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Antibacterial Block Copolymer Thin Films and Growth Characteristics of HeLa cells

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Abstract of the Thesis

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The large number of devices used in medical applications have to be protected from microbial attacks as it leads to serious harm. Further, the current methods of tackling this problem involve using chemicals such as disinfectants which are harmful to us humans. This thesis aims to study the effectiveness of block copolymer thin films against microbes without using traditional harmful methods. We have attempted to prepare anti-bacterial thin films which can kill the bacteria using their surface morphology and thus eliminate the harm posed to us.

In the second part of this thesis, we studied the growth characteristics of HeLa cells in hydrogel and the effect of titanium dioxide on them. HeLa cells are widely used in biomedical research and have played a key role in various scientific studies such as the development of polio vaccine. We prepared hydrogels of different concentrations and observed the growth of HeLa cells at different interval.

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Anti-bacterial block copolymer thin films

Chapter 1: Introduction

Theory

An increasing number of synthetic materials are being studied for their applications in biomedical devices. Devices such as heart valves, pacemakers, prosthetic limbs and dental implants use a variety of biomaterials. These biomaterials interact with the biological systems through their surfaces and as such, it is extremely important that we study and control the surface properties of these materials [1]. The combined knowledge of molecular biology and the biophysical correlates of material surface properties can help us better understand the local interactions between cells and their immediate microenvironments [2]. Polymers allow for the maximum flexibility to set up *in vitro* models. As such, polymeric thin films are very attractive for biomedical applications [3].

In micro-phase separated block polymers, the thin films form periodic structures on the order of tens of nanometers with the domain size depending on the molecular weight. At a certain temperature termed, order to disorder transition temperature, T_{ODT}, the micro-phase of block copolymer either separated into individual micro-domains or the domains mix. The self-assembly of block copolymers into well-defined morphologies is useful in numerous applications [4]. We can control the orientation in block copolymer micro domains. Long range ordered nanostructures can be generated using applying external fields, like solvent fields. This effect of shear to achieve long range order in block copolymer thin film forming perpendicular lamellae has been studied by Saswati Pujari et al. [5].

The control of growth of microbes is a very important issue in modern society. Plants and animals have developed a variety of mechanisms to counter the microbial infection. However, the large number of objects in our day to day use lack any defense mechanism against microbial growth. This is a serious problem for biomedical applications. To counter this we can either use sterilization or anti-microbial surfaces. Anti-microbial surfaces prevent the growth microbial biofilm. These either repel the microbes so they cannot attach themselves to the surface or kill the microbes [6]. Although currently, the majority of anti-microbial surfaces employ polymers with active functional groups, block copolymers with their unique surface properties can also be used.

G. Coulon et al. studied the formation of islands and holes morphology in symmetric diblock copolymers. They found that in such diblock copolymer thin films, the interactions of the blocks with the interfaces generates a multilayered structure with the lamellar micro domains oriented parallel to the substrate. As a result of this, the film thickness at equilibrium is quantized:

$$t_e = (n + ½) L$$

where,

t_e – thickness,

n – integer ≥ 0

L – lamellar period

And when the thickness does not correspond to the t_e islands or holes are formed on the surface, the height or depth of which is equivalent to L [7, 8].

When such a diblock copolymer thin film is applied on a surface, it will act as an effective anti-microbial surface. The island and holes morphology will ensure that the bacteria are killed and it has the benefit of not being harmful to humans like the disinfectants currently used.

Objective

This thesis aims to study the formation of islands and holes on the surface of symmetric diblock copolymers and to test their effectiveness against bacteria. We chose PS-P_2VP (polystyrene- poly -2- vinyl pyridine) and PS-PMMA (polystyrene-poly methyl methacrylate) to prepare the thin films. We used an ellipsometer to measure the thickness of the films and used AFM (Atomic Force Microscopy) to observe the surface morphology.

Chapter 2: Experimental Work

Equipment and Materials

Silicon wafer was used as the substrate. Single wavelength ellipsometry was used to determine the thickness of the obtained film. PS-PMMA and PS-P_2VP were directly obtained from the market and were used as is. Deionised (DI) water, hydrogen peroxide and sulfuric acid were also used.

Procedure

We started with the PS-P_2VP block copolymer. We dissolved it in toluene and then diluted the solution to obtain concentration of 7 mg/ml.

We washed the silicon wafer using DI water, hydrogen peroxide and sulfuric acid in the ratio 3:1:1

Then the wafer was placed on the electro spinning machine and two drops of the PS-P_2VP solution were placed on it.

The spin casting was carried out at different speeds to prepare films with different thickness.

The same procedure was followed to prepare PS-PMMA thin films. However, the concentration of the solution prepared was 8 mg/ml. and 12 mg/ml.

The samples were annealed at 170°C for 3 days. Then the surface morphology was observed using AFM.

Results

The thickness obtained for PS-P_2VP samples and the corresponding surface morphology as seen in AFM are given below:

Speed - 2500 rpm	Time - 30s.	
Sample	Thickness A°	
	1	2
1	352 A°	345 A°
2	357 A°	360 A°

Table 2.1: Spin casting parameters and thickness obtained. Trial I

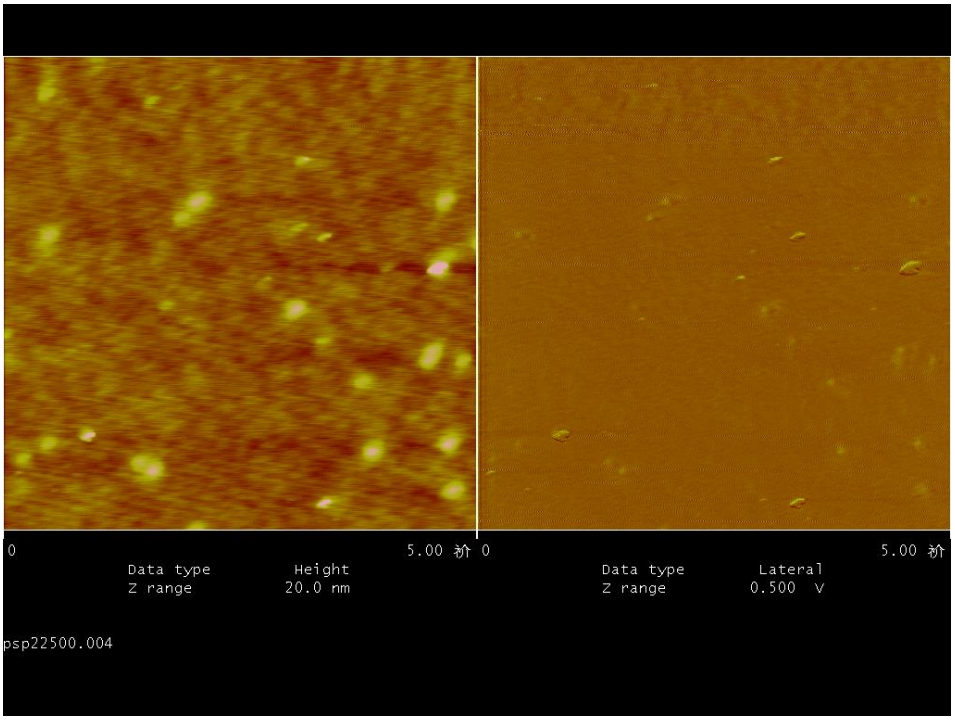

Figure 2.1: PS-P_2VP 2500 rpm Sample 1

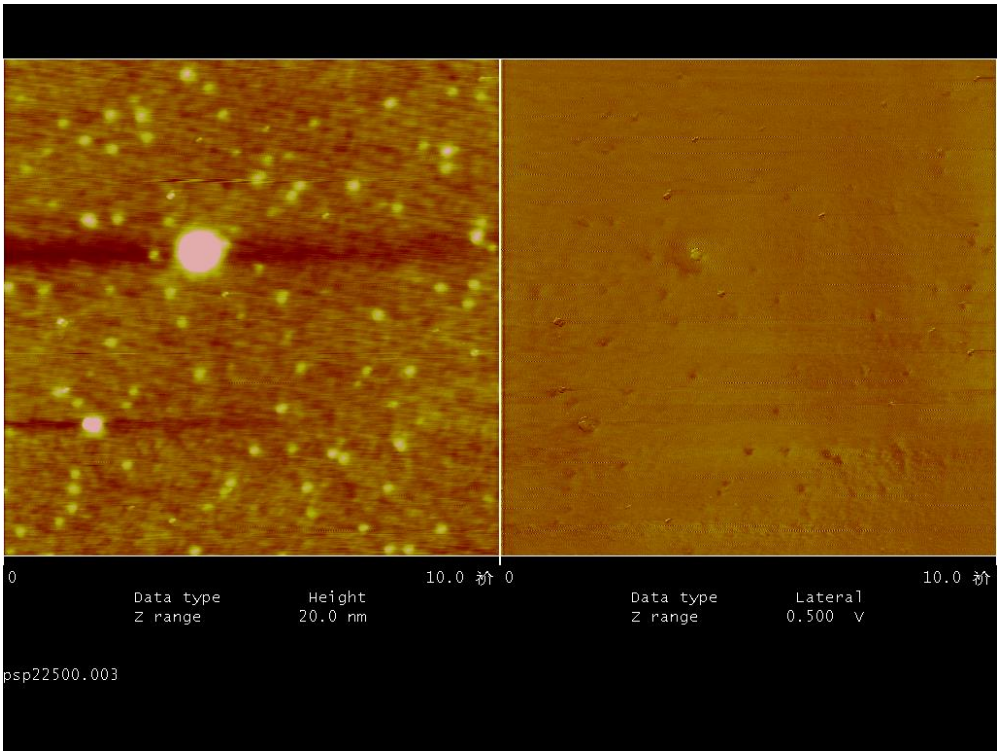

Figure 2.2: PS-P_2VP 2500 rpm Sample 2

Speed - 1500 rpm	Time - 30s.	
Sample	Thickness A°	
	1	2
1	373 A°	366 A°
2	390 A°	375 A°

Table 2.2: Spin casting parameters and thickness obtained. Trial II

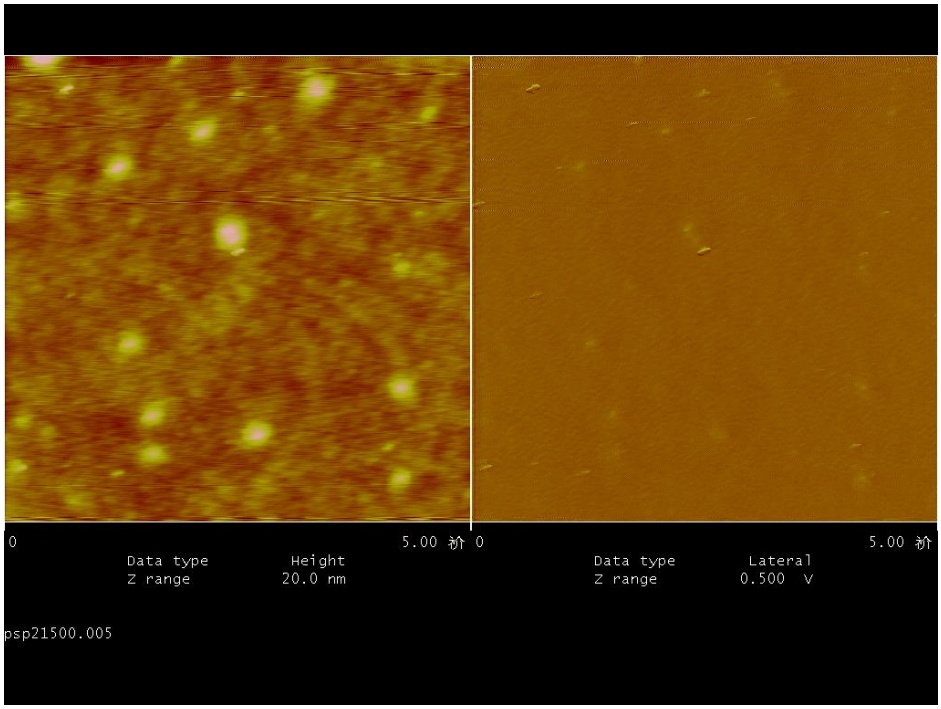

Figure 2.3: PS-P_2VP 1500 rpm Sample 1

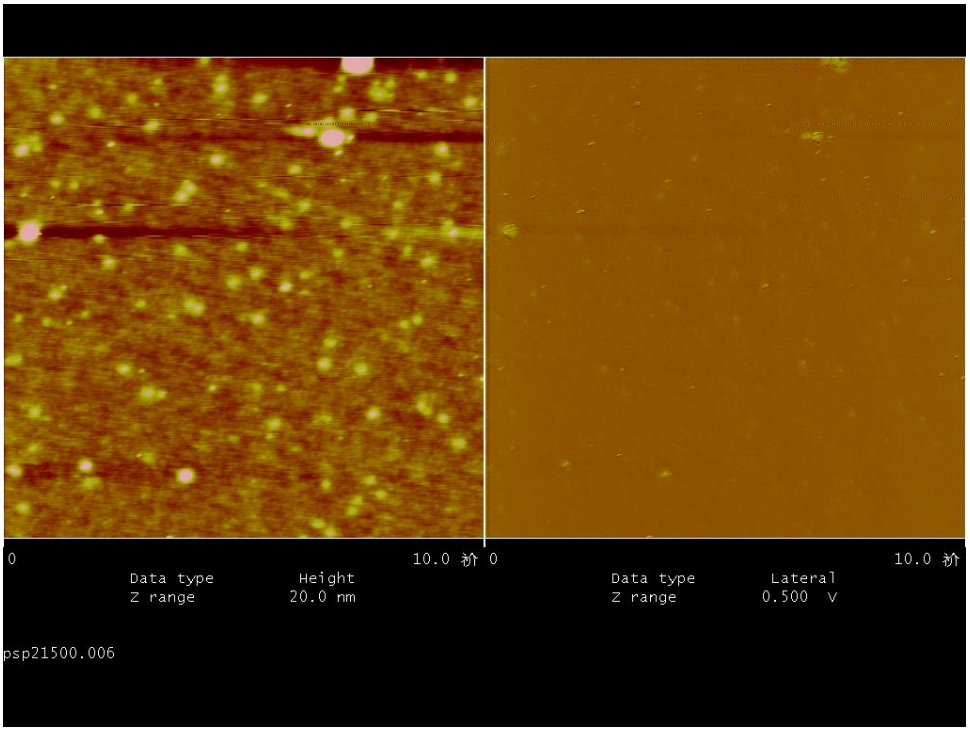

Figure 2.4: PS-P_2VP 1500 rpm Sample 2

Speed - 1200 rpm	Time - 30s.	
Sample	Thickness A°	
	1	2
1	407 A°	416 A°
2	408 A°	415 A°

Table 2.3: Spin casting parameters and thickness obtained. Trial III

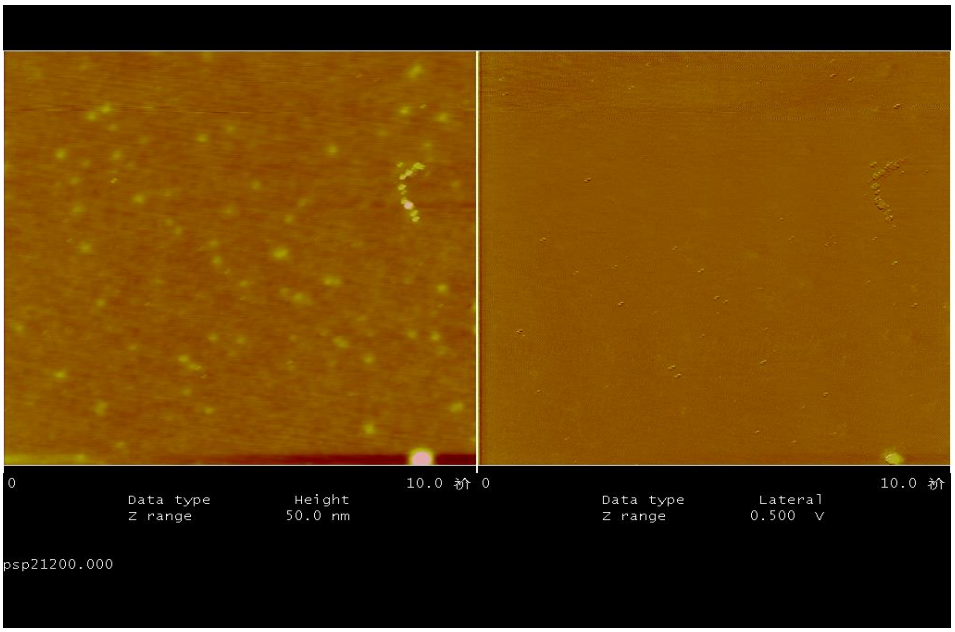

Figure 2.5: PS-P_2VP 1200 rpm Sample 1

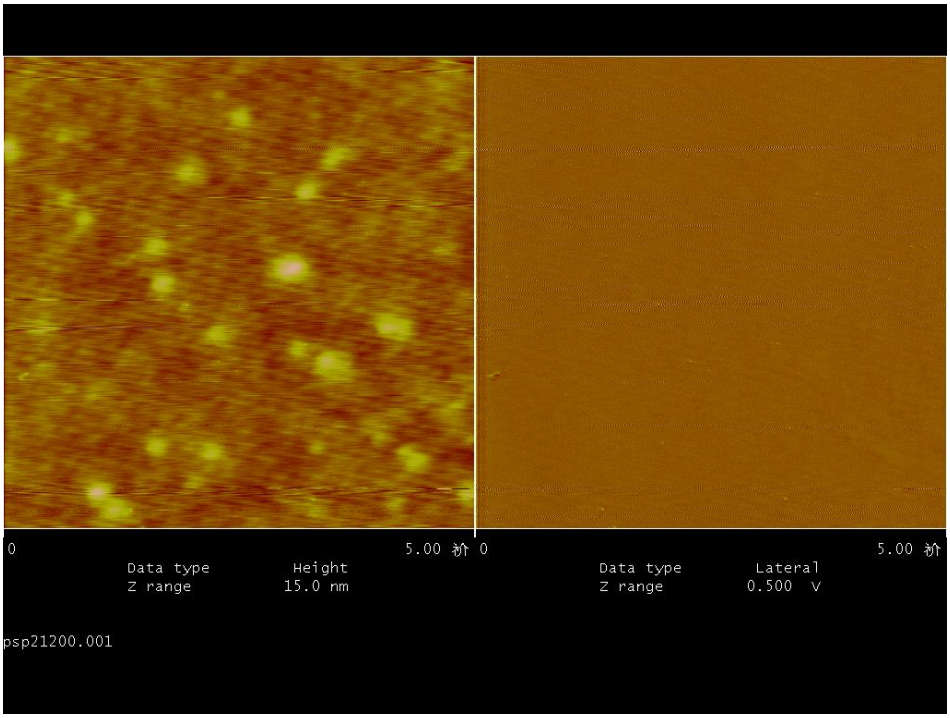

Figure 2.6: PS-P_2VP 1200 rpm Sample 2

The results for the PS-PMMA samples are shown below. The AFM images of the surface were taken under tapping mode.

Speed - 1500 rpm	Time - 30s.	
Sample	Thickness A°	
	1	2
1	441 A°	439 A°
2	449 A°	450 A°

Table 2.4: Spin casting parameters and thickness obtained. Trial I

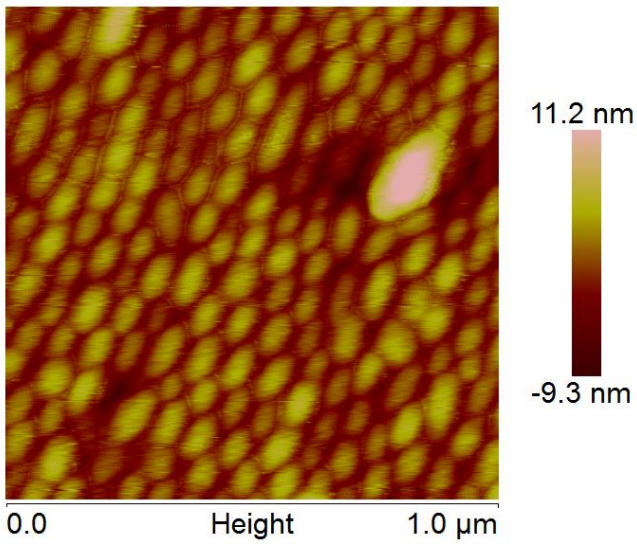

Figure 2.7: PS-PMMA 1500 rpm Sample 1

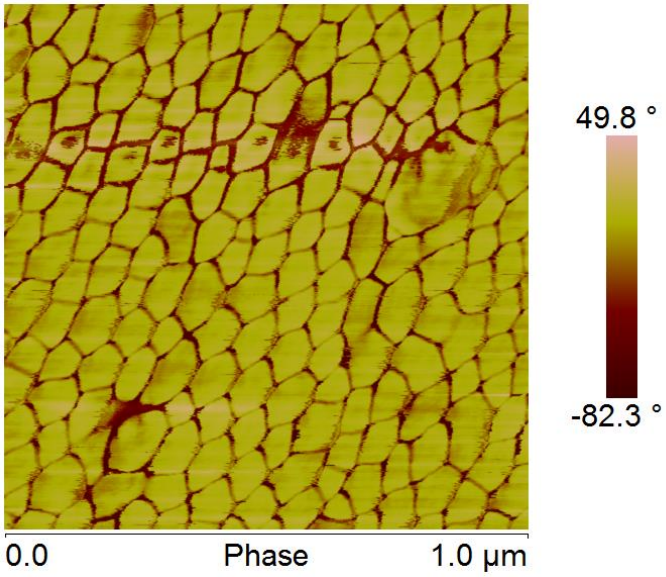

Figure 2.8: PS-PMMA 1500 rpm Sample 2

Conclusion

As is clear from the above images, the desired islands and holes morphology is not observed. Instead is a lamellar morphology is obtained. This indicates that the process parameters used are unsuitable. We will try again by modifying the speed and time for spin casting as well as the annealing time and temperature, to obtain the desired morphology for anti-bacterial applications.

Growth characteristics of HeLa cells

Chapter 3: Introduction

Background

The HeLa cell line is the oldest, most commonly used permanent human cell line. It originated from the cervical cancer tumor of patient Henrietta Lacks, after which it is named. The cells have been used widely, and notably were used to develop the polio vaccine [9, 10]. Being the first human cell line established, HeLa cells have been pivotal to several modern studies. They were used to study the infection in human cells due to environmental perturbations by Ludwig H. et al. and genetic disorder by Jaluria P. et al. [11, 12]. Pelkmans L. et al. used them to study endocytosis [13].

In tissue engineering, due to the fast pace of development, we can now repair or replace damaged tissue in organs. This is a rapidly growing and exciting field. One of the key components in this field are the cell scaffolds. They are especially important as they should have similarities to the natural extra cellular matrix and their surface should permit cell adhesion and growth [6]. Hydrogels, due to their excellent biocompatibility, are widely used in this application. They consist of hydrophilic polymers or copolymers cross linked to form a three dimensional structure and can absorb large amounts of water [14, 15].

Titanium dioxide is a naturally occurring oxide of titanium. Although the bulk of it is used as a pigment in paint, its biomedical properties are gaining interest. This is due to its excellent biocompatibility, high chemical stability and low toxicity [16]. Titanium dioxide has been demonstrated to enrich phosphopeptides for phosphoproteome analysis by Li-Rong Yu et al. Additionally titanium dioxide can be used to kill cancer cells [17].

Objective

This thesis aims to study the growth characteristics of HeLa cells under different conditions. We prepared hydrogels with different ratio of its constituents and kept them incubated for different number of days. Then we observed the cells using confocal laser scanning microscopy.

Chapter 4: Experimental Work

Equipment and materials

The hydrogel was prepared using porcine Type A gelatin and Microbial Transglutaminase (mTG). HeLa P8 cells were used.

The cell growth medium used was Dulbecco's modified eagle medium (DMEM) with reference number 11965-092.

Dulbecco's phosphate buffered saline (DPBS) with reference number 14190-144 was also used.

Fetal Bovine Serum (FBS) was used to provide essential proteins for cell growth and penicillin/streptomycin (Pen/Strep) was used to prevent the growth of bacteria.

0.05% Trypsin-EDTA solution was used to trypsinize the cells. 0.4 % Triton X-100 solution was used to permeabilize the cells.

Propidium iodide (PI) (Red Dye) and Alexa Fluor AF488 (Green Dye) were used as dyes while 7% Formaldehyde was used to fix the cells.

Leica TCS-SP2 microscope was used to observe the stained cells and IEC HN-SII centrifuge was used to centrifuge the cells. The hemocytometer was used to count the cells and the cell concentration was then calculated.

We use titanium dioxide in its mineral form- Rutile and Anatase.

Procedure

Part I –

10% wt./vol. solution of the gelatin was prepared in DI water. We need to heat it to 65°C for the gelatin to dissolve in the water.

10%wt./vol. solution of the mTG was prepared in DI water. It is easily dissolved at room temperature itself.

Then the solutions were sterilized using filters and 5ml syringes.

We prepared three six well plates of the hydrogels 1.25 ml. in each well, mixed in the ratios 1:25 and 1:75 of mTG : Gelatin and kept them in the incubator for 24 hr. at 37°C.

Then the hydrogels were heated in the rocker at 65°C for 15 min. to deactivate the mTG.

Part II –

The cell growth medium was obtained by preparing a solution of 10% FBS and 1% Pen/Strep in DMEM. The cells were placed in a 15 ml. tube and kept in the incubator for 24 hr.

After that 4 ml. of the 0.05% Trypsin solution was added to the cell growth medium and kept in the incubator for 5 min. Then the solution was removed and 4 ml. of DMEM was added.

The cells were centrifuged at 1000 rpm for 10 min. after which the medium was again removed and the cells were mixed and diluted with 4 ml. of the medium.

Using a hemocytometer the number of cells was counted and the concentration of the cells was then calculated.

The concentration was found to be 0.052cells/ml.

52 µl. was added to each of the three plates and they were placed in the incubator for 24 hr., 48 hr. and 72 hr.

After 24 hr. add 40 µl. of Anatase to two wells, one which has the ratio 1:25 and the other 1:75, and add 40 µl. of Rutile similarly.

The other two wells act as the control specimen.

Part III –

The medium was removed from the wells and they were washed with DPBS.

Then the 3.7% formaldehyde solution was used to fix the cells.

After 15 min. the formaldehyde was removed and the wells were washed twice with DPBS.

After the DPBS was removed, we added 1 ml. of the 0.4% Triton X-100 solution to each well.

The plate was kept at room temperature for exactly 7 min. to allow the cells to permeabilize.

Then the 0.4% Triton X-100 solution was removed and the wells were washed with DPBS twice.

Part IV –

When adding the dye take care not to expose it to light.

Prepare a solution of PI in DPBS in the ratio 1:200

Add 1 ml. of the solution to each well and keep the plate at room temperature for exactly 2 min. 30 s.

Remove the dye and wash twice with DPBS.

Then we prepare the solution of AF in DPBS in the ratio 1:300

Add 1 ml. of the solution to each well and keep the plate at room temperature for 20 min.

Remove the dye and wash twice with DPBS.

Then enough DPBS was added so that the cells would remain hydrated and the plate was kept in the refrigerator.

This process was repeated for each of the plates.

Part V –

After the staining, we used confocal microscopy to obtain images of each of the plates.

About 100 pictures were taken of the cells from each of the samples.

The pictures were then analyzed using the software ImageJ.

Results

After analyzing the images, we can observe that the average cell density increases for the sample increases from 6993 cells/cm^2 on day 1 to 9705 cells/cm^2 on day 2 to 11356 cells/cm^2 on day 3.

By studying these characteristics we can attempt to better understand the effect of the hydrogel matrix and titanium dioxide system on HeLa cells.

Figures and tables

The corresponding data and the graph for the Rutile sample in hydrogel of ratio 1:75 are as shown:

Sample- R 1 to 75 1

Day	1 image cell voxels(pix^2)	# cells in image	Avg area of cell (pix^2)	Area in stack	# Cells in stack	# Images in stack	Avg cell density (Cells/cm^2)	Standard Error
1	15722	37	424	1475800	3480	87	6993	41.80909
3	17656	32	551	3436099	6236	96	11356	89.67162
2	18649	53	351	1968323	5607	101	9705	109.8271

Table 4.1: Growth rate of the sample R 1 to 75 1 analyzed using ImageJ software

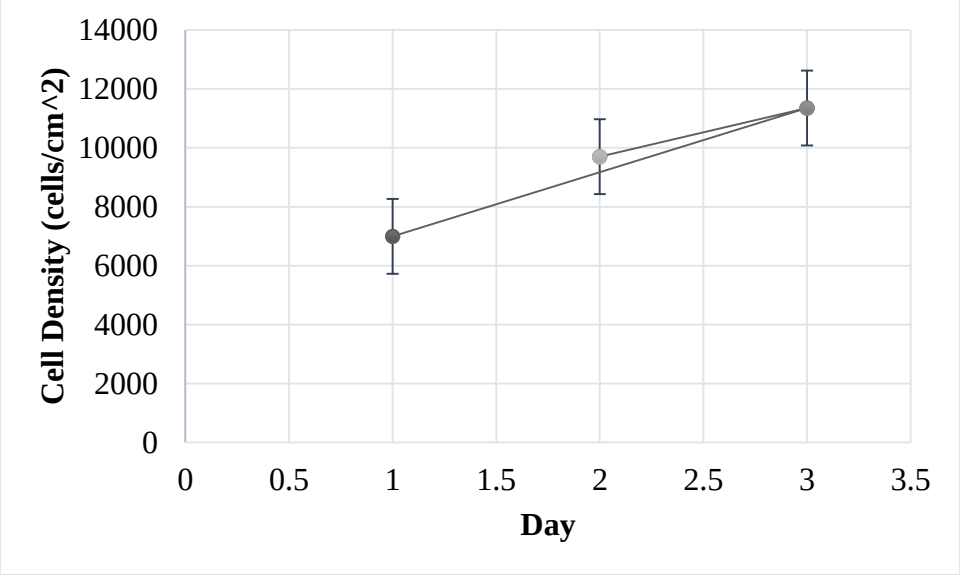

Figure 4.1: Growth curve for the sample R 1 to 75 1

The images for the sample R 1 to 75 1 under confocal microscopy-

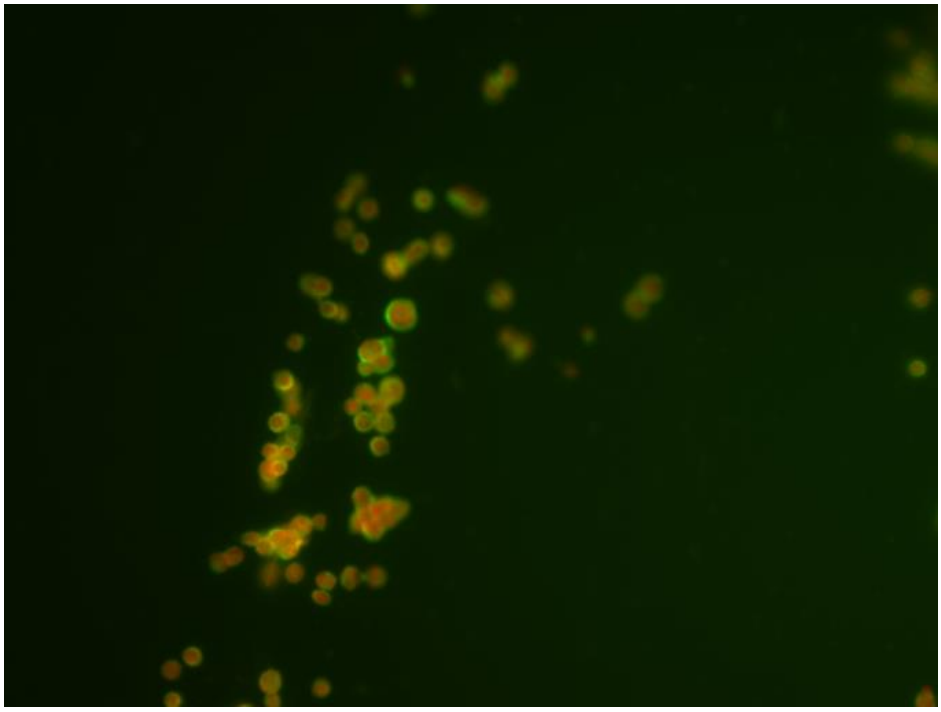

Figure 4.2: R 1 to 75 1 comp 1

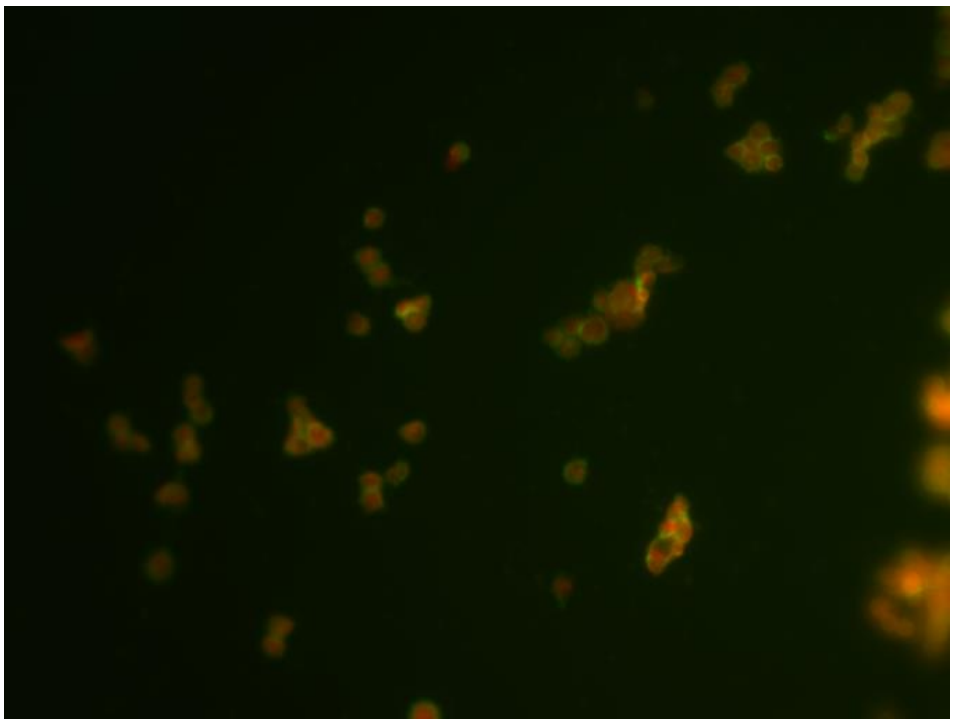

Figure 4.3: R 1 to 75 1 comp 2

The structure of Rutile and Anatase-

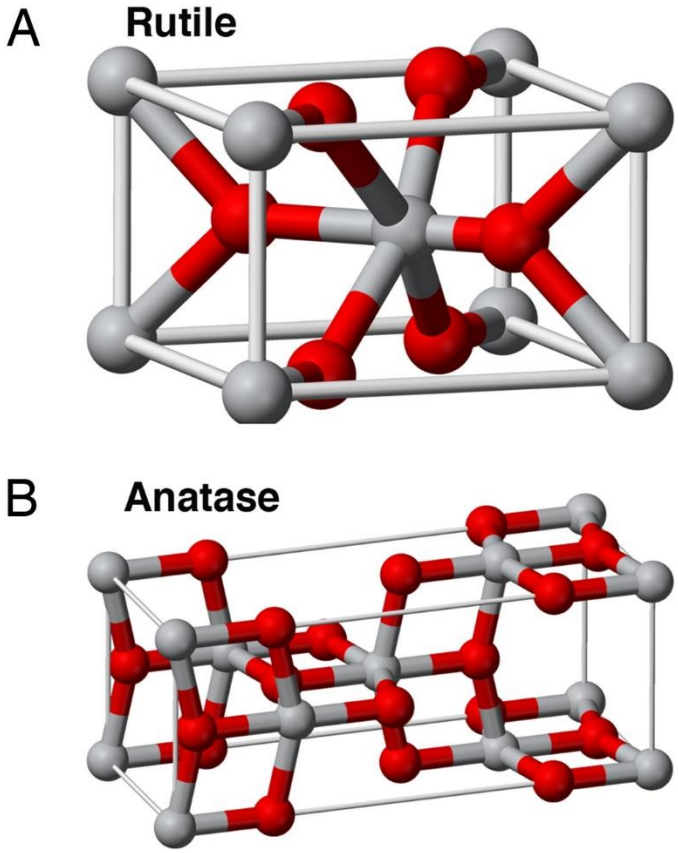

Figure 4.4: Bulk crystalline structure of Rutile and Anatase. Titanium atoms are in red and oxygen atoms in gray [9].

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