RPM), though at low speed the release rate increase is sharper than that at high speed. At low concentration of drug (0.98 % w/w) there is no relative change in the drug release rates, but at high concentration (2.0 % w/w), the release rate decreases with increase in homogenizer speed.

4.1.3.2.2 Time Interval 1 hr – 5 hrs

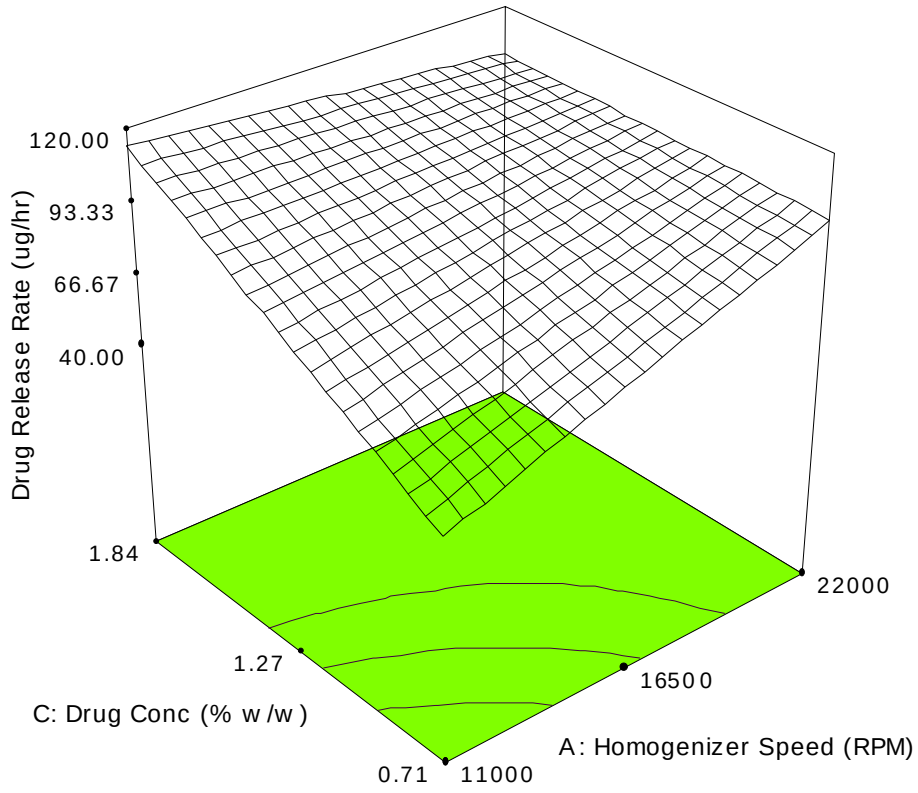


Fig 4.14 Design of Experiments study of Drug Release Rate at blender speed of 1120 RPM for a time interval 1 hr – 5 hrs

For interval 1 hr – 5 hrs, the drug release rate increases sharply at low speed of homogenizer (11000 RPM) but at high speed (22000 RPM), there is no relative change in release rate. On the other hand at low drug concentration (0.71 % w/w), the drug release rate increases sharply with increase in homogenizer speed, but at high drug concentration (1.84 % w/w) there is no relative change in the release rate. There is a synergy between two variables, drug concentration and homogenizer speed.

4.1.3.2.3 Time Interval 5 hrs – 24 hrs

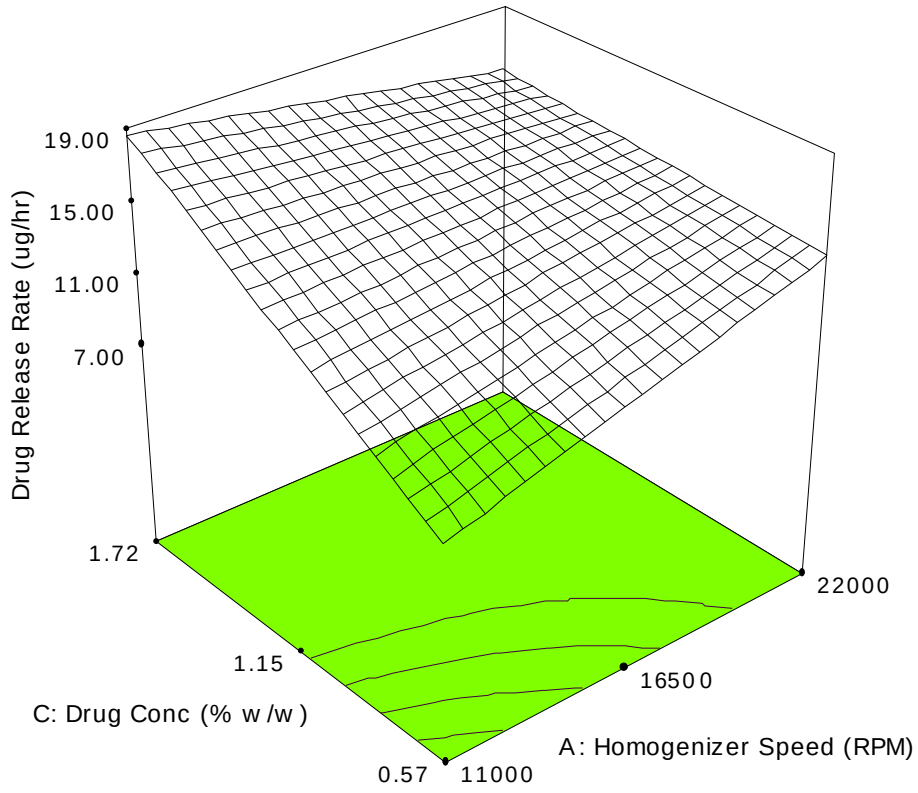


Fig 4.15 Design of Experiments study of Drug Release Rate at blender speed of 1120 RPM for a time interval 5 hrs - 24 hrs

For time interval 5 hrs – 24 hrs the same synergism is seen between two variables drug concentration and homogenizer speed. The trend of drug release rate for this interval is same as that for 1 hr- 5 hrs though overall release rates are lower.

4.1.3.2.4 Time Interval 24 hrs – 144 hrs

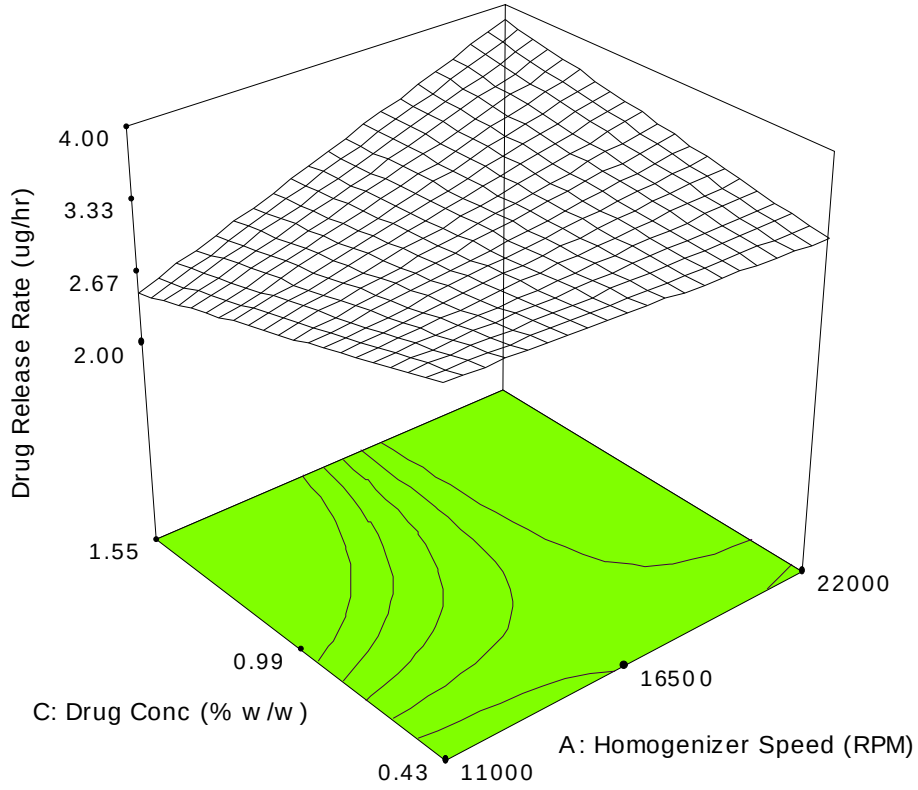


Fig 4.16 Design of Experiments study of Drug Release Rate at blender speed of 1120 RPM for a time interval 24 hrs -144 hrs

From the 3 D graph, it's clear that, there is a synergy between variables drug concentration and homogenizer speed. The drug release rate decreases with increase in drug concentration and homogenizer speed at low homogenizer speed (11000 RPM) and low drug concentration (0.43 % w/w) respectively. On the other hand, the release rate increases with increase in drug concentration and homogenizer speed at high homogenizer speed (22000 RPM) and high drug concentration (1.55 % w/w).

4.1.3.3 Influence of variable" Drug Concentration" on Drug Release Rate at Drug concentration 1.25 % w/w

4.1.3.3.1 Time interval 1hr – 5 hrs

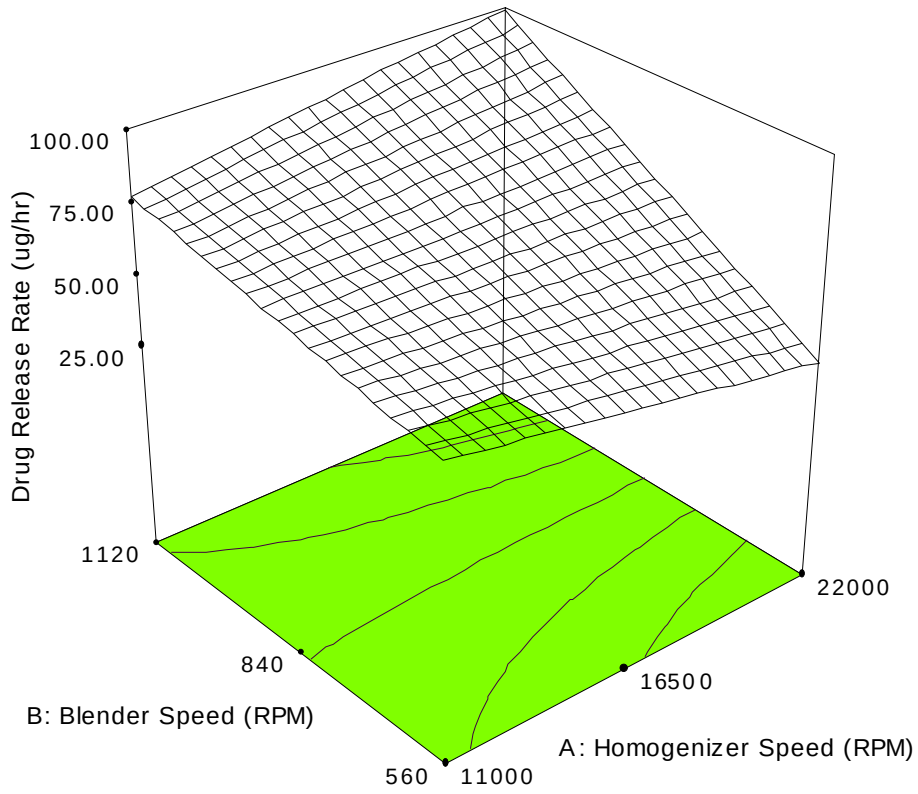


Fig 4.17 Design of Experiments study of Drug Release Rate at drug concentration of 1.25 % w/w for a time interval 1 hr - 5 hrs

For interval 1hr – 5 hrs, at 1.25 % w/w concentration of drug, there is relatively no change in drug release rate at all speeds of blender, at low homogenizer speed (11000 RPM), but at high speed (22000 RPM), the release rate increases sharply with increase in blender speed. At low blender speed (560 RPM), the drug release rate slightly decreases with increase in homogenizer speed, but at high blender speed (1120 RPM), the release

rate increases with increase in homogenizer speed. The variables of blender speed and homogenizer speed have a synergy at this interval.

4.1.3.3.2 Time interval 5hrs – 24 hrs

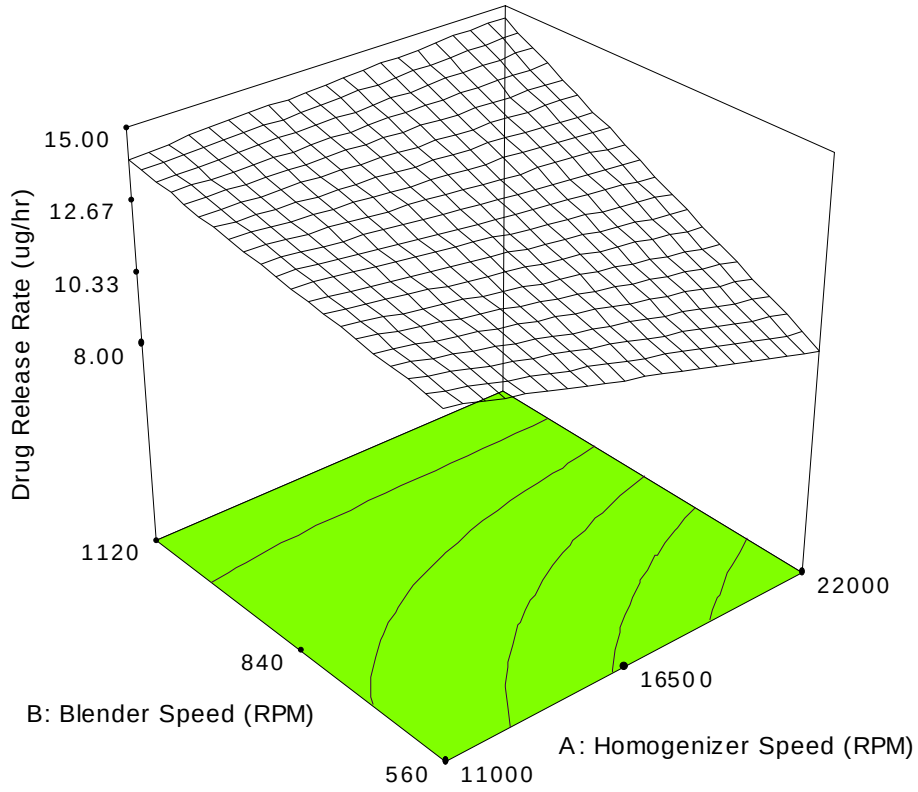


Fig 4.18 Design of Experiments study of Drug Release Rate at drug concentration of 1.25 % w/w for a time interval 5 hrs - 24 hrs

For interval 5 hrs – 24 hrs, the trend of drug release rates is similar to that obtained at interval 1hr – 5 hrs, only difference is, the release rates are smaller for this interval than for the interval of 1hr – 5 hrs.

4.1.3.3.3 Time interval 24hrs – 144 hrs

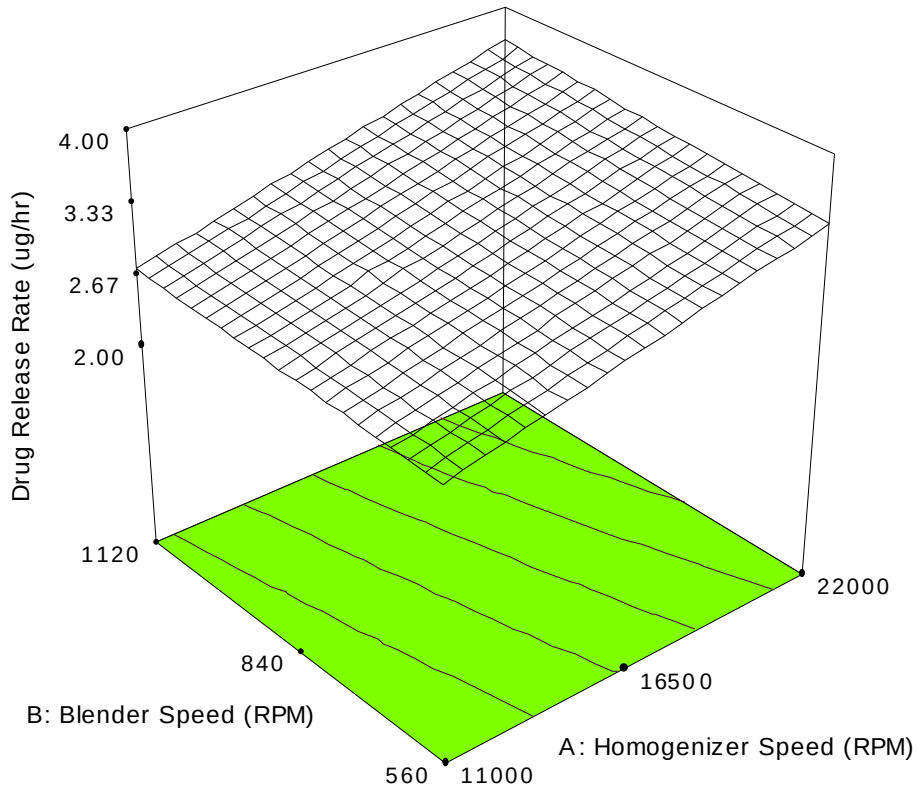


Fig 4.19 Design of Experiments study of Drug Release Rate at drug concentration of 1.25 % w/w for a time interval 24 hrs - 144 hrs

From the graph, it's clear that, for interval 24 hrs – 144 hrs, the drug release rate increases with increase in homogenizer speed at all speeds of blender.

4.1.3.4 Influence of variable" Homogenizer Speed" on Drug Release Rate

It was observed that, the influence of homogenizer speed on Drug release rate was only during the interval of 0 hr – 1hr.

4.1.3.4.1 Homogenizer speed of 11000 RPM

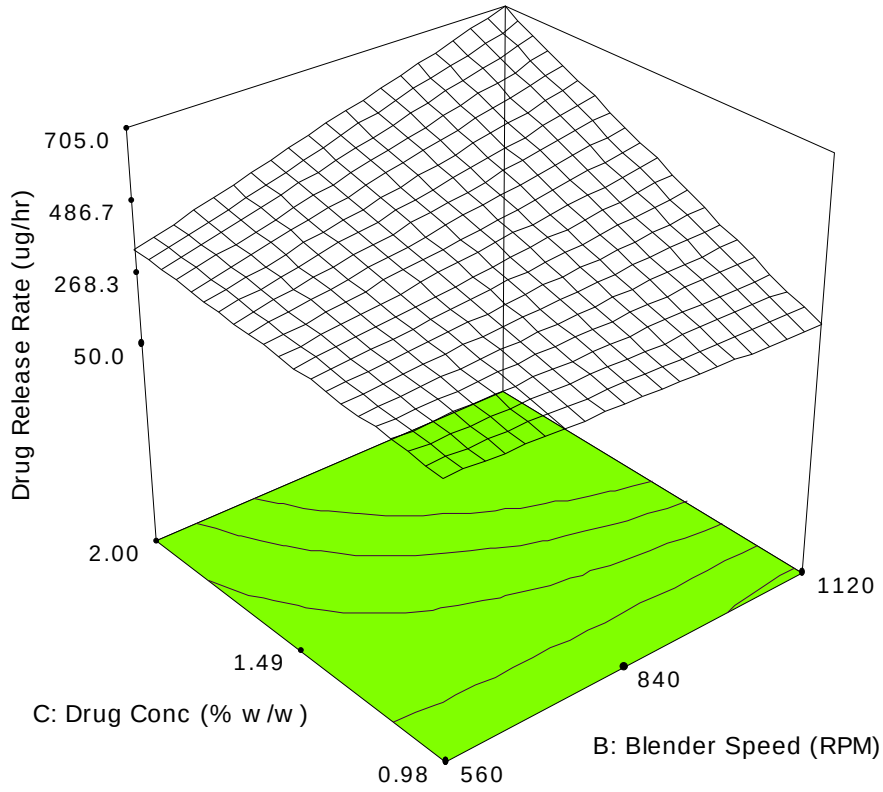


Fig 4.20 Design of Experiments study of Drug Release Rate at homogenizer speed of 11000 RPM for time interval 0 hr - 1 hr

At a homogenizer speed of 11000 RPM, there is a synergy between the variables drug concentration and blender speed. The drug release rate increases sharply at high

drug concentration (2.0 % w/w) and high blender speed (1120 RPM), with increase in blender speed and drug concentration respectively.

4.1.3.4.2 Homogenizer speed of 22000 RPM

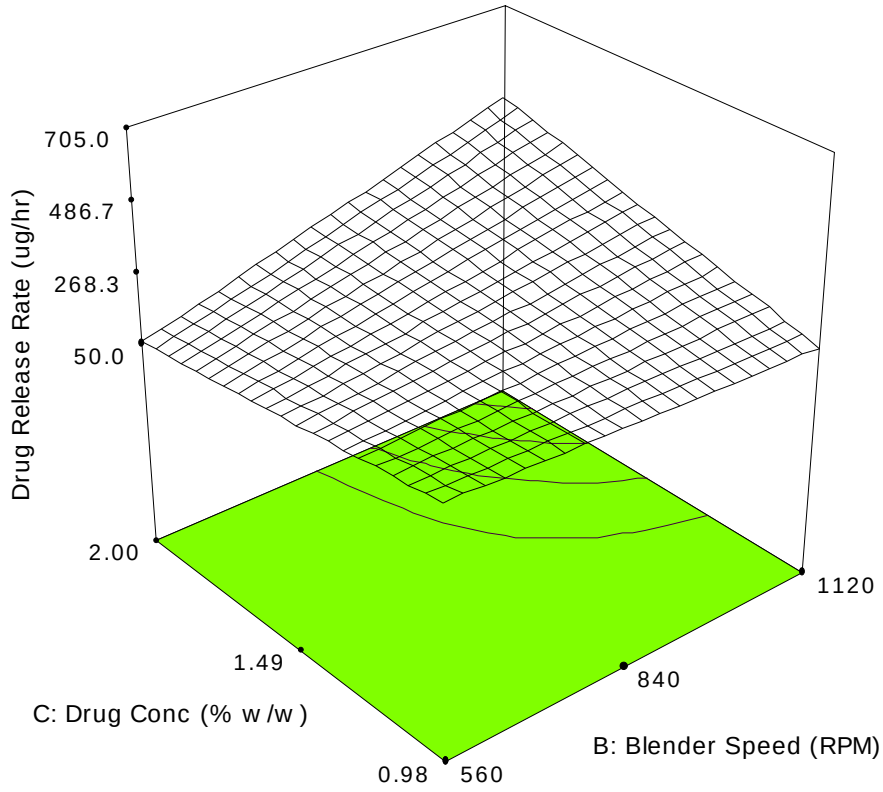


Fig 4.21 Design of Experiments study of Drug Release Rate at homogenizer speed of 22000 RPM for time interval 0 hr - 1 hr

At homogenizer speed of 22000 RPM, the trend of drug release is same as that obtained at 11000 RPM. Only the difference is; overall drug release rate decreases at 22000 RPM.

4.1.3.5 Cube graphs obtained at various time intervals

4.1.3.5.1 Time interval 0 hr – 1 hr

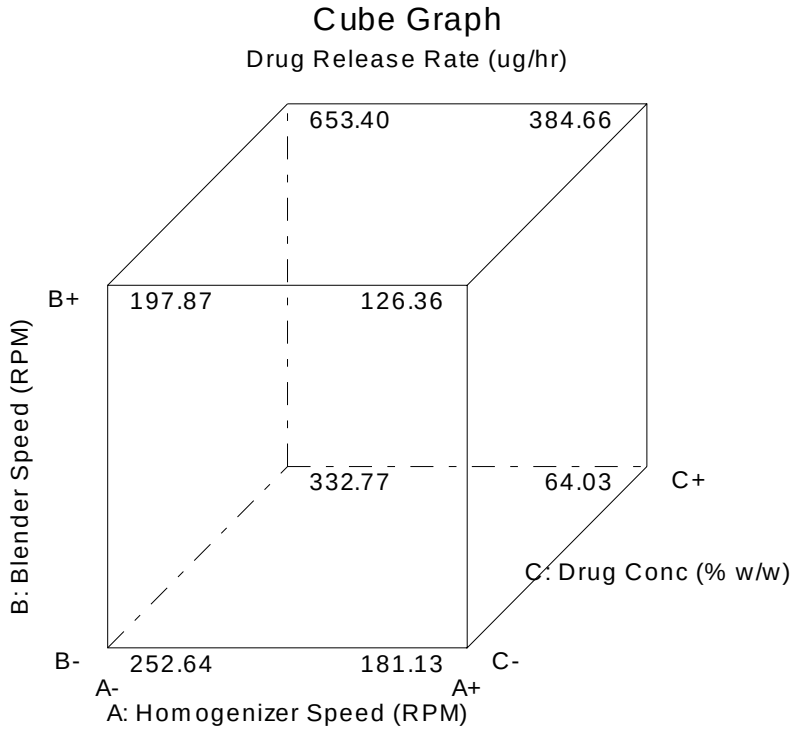


Fig 4.22 Design of Experiments study of Drug Release Rate for time interval 0 hr - 1 hr

The cube graph correlates all three variables, blender speed, homogenizer speed, and drug concentration. From the cube graph, it can be seen that, during time interval 0 hr – 1 hr the drug release rate is high and is varying from 64.03 µg/ hr to 653.40 µg/ hr. It can be said that, during this interval, drug release rate changes significantly.

4.1.3.5.2 Time interval 1 hr – 5 hrs

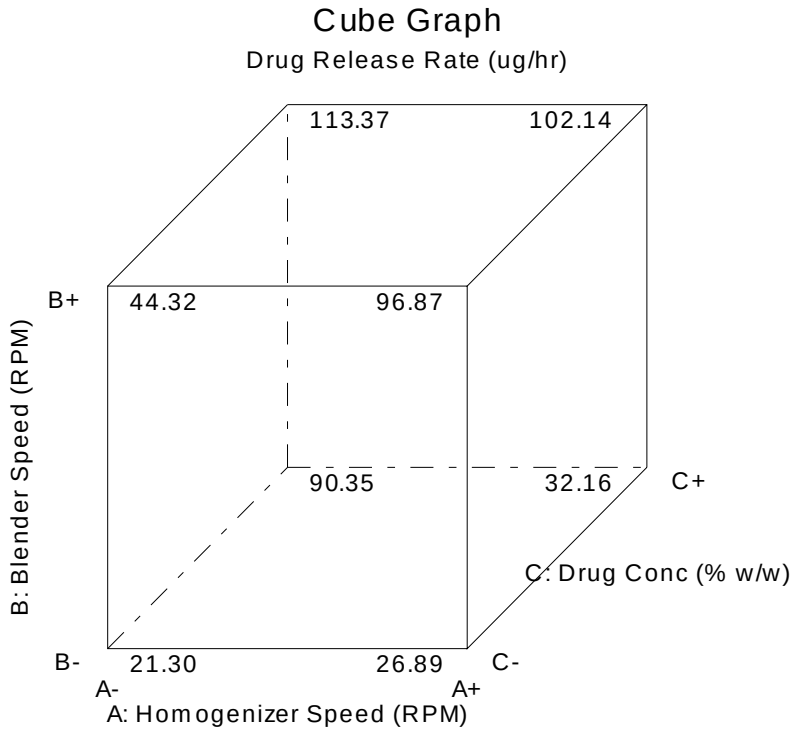


Fig 4.23 Design of Experiments study of Drug Release Rate for time interval 1 hr - 5 hrs

From the figure it can be seen that, during this time interval the drug release rate changes from 21.30 µg/ hr to 113.37 µg/ hr. During this interval, it can be observed that blender speed and drug concentration dominates the drug release rate.

4.1.3.5.3 Time interval 5 hrs – 24 hrs

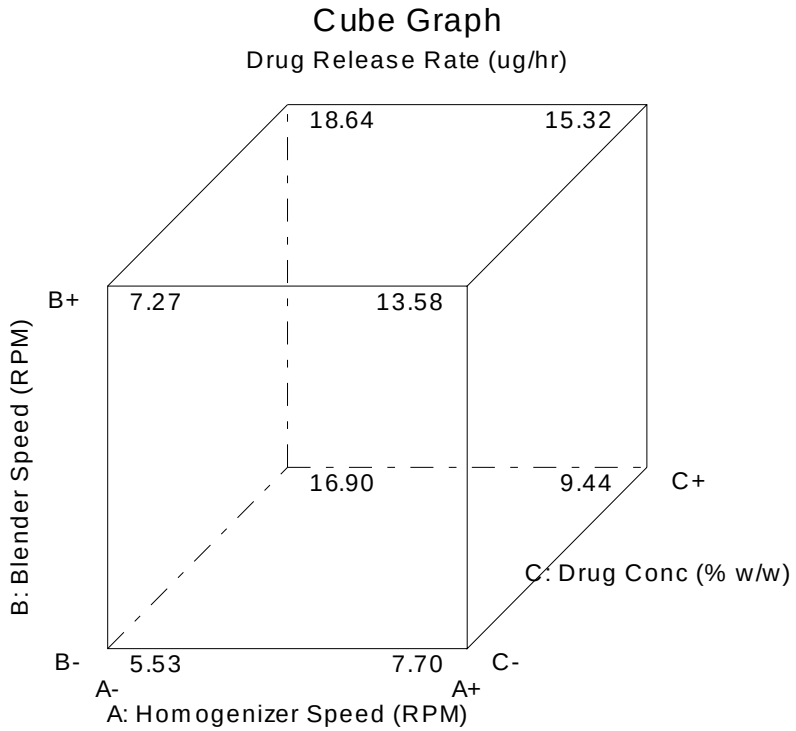


Fig 4.24 Design of Experiments study of Drug Release Rate for time interval 5 hrs - 24 hrs

From the figure it can be observed that, during this interval, the drug release rate changes from 5.53 µg/ hr to 18.64 µg/ hr. In this interval also, it can be said that, blender speed and drug concentration has greater impact on drug release rate.

4.1.3.5.4 Time interval 24 hrs – 144 hrs

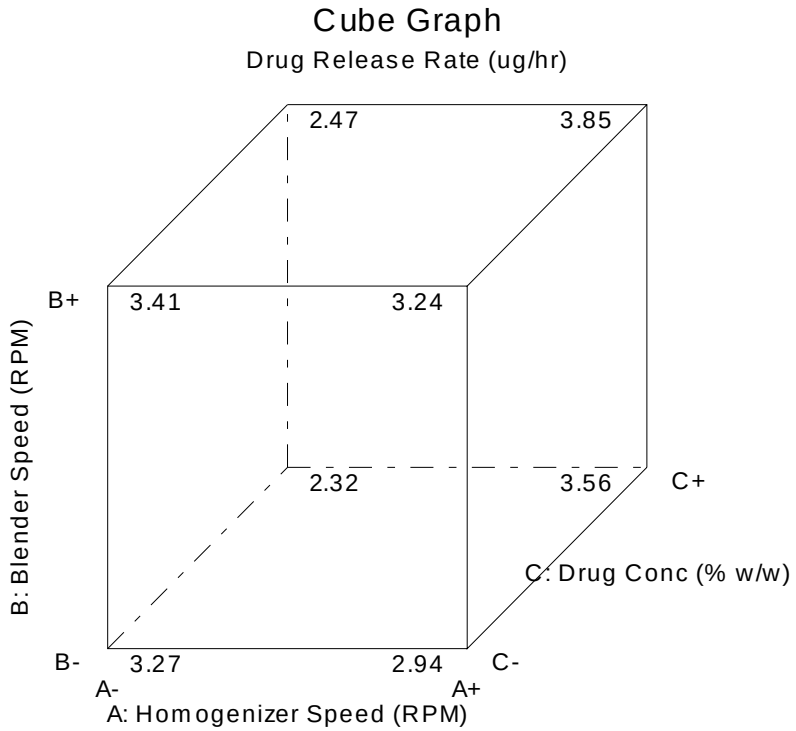


Fig 4.25 Design of Experiments study of Drug Release Rate for time interval 24 hrs - 144 hrs

From the cube diagram it can be seen that, the drug release rate varies from 2.32 µg/ hr to 3.85 µg/ hr with change in values of variables homogenizer speed, blender speed and drug concentration. It can be stated that, during time interval 24 hrs – 144 hrs the drug release rate does not changes significantly.

4.2 Layered Scaffolds

4.2.1The structure of layered scaffolds

The scaffolds had three layers. The amount of PLLA solution and PVA solution in top and bottom layers can be varied. The amount of emulsion in top and bottom layers determined the thickness of these layers. There was no physical boundary between the layers. The blender speed controlled the pores size and the porosity of the scaffolds. The pore sizes and porosity in all the layers is estimated to be same, as all the layers were made at the same speed of blender. The scaffold can be shown schematically as follows:

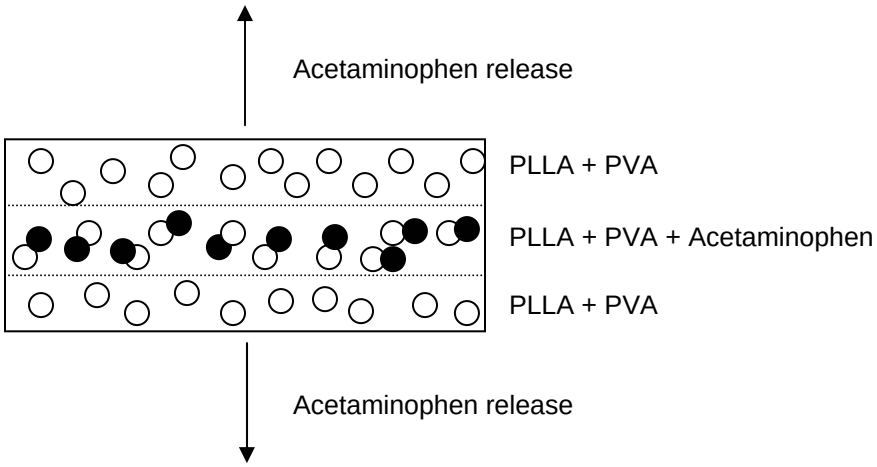


Fig 4.26 Schematic of Layered scaffold structure, with Acetaminophen particles shown as dark circles associated with PVA as hollow circles & remaining portion as PLLA & pores

From fig 4.23, it can be seen that, Acetaminophen can be released from the scaffold only through top and bottom layers. All other four faces of the scaffold were

coated by PLLA. The scaffold formed as a uniform blend of three layers without any boundary.

4.2.2 Drug release study 1

The amount of drug present into DPBS solution was obtained from the concentration of Acetaminophen into the solution. The cumulative release of drug from the scaffold can be given as follows:

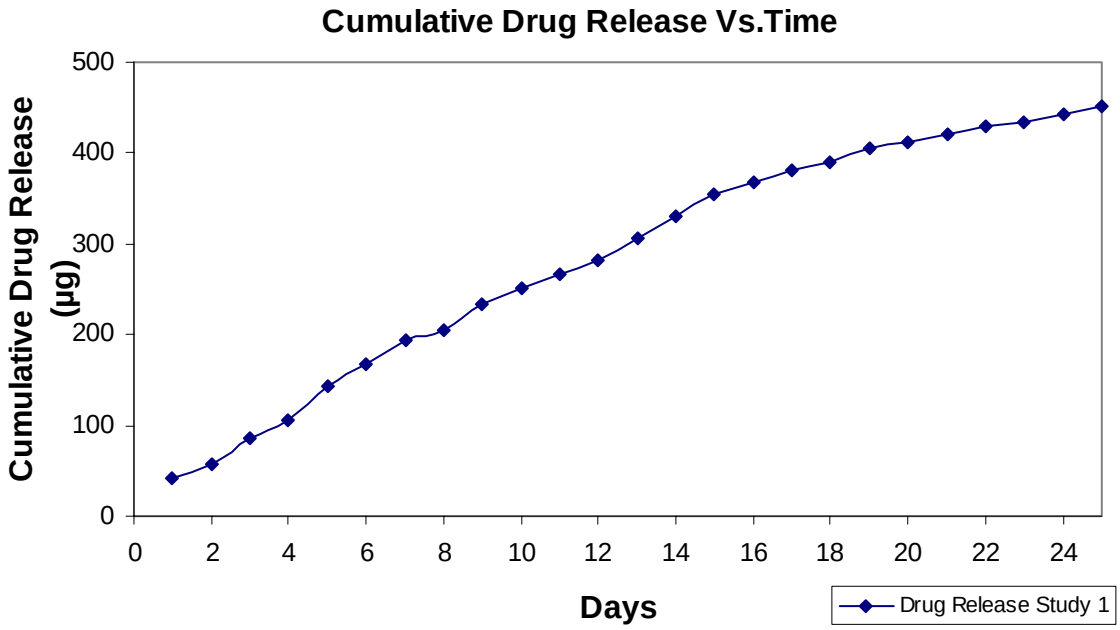


Fig 4.27 Cumulative drug release Vs. Time for Drug Release Study 1

From the graph, it can be seen that, the drug release was almost a linear process invariable of the concentration of drug present into the scaffold. It was also observed that, there was no or very small burst release. The total amount of Acetaminophen present into the sample was 938.3886 µg.

The percentage of drug released with time can be given as follows.

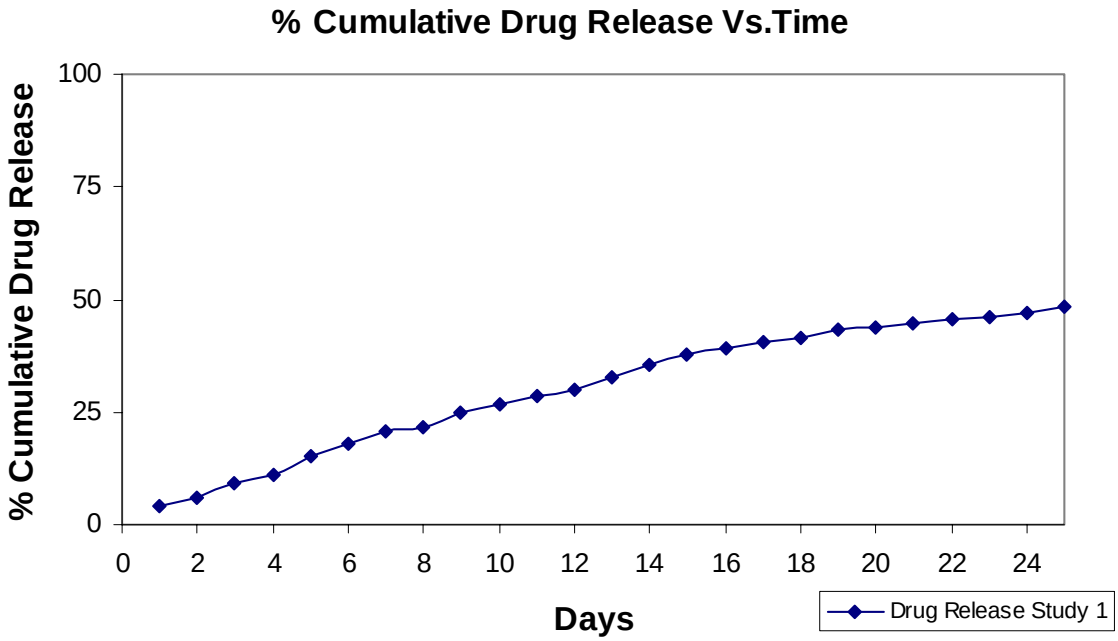


Fig 4.28 % Cumulative drug release Vs. Time for Drug Release Study 1

From the graph, it can be seen that, up to 25 days, only ~ 50 % of drug was released. Such trend can be considered as long term drug release. The drug release rate was calculated by dividing the total amount of drug released up to a specific time by time in terms of days. The formula can be given as follows.

$$R = \frac{D}{T}$$

Where:

R Drug Release Rate (µg/ day)

D Cumulative Drug Released at Time ***T*** (µg)

T Time, when the Drug Release was measured.

By using given formula, the drug release rate was calculated and can be plotted as follows:

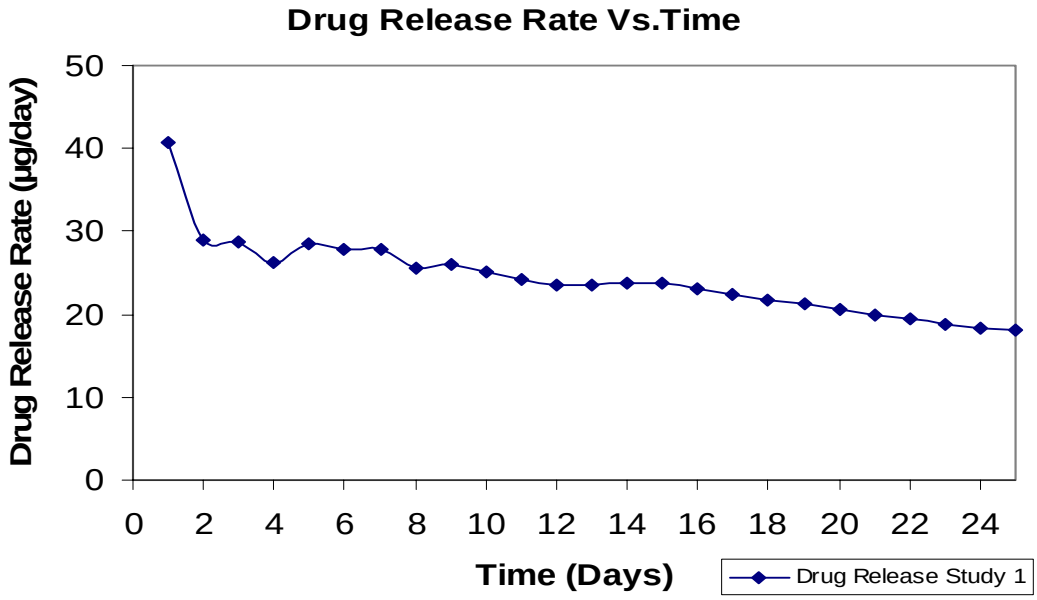


Fig 4.29 Drug release Rate Vs. Time for Drug Release Study 1

From the graph, it can be seen that, the drug release rate decreased linearly with time. On first day, ~ 40 µg of drug was released but from second day, the release rate dropped to ~ 28 µg. Hence it can be stated that, there was a relatively small burst release on first day. From 2nd day to 25th day, the release rate was fairly in between the range of 28 µg/day – 20 µg/day.

As the drug is released from the scaffold; the total amount of drug present into the scaffold changes. Hence it was studied, how the amount of drug present into the scaffold affects further drug release. At each reading or check point, the amount of drug released from the scaffold and amount remaining in the scaffold was calculated. From this information percentage of drug released from the scaffold was found and can be given as

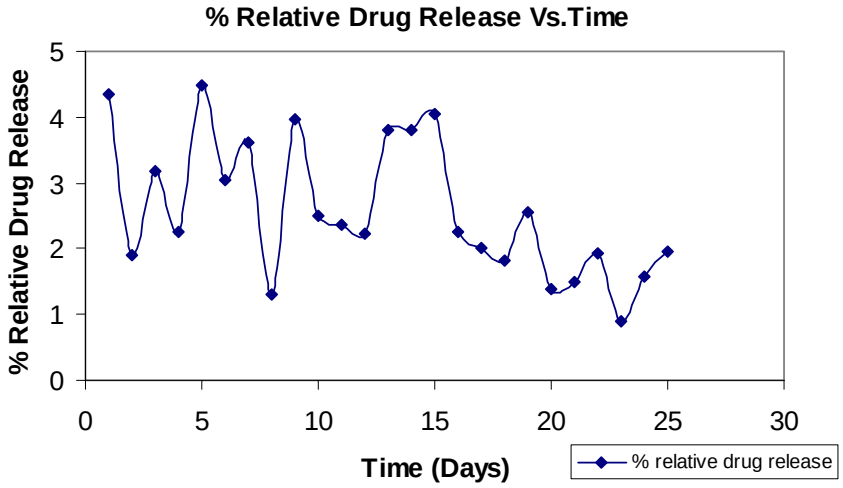
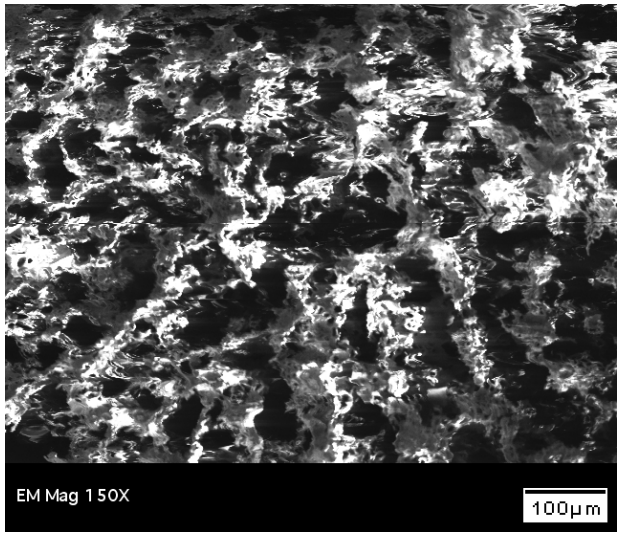


Fig 4.30 % Relative Drug Release Vs. Time

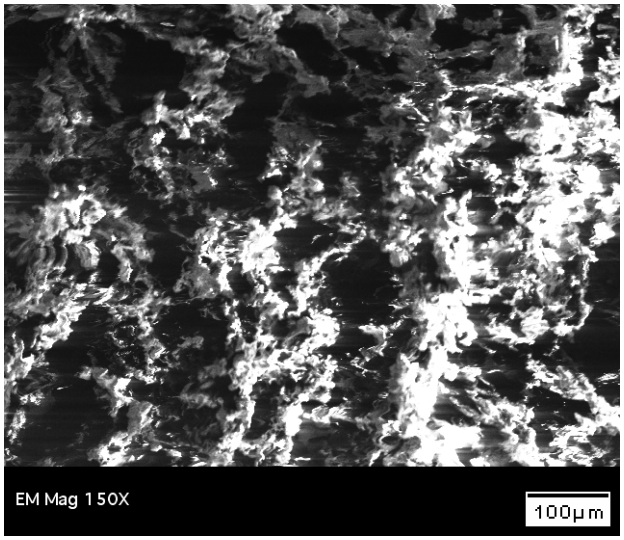
The process is named as percentage relative drug release. From the graph, it can be seen that, on first day ~4.4 % drug is released. On 2nd day ~1.9 % drug is released, means it decreased as compared to 1st day release. On 3rd day ~3.2 % drug is released, means it increased as compared to 2nd day release. Such relationship is observed through out the experiment, there are fluctuations in % drug release, almost every alternate day. This type of release is a kind of negative feedback system. Hence it can be observed that, in this study, there is little or no burst release as such and drug release is relative to the amount of drug present into the scaffold at a particular time. Though there is increase and decrease in % drug release, the amount in change is decreased with time, which meant that overall drug release rate decreased with time.

4.2.3 Drug release study 2

4.2.3.1 SEM analysis of the Scaffolds



(a)

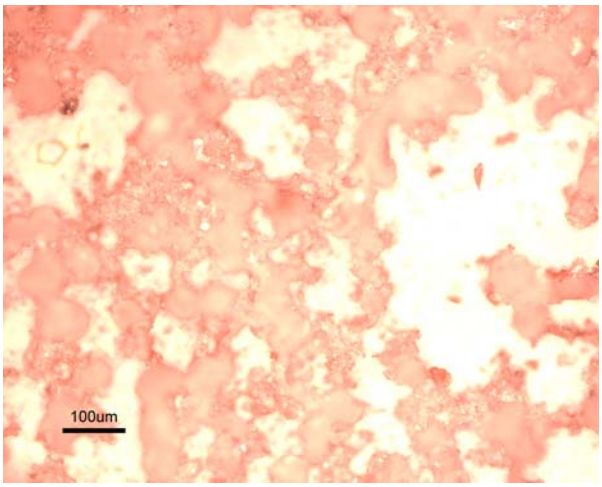


(b)

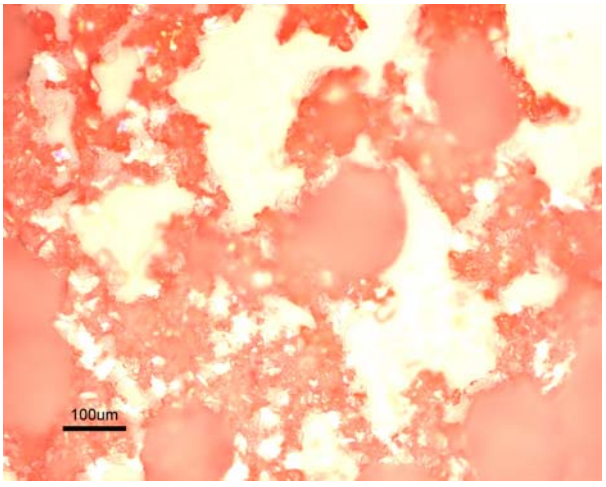
Fig 4.31 SEM pictures of layered scaffolds prepared with (a) Top & Bottom layers with 1.0 % w/v PVA solution and Middle layer with 1.0 % w/v PVA solution having 1.0 % w/v Acetaminophen, all layers prepared at 560 RPM speed of Blender (scaffold 1) (b) Top & Bottom layers with 1.5 % w/v PVA solution and Middle layer with 1.5 % w/v PVA solution having 1.0 % w/v Acetaminophen, all layers prepared at 560 RPM speed of Blender (scaffold 2).

4.2.3.2 Cross Section Analysis of the Scaffolds

For an ideal scaffold, not just pore size and porosity are important but also the connectivity of pores. To analyze the connectivity of the scaffolds, the cross sections of the scaffolds were analyzed.



(a)



(b)

Fig 4.32 Cross section picture of (a) 1.0 % PVA LO (scaffold 1), (b) 1.5 % PVA LO (scaffold 2) taken by optical microscope. The scale is 100 µm

It was seen that, both the scaffolds had a pore size up to 300 µm, though the distribution of pores is slightly different in both the scaffolds. The pores seemed to be well interconnected. The three layers of the scaffold can not be differentiated, which made sure that, all three layers formed a scaffold as a whole uniform structure.

4.2.3.3 Scaffolds porosity measurement

The porosity of the scaffolds was calculated from the density, volume and mass of all ingredients of the scaffolds. The density of PLLA, PVA and Acetaminophen was 0.4581 gm/ml, 1.25 gm/ml and 1.2083 gm/ml respectively. The scaffolds were numbered as **1** – 1 % PVA LO and **2**- 1.5 % PVA LO. The porosity of both the scaffolds can be given as follows.

Table 4.8 Relative Mass and Volume of all Ingredients and Porosity of Scaffolds

S.N.	**Total Mass of sample (gm)**	**Total Volume of sample (ml)**	**Relative Mass (gm)**			**Relative Volume (ml)**			**Porosity (%)**
			PLLA	**PVA**	**Drug**	**PLLA**	**PVA**	**Drug**	
1	0.0125	0.1004	0.01202	0.00036	0.00012	0.0262	0.0002	0.0001	73.5
2	0.0190	0.1211	0.01800	0.00081	0.00018	0.0392	0.0006	0.0001	66.9

4.2.3.4 Acetaminophen Release Study

The cumulative Drug release from both the scaffolds can be given as follows:

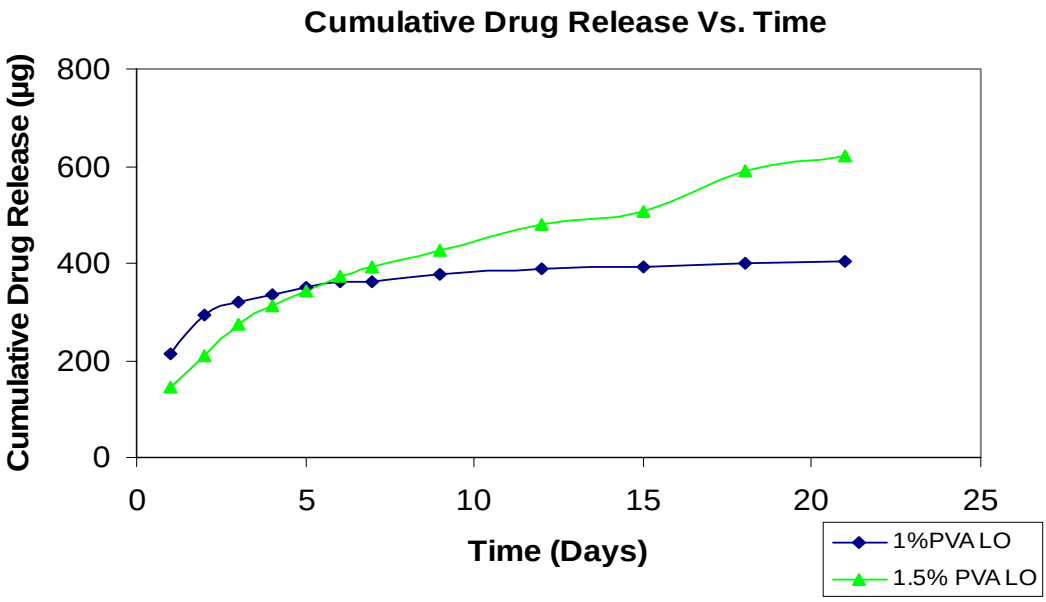


Fig 4.33 Cumulative Drug release Vs. Time for Drug Release Study 2

The curves are named after the constituents from which the scaffolds are made. "LO" stands for low speed of Blender (560 RPM). Both the scaffolds were made from Acetaminophen solution with 1 % w/v concentration. From the graph it can be seen that, both the scaffolds had almost a linear drug release, though the rate was different. Up to 6^{th} day, the cumulative drug release from scaffold 1 was higher than scaffold 2 and later it lowered. It can be seen from fig 4.22 that scaffold 1 has large proportion of bigger pores and are thoroughly distributed than scaffold 2, as a result the drug from scaffold 1 was released faster than scaffold 2 in the beginning. Though the scaffolds had same drug concentrations, the actual amount of drug was different in the samples taken from the

scaffolds. A difference in drug release resulted in concentration variation of drug in the samples which affected further drug release process from the scaffolds.

The total amount of Acetaminophen in 1 % PVA LO (scaffold 1) and 1.5 % PVA LO (scaffold 2) was 859.6154 µg and 1139.336 µg respectively. The percentage of total amount of drug released with time can be given as follows for both the scaffolds.

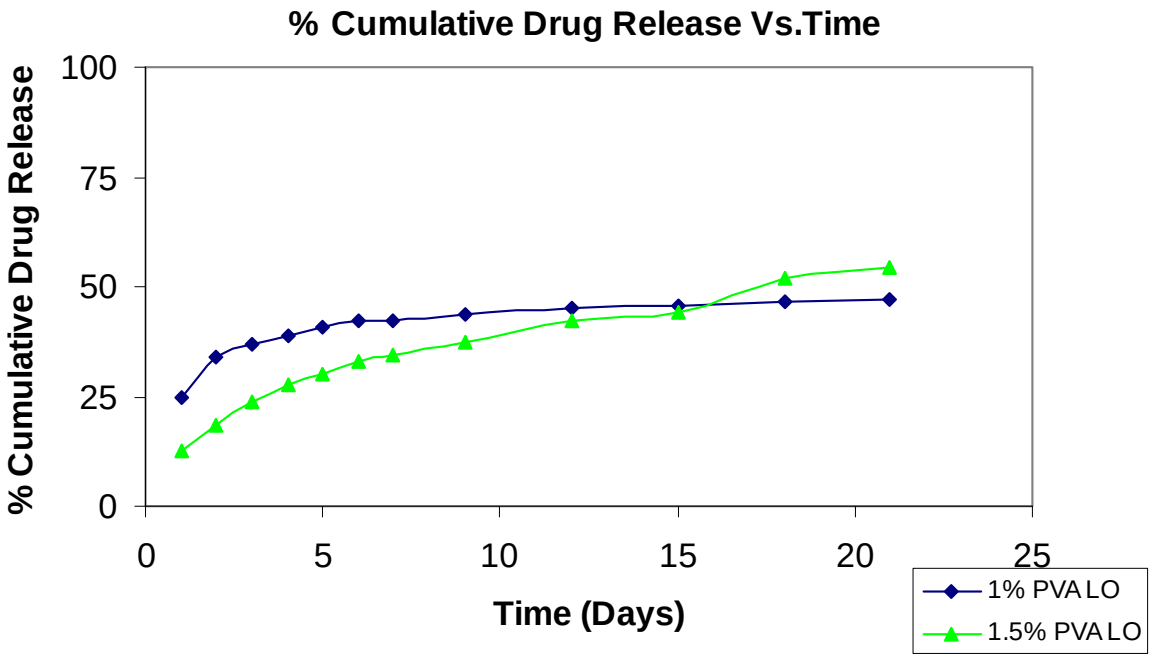


Fig 4.34 % Cumulative Drug release Vs. Time for Drug Release Study 2

From the graph it can be seen that, by 21st day, both the scaffolds have released ~ 50 % of drug. Up to 15th day, scaffold 1 had a greater percentage of cumulative drug release than scaffold 2. It can be stated that, scaffold 1 has higher content of bigger pores than scaffold 2. It was also noted that, scaffold 1 has greater porosity than scaffold 2, and hence up to 15th day, scaffold 1 has higher percentage of drug released than scaffold 2.

From the cross section pictures and porosities of the scaffolds, it was acceptable that scaffold 1 would release drug faster than scaffold 2.

The drug release rates for both scaffolds were calculated and can be given as follows.

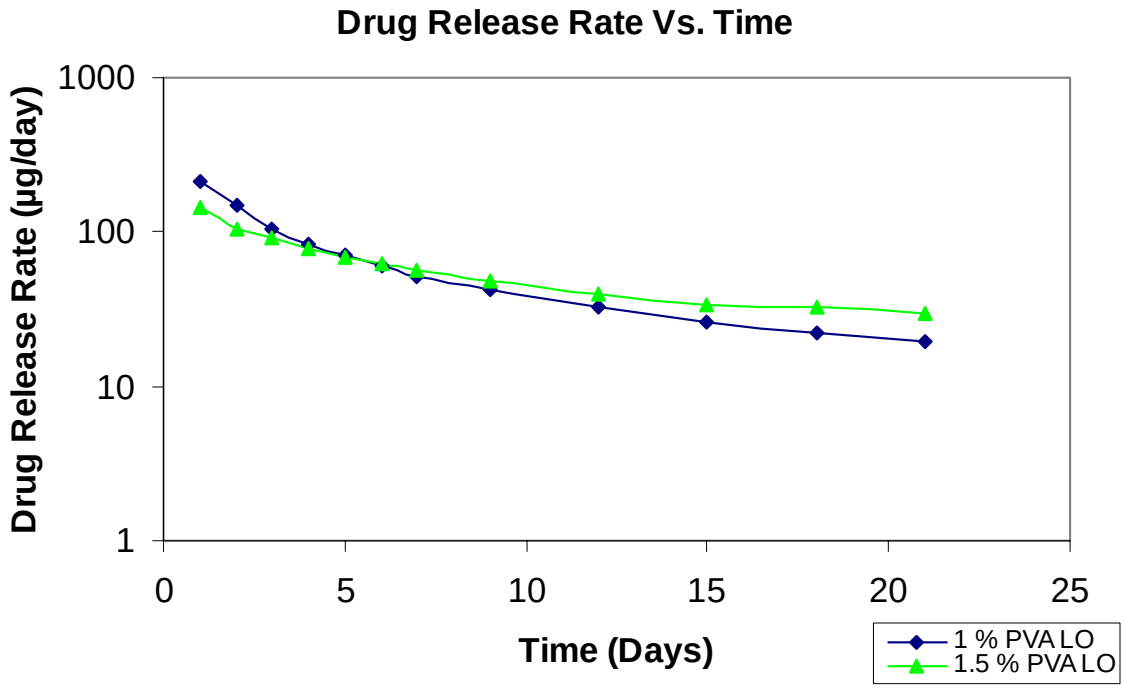


Fig 4.35 Drug release rate Vs. Time for Drug Release Study 2

From the graph, it's clear that, till 5th day, the scaffold 1 had higher drug release rate than scaffold 2. On 6th day, both the scaffolds had fairly same release rates, but from 7th day onwards scaffold 2 had greater release rate than scaffold 1. As explained earlier, a difference in drug release from the scaffolds, because of structural or morphological difference in scaffolds, resulted in different drug concentrations in the scaffolds. As the concentration of drug in scaffold 1 has lowered faster than scaffold 2, it can be estimated that the drug release rate of scaffold 1 will reduce faster than scaffold 2.

The effect of amount of drug present into the scaffold at a particular time on further drug release was studied. The procedure described in Drug Release Study 1 was followed in this experiment as well. The resultant graph can be given as follows.

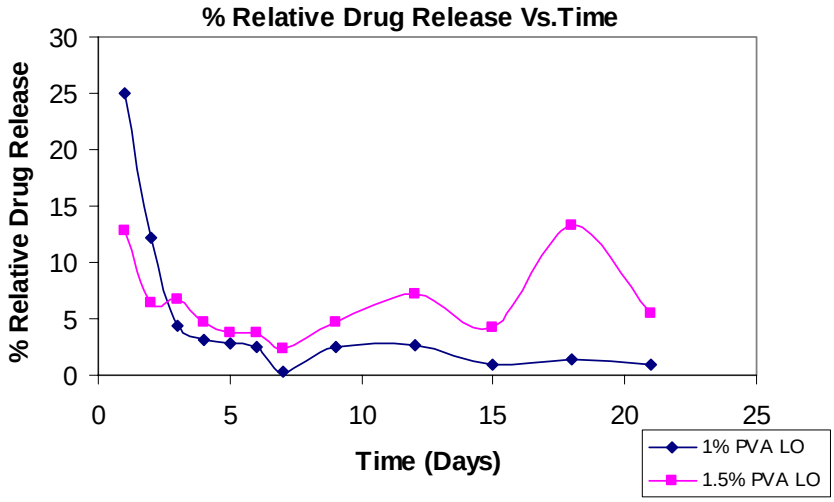


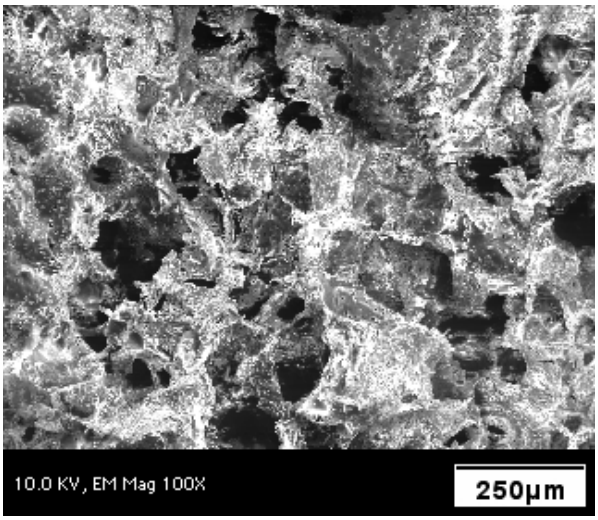
Fig 4.36 % Relative Drug Release Vs.Time

From the graph, it can be seen that, the trend of % relative drug release for both the scaffolds is almost same. In case of scaffold 1, till 3rd day, % relative drug release decreased sharply and till 7th day, rather slowly then it increased till 13th day and again decreased on 15th day. Later till 18th day it increased and then decreased till 21st day. It can also be seen that, till 3rd day, this scaffold has released significant amount of drug, hence there is relatively high burst release from this scaffold. In case of scaffold 2, it can be seen that, till 2nd day, the % relative drug release decreased then it slightly increased till3rd day and again decreased till 7th day. Later it increased till 13th day and decreased till 15th day. It again increased till 18th day and then decreased till 21st day. It can be seen that, % relative drug release on 18th day is greater then that on 1st day, hence this scaffold has no burst release as long as % relative drug release is concerned. It is also observed that scaffold 2 has higher drug release rate than scaffold 1, at least after 3rd day.

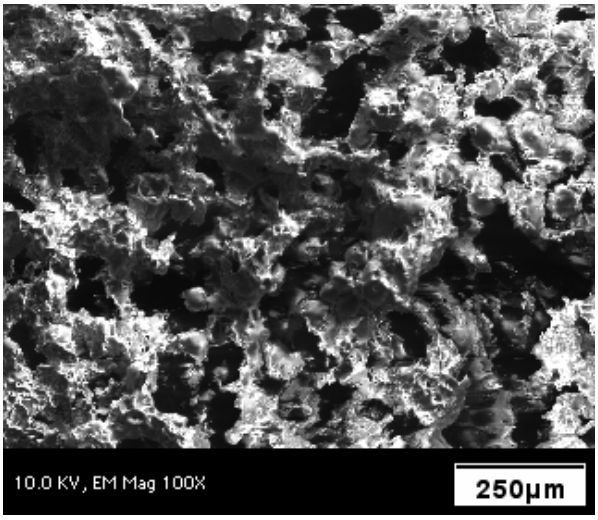
4.3 Cells culture on scaffolds

4.3.1 Pore size and Porosity of the Scaffolds

The scaffolds were observed under SEM. The SEM pictures of the scaffolds are given as follows.

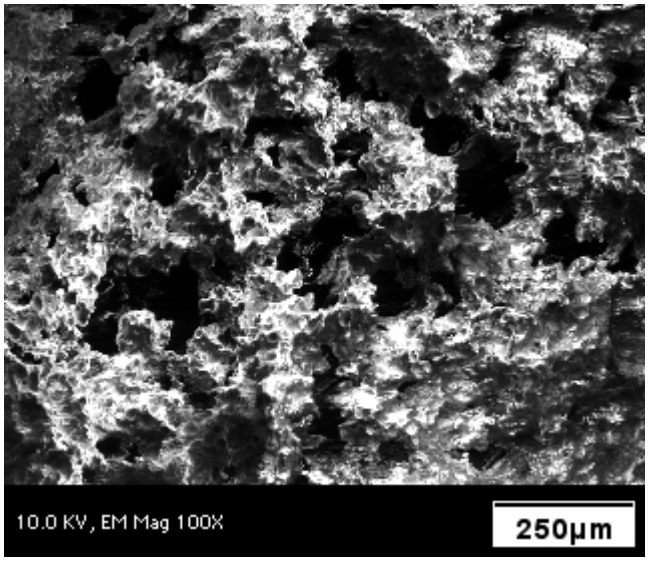


(a)

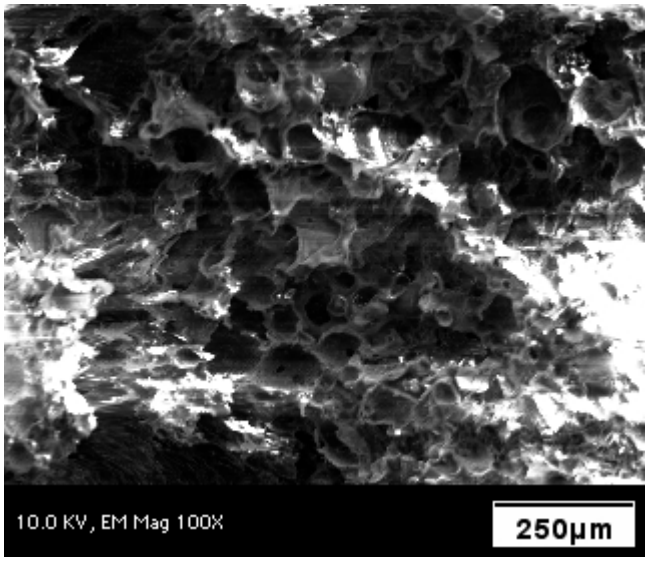


(b)

Fig 4.37 SEM picture of scaffold prepared with (a) 0.5 % PVA concentration solution at Low speed (560 RPM) of blender, (b) 0.5 % PVA concentration solution at High speed (1120 RPM) of blender



(a)



(b)

Fig 4.38 SEM picture of scaffold prepared with (a) 1.5 % PVA concentration solution at Low speed (560 RPM) of blender, (b) 1.5 % PVA concentration solution at High speed (1120 RPM) of blender

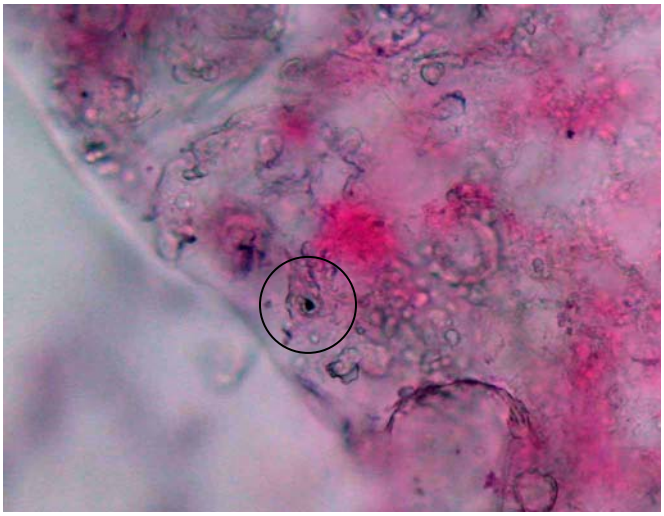
From SEM pictures, it can be seen that all the scaffolds have pores having size in the range of 25 to 250 µm.

The porosity of these scaffolds was calculated by using the same principle of density and volume of the ingredients of the scaffolds. The density of PLLA: PGA copolymer, PVA was 1.27 gm/ml, 1.25 gm/ml respectively. The scaffolds are numbered as 1- 0.5 L, 2- 0.5 H, 3- 1.5 L, 4- 1.5 H. The porosity of the scaffolds can be given as follows.

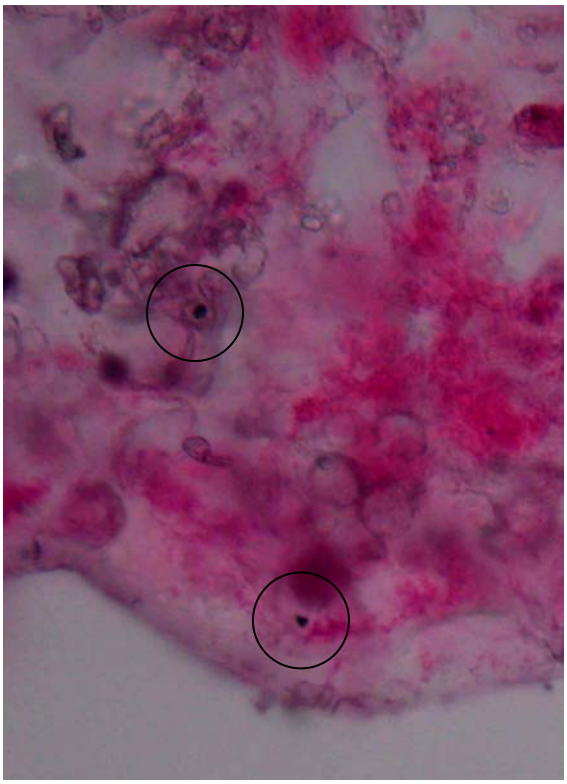
Table 4.9 Relative Mass and Volume of all ingredients and Porosity of Scaffolds

S.N.	Total Mass of sample (gm)	Total Volume of sample (ml)	Relative Mass (gm)		Relative Volume (ml)		Porosity (%)
			PLLA: PGA	PVA	PLLA: PGA	PVA	
1	0.0051	0.0718	0.005024	0.000075	0.00395	0.00006	94.41
2	0.0126	0.1236	0.012413	0.000186	0.00977	0.00014	91.97
3	0.0084	0.0857	0.008038	0.000361	0.00632	0.00028	92.28
4	0.0023	0.0406	0.002200	0.000099	0.00173	0.00007	95.54

4.3.2 Histological analysis of the scaffolds

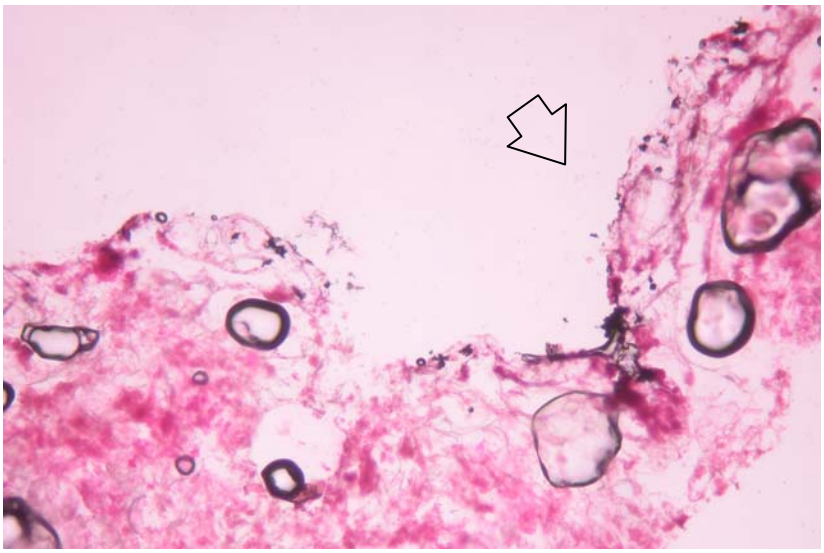


(a)

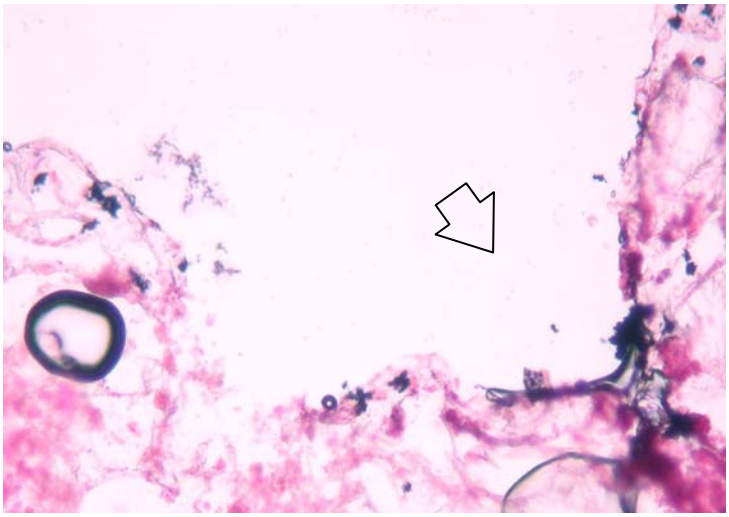


(b)

Fig 4.39 (a) and (b) Scaffold 1 (0.5 L), H & E stained, seen under optical microscope. Cells are encircled

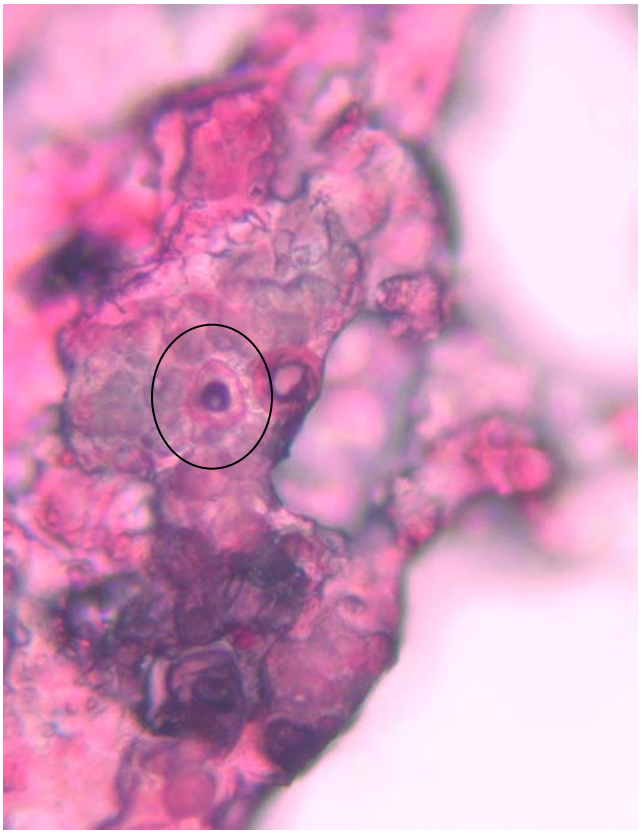


(a)

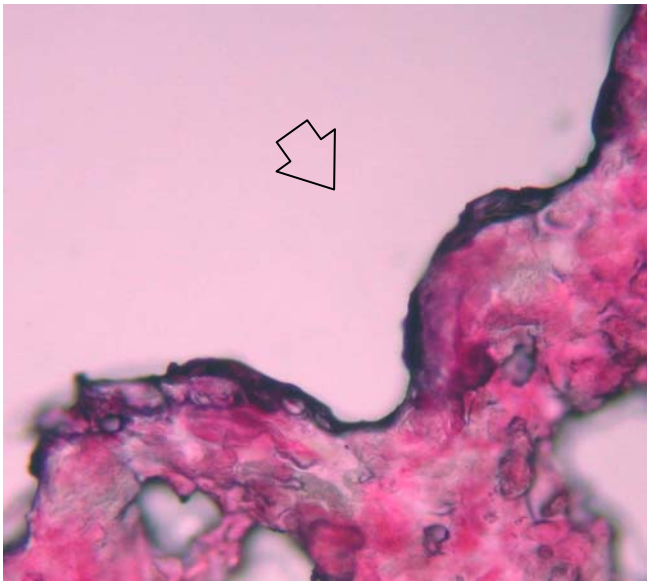


(b)

Fig 4.40 (a) and (b) Scaffold 2 (0.5 H), H & E stained, seen under optical microscope. Arrow shows the top side of the scaffold having cells. The cells are seen as dark spots



(a)



(b)

Fig 4.41 (a) and (b) scaffold 3 (1.5 L), H & E stained, seen under optical microscope. (a) Cell is encircled. (b) Top side of the scaffolds with cells as dark spots, shown by arrow.

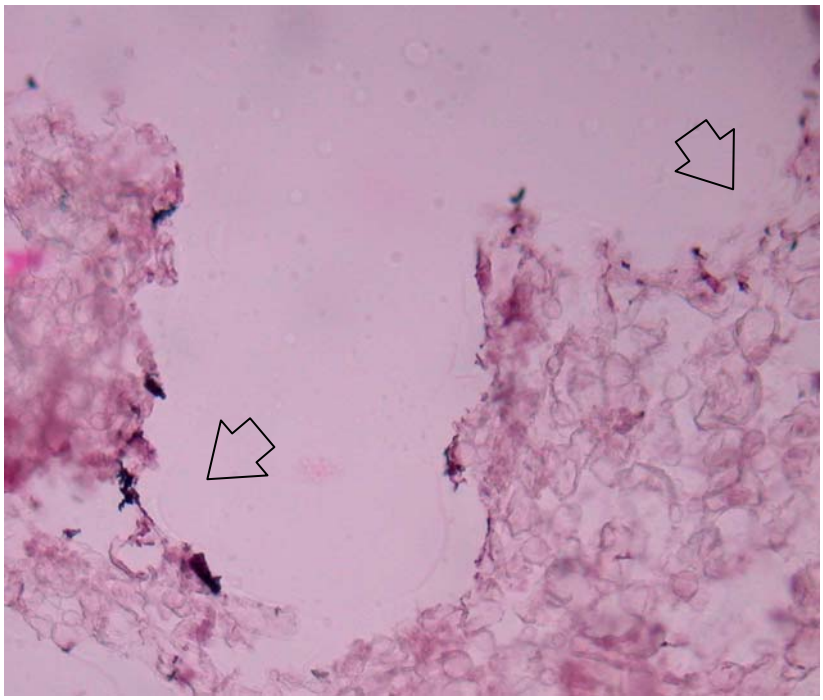


Fig 4.42 Scaffold 4, H & E stained, seen under optical microscope. The top side is shown by arrows. Cells are seen as dark spots.

From the pictures, it can be seen that the cells are concentrated on the top surface of the scaffolds, which is very natural. As the cells are seeded on the surface of the scaffolds, the surface area of the scaffold is first occupied by the growing cells and as the time passes and cells do not find any area on the surface, they start migrating into the pores of the scaffolds. From fig 4.39 (b) it can be seen that there are two cells, one at the edge of the cross section of the scaffold and other a little interior in the section, which indicates that, some cells migrated into the pores of the scaffolds. The same thing can be observed in fig 4.41 (e) and (f).

From fig 4.40 and 4.42, it can be observed that, large number of cells was concentrated on the surface and there was no cell seen in the interior of the scaffold, which can mean that, there was enough area on the surface for the cells for attachment. It should be noted that, the pictures just show the cells present into a particular cross section. There can be some cells attached to the pores of the scaffolds but are not present into the given cross section.

It can be concluded that, the polymer presented a suitable ground for cells adhesion and cells remained attached. There might be some cells which died over time and lost attachment. Hence this experiment gave sufficient footing for cells culture on scaffolds having bioactive chemicals or growth factors embedded in it. The layered scaffolds can be efficiently used for cells culturing and can release growth factors for longer time at a predetermined rate.

CHAPTER 5

DISCUSSION

5.1 Polymer Microspheres Dispersed Scaffolds

The controlled presentation of specific molecular cues, at the appropriate time and location is an underlying objective of many tissue engineering approaches [12]. Microspheres with encapsulated proteins can be embedded within synthetic polymers to prolong the proteins release [12]. In this study, an attempt has been made to incorporate microspheres into the scaffolds and their drug release rate was determined. The drug was associated with PVA. Being hydrophilic and soluble in water, PVA dissociates from drug as it comes in contact with water and the drug is released into the medium.

When molded into 3-D constructs, the drug delivery capacity of microspheres is coupled with the structural support afforded by the scaffolds [12]. Although the drug release profile from microspheres depends on it's composition it also depends on its formation in a scaffold [12]. In case of scaffolds, in which PVA + Acetaminophen solution was blended with PLLA solution at low homogenizer speed (11000 RPM) like scaffold 1 (P0.5, A0.5, H11K, B560) and Scaffold 2 (P0.5, A0.5, H11K, B1120) the percentage of cumulative drug release is higher. In case of scaffolds prepared using high homogenizer speed (22000 RPM), like scaffold 4 (P1.5, A1, H22K, B1120), scaffold 6 (P1.5, A1, H22K, B560) and scaffold 8 (P0.5, A0.5, H22K, B560), the percentage of

cumulative drug release is lower. The low speed of homogenizer resulted in bigger microspheres and high speed resulted in smaller ones.

Drug release not only depends on the pore size but also porosity of the scaffold. The molecular weight of polymer has a large effect on porosity of the scaffolds. A polymer with a high molecular weight does not form scaffold with high porosity as the same polymer with a lower molecular weight [13]. In this study, PLLA which was used as a polymer had high molecular weight (~110 KD). Hence it can be assumed that, the scaffolds were not highly porous, though the pore size was in the range of 25 to 250 µm. As the microspheres were embedded into the polymer, it is likely that, bigger microspheres are readily accessible to water as compared to smaller microspheres. Smaller microspheres have greater chances of getting coated on all sides as compared to bigger ones and drug release from these microspheres is possible only when the polymer of scaffolds degrades.

The most influential factor in this study was found to be blender speed. As discussed earlier, the drug release from the scaffolds was possible due to the access of water to the microspheres. In case of scaffolds, which are prepared using high blender speed (1120 RPM) like scaffold 2 (P0.5, A0.5, H11K, B1120) and scaffold 4 (P1.5, A1, H22K, B1120), it can be seen that, these scaffold have pores uniformly distributed throughout the mass of scaffold, which is again clearly seen in fig 4.1 (b) and 4.2 (a). These scaffolds maintained higher percentage of drug release till first 48 hours of the

experiment, later onwards, scaffold 4 has declined its drug release. This might have caused because it was fabricated with higher homogenizer speed than scaffold 2.

In case of scaffolds prepared with high drug concentration (1.0 % w/v), which is indeed associated with high PVA concentration (1.5 % w/v) like scaffold 6 (P1.5, A1, H22K, B560) and scaffold 7 (P1.5, A1, H11K, B560), these scaffolds have lower percentage of drug release than all other scaffolds after 48 hours of experiment. Though these scaffolds have higher drug concentration, as explained earlier, blender speed seems to be influential in these scaffolds. Being prepared at low blender speed (560 RPM), these scaffolds might lack pores connectivity and hence declined drug release. In case of scaffold 4 (P1.5, A1, H22K, B1120), though it is prepared at high blender speed and has high drug concentration, because of high homogenizer speed it has smaller microspheres and hence the drug release has declined after 48 hours. There might be effect of PVA concentration as well on the structures of these scaffolds. It can be estimated that, the viscosity increases with concentration of PVA. As solutions with low viscosity can be easily homogenized than solutions with high viscosity, these scaffolds might not have uniform emulsion of (PVA + Drug) solution and PLLA solution.

In case of scaffold 6 (P1.5, A1, H22K, B560), which is prepared at low blender speed, high homogenizer speed and has high PVA concentration has the lowest drug release in all the scaffolds studied. From the above mentioned probabilities, it is clear that, this scaffold must be having the least release rate.

It is seen that, the percentage of drug release from the scaffolds is varying from ~ 25 % to ~98 %. The release profile of a drug from the scaffolds depends on the degradation rate of the scaffold polymer. The degradation rate of the polymer depends on the molecular weight [13]. Because of high molecular weight of PLLA, the polymer almost remained intact during 6 days of study. Hence the microspheres which were completely enclosed by PLLA were unable to come in contact with water; therefore the drug could not be released. This is more obvious in case of scaffolds prepared at low speed of blender and high speed of homogenizer, like scaffold 6 (P1.5, A1, H22K, B560), scaffold 4 (P1.5, A1, H22K, B1120), and scaffold 7 (P1.5, A1, H11K, B560), the percentage of drug release is smaller than other scaffolds.

5.2 Layered Scaffolds

The scaffolds made by this procedure had a long drug release period. The reason was, there was no drug in immediate contact with the medium and the drug was released only from two surfaces. From two drug release studies, it was observed that, there is a great influence of Blender speed and the amount of emulsion used to make top and bottom layers.

Blender speed, as mentioned earlier, controls the pore size and porosity. The drug from the middle layer can be released only through the channels formed by dissolving PVA in top and bottom layers and pores present in the same layers. The blender speed was inversely proportional to the pore size. For higher drug release rate, larger pores are

beneficial and hence the lower speed of blender. The other option is using various PVA concentrations in top and bottom layers.

In drug release study 2, two scaffolds having different PVA concentrations had different drug release rates. PVA concentration affects the viscosity of the solution. From fig 4.24 it can be seen that, both the scaffolds have relatively similar structure. In case of scaffold 1 (1 % PVA LO), the % cumulative drug release was greater than scaffold 2 (1.5 % PVA LO) till 15^{th} day. But onwards, it was observed that, scaffold 2 has increased drug release than scaffold 1. The reason could be, because scaffold 1 has lesser amount of PVA than scaffold 2, it readily dissolved in water and let water reach middle layer faster than scaffold 2. Because scaffold 1 released greater amount of drug than scaffold 2, the concentration of drug in both the scaffolds changed with time. Hence after 15^{th} day, scaffold 1 has declined drug release. For scaffold 2, having greater amount of PVA, it took more time for PVA to dissolve in water and hence drug released at lower rate. But because it could maintain higher concentration of drug as compared to scaffold 1, it has a sustained drug release even after 15^{th} day.

From table 4.8, it can be seen that, scaffold 1 is more porous than scaffold 2, which is indeed implicates the connectivity of the pores. Scaffold 1 has higher connectivity than scaffold 2, and as a result less tortuosity than scaffold 2. The overall effect of lesser PVA content and higher porosity, scaffold 1 released drug faster than scaffold 2, which has higher PVA content and low porosity.

5.3 Cells Culture on Scaffolds

From microscope pictures of the cross sections of the scaffolds, it was seen that, there is high concentration of cells on the surface, on which the cells were seeded and few in the interior of the scaffolds, which can be estimated from the time for which cells were cultured. In this experiment the cells were cultured for 10 days, this might not be a sufficient time for cells to penetrate into the interior of the scaffolds, but since some cells are observed in the interior of cross sections, it can be seen that, some cells did penetrate into the scaffolds pores.

The scaffold had PVA in them but the cells were cultured on the scaffolds which were kept into the medium for 3 days. As PVA dissolves readily in water or medium having water, in mean time PVA might have lost into the medium, as a result interaction of cells with PVA could not analyzed.

Staining procedure requires washing with many chemicals and water several time, it is assumed that, not all cells remain attached to the scaffolds in this procedure, hence few cells are seen under microscope.

Finally, it can be concluded that, in the case of microspheres dispersed scaffolds, the drug release rate was high and hence scaffolds made by this method are suitable for short term drug release and high release rate. The major limitation of these scaffolds was burst release. It was observed that, on first day, these scaffolds released significant amount of drug. It is expected that, by increasing blender speed beyond 1120 RPM, the

pore size will reduce and less amount of drug will be released. It is also expected that, the pore size of the scaffolds should be appropriate for cells growth.

In case of layered scaffolds, it was observed that, these scaffolds were good for long term drug release. The drug release rate was smaller as compared to microspheres dispersed scaffolds; however it could not be determined which factor was dominating for these scaffolds. The scaffolds obtained by this method were having good pores connectivity. It is recommended to work more specifically on thickness of the top and bottom layers and blender speed. It is expected that, with increase in PVA concentration in all the layers, drug release rate will increase. At this moment, it could not be determined whether drug concentration has any effect on drug release rate. It is recommended to make all the layers at the same speed of blender so that, uniform pore size and porosity will be obtained. The limitation of this scaffold was that, the drug was allowed to release only from top and bottom layers and other four surfaces were coated by polymer.

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BIOGRAPHICAL INFORMATION

Mayur B. Uttarwar was born in Maharashtra, India, in August 1981. After completing bachelor's education in Computer Science from University of Pune, India, he joined UT Arlington in fall 2004. In UT Arlington, he worked on "Drug Releasing Scaffolds", which is described in details in this manuscript, apart from that, he worked on designing a "Drug Delivery Device" which can deliver drug for variable time periods by releasing drug at a specific rate for each time period. The device is flexible to incorporate multiple drugs and their release rates can be altered. He is looking forward to do Ph.D. in this field.