Smart Microstructures for Hyperthermic Therapy

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ABSTRACT

In this study the microstructures of polycaprolactone- Fe_3O_4 (PCL-FO) composites were fabricated for hyperthermic therapy. The spin coating and electrospinning techniques were used to fabricate PCL fabricate PCL-xFO (x= concentration of FO nanoparticles) thin films and nanofibers respectively. Various characterization techniques like scanning electron microscopy (SEM), atomic force microscopy (AFM), vibrating sample magnetometry (VSM), Contact Angle Goniometry, and X-Ray Diffraction were deployed. The cytocompatibility of PCL-xFO microstructures were analysed with "HeLa" cell line. It is found that the viability of HeLa cells grown on PCL-xFO microstructures after 24 h of incubation was increased with an increase in the concentration of the FO nanoparticles. Interestingly, in the presence of external alternating magnetic field (AMF) these microstructures generated heat and results in decrease in cell viability.

Keywords: Electrospinning, Alternating magnetic field, Polycaprolactone.

1. INTRODUCTION

New techniques as well as devices have evolved for the treatment of cancer from the past few years. Earlier conventional treatments for cancer involved different techniques like minimally invasive surgery, chemotherapy and radiation therapy etc, also certain cancer vaccines like human papilloma virus (HPV), which was approved in June 2006 by Food and Drug Administration (FDA) were used. As reported cancer treatment through chemotherapy involves many health risks like side effects followed by hair loss, rashes, damage to the normal cells surrounding the cancer tissue, also in certain cases liver and heart related problems reported. In Radiation therapy, exposure to high energy X-Ray radiations which also causes side effects like Fatigue, Skin irritation on the exposed area, Nausea and diarrhoea. All these health related issues due to the conventional cancer treatments, therefore frequently raised the need for targeted drug delivery systems so as to reduce the health risks by targeting only the cancer tissues.

Looking few years back shows the different biocompatible polymers as the centre of attraction to many Scientists for the fabrication of such kind of micro devices that can travel through the natural pathways inside the body detect the tumour and could effectively destroy them leaving the normal cell structures unaltered. In this regard, the PCL-xFO microstructures were fabricated and tested with HeLa cells. Such microdevices Recently Magnetic nanoparticles (MNP's) have emerged in the field of nanomedicines and nanodevices which showing its wonderful biomedical applications including Diagnosis and imaging etc. Also mentioning it's another great application i.e. magnetic Hyperthermia which is the heat generation property of (MNP's) on the application of alternating magnetic field to them. [1] For the hyperthermia experiment it's needed to incorporate material in the films and fibres that can generate heat using hysteresis loss or Neel relaxation. In the recent few years MNP's have found to be applicable in the medical field for enhancing the images acquired by the MRI.

These MNP's are expressed by the magnetotactic bacteria naturally in the form of magnetosomes which was used as magnetic microbots primarily by(Martel et al. 2007) where they studied the swimming action as well as its response to the external magnetic field which they called as magnetotaxis for which they selected MC-1MTB strain for their experiments, which they could load with the drug molecule and send them inside the body and could track them with the application of external magnetic field.[4]

2. EXPERIMENTAL PROCEDURES

2.1. Fabrication of Thin Films and Fibrous substrates

Thin films of concentration 12% $^{W}/_{v,}$ polycaprolactone (PCL) for 2ml of solution is prepared by adding 0.24gm of PCL beads in 2ml of Trifluroethanol (TFE) solvent. This completes with the formation of base material for the cell adhesion, but for making the base material magnetically active addition of Iron oxide MNP's is to be done. So starting with different concentrations of Iron oxide MNP's at different rotations per minute (rpm).

ION particle concentration in PCL (w/w)%	Rotations per minute(RPM)
10%	1000, 3000, 6000 For all.
20%	
30%	
40%	
60%	

Figure 1. Table showing Different concentrations of Iron oxide MNP's in PCL solution at Different rpm.

After preparing the sample solution the spin coating is done on Aluminium substrate using Spin coater machine at different rpm for 30 seconds. Spin coating resulted in the creation of thin films of PCL Iron oxide MNP's composite evenly spread on the aluminium substrate.
Surface characteristics were determined by atomic Force Microscopy and SEM .

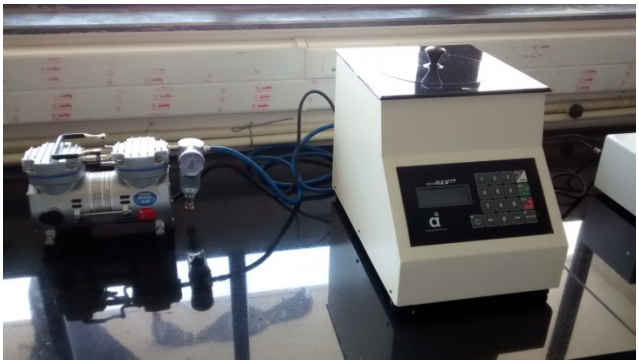


Figure 1. Spin coating machine.

For the determination of the thickness of the fabricated films optical profilometery was done by spin coating the PCL-xFO composites on the glass substrates to avoid any error due to uneven substrate. Fibrous structures were fabricated using Electrospinning technique using the same 2ml of 12%^{W}/v PCL. There are many aspects that should be kept in mind during the fabrication of fibres like fibre thickness, deposition time etc. Diameter of nano fibres is one of the major parameter that should be optimized so as to properly internalize the MNP's within the fibre so that the MNP's are not on the surface of the fibres, **Following samples were Electrospun:-**

MNP concentration (w/w)%	**Flow rate (ml/hr)**	**Voltage applied (kv)**
PCL	0.8	12, 15
10%		
20%		
30%		

Figure 2. Table showing parameters at which samples were Electrospun

Figure 2. Electrospinnig machine.

2.2. "HeLa" Cell line culture

HeLa cell type is the immortal cell line and one of the oldest human cell line commonly used in various research fields derived from cervical cancer cell. [2]

The cells were grown in $25cm^2$ cell culture flask using Dulbecco's Modified Eagle Medium (dmem, himedia laboratories pvt lt. mumbai) followed by addition of 10%Fetal Bovine Serum (fbs, sigma aldrich, usa) and 1% penicillin/streptomycin solution and was filtered using syringe filter and kept in the incubator (incubator, Eppendorf) at 37^0C with the maintained level of 5% Co_2 inside it. Once the cells are confluent, say 80-90% then the Cells were trypsinized using trypsin (0.25% trypsineEDTA solution, SIGMA ALDRICH) and transferred to the fresh flask containing medium.

2.2.1. Cell Revival

Vial containing 1ml of HeLa cell suspension that was kept frozen in the liquid Nitrogen at (-196^0C) is been taken out, and prior of transferring the cell suspension it has to be thawed so as to bring the temperature of the frozen cells to the cell culturing temperature i.e. 37^0C. After that 1ml of pre-warmed media is added drop by drop to the vial (without G418 and Amp.B). The media is gently mixed and transferred into the fresh tube. Now 9ml of medium is added and set it for the centrifugation at 600rpm. After centrifugation discard the supernatant gently and dissolve the cell pellet in 3ml of medium (without G418 and Amp.B), and finally transfer it to the culture flask containing 2ml of medium.

2.3. Cell viability assay (MTT)

For estimating the growth of cells on the fibres and films was done by MTT assay. To perform MTT assay set up the culture well plates and the desired amount of films and fibres were seeded and place into the culture wells. After the cell start growing carefully media was removed. 0.5ml of MTT solution was added to the sample containing wells followed by incubation for 2 hours. After 2 hours MTT solution was removed and 0.5ml of Dimethyl sulphoxide (DMSO) was added to the culture wells. After addition of DMSO the solution was transferred into another culture well plate and the absorbance was recorded using spectrophotometer at 570nm.

2.3.1. Alternating Magnetic Field (AMF) Heating

To quantify the Hyperthermic effect produced by the films and fibres, the samples were primarily exposed to the external alternating magnetic field without seeding the HeLa cells on them, so as to first make out the observations regarding their heat generation ability. As the both samples were containing Iron Oxide MNP's which are paramagnetic in nature will always respond to the alternating magnetic field resulting in the formation of Eddy currents on the surface of MNP's which are ultimately responsible for the heat generation in the samples. For the conformation the samples were cut into small $1cm^2$ patches, and were peeled off from their substrates. Small peels of samples were put into the centrifuge tubes containing 1ml of 1X Phosphate Buffer Saline (PBS) and the tubes were held inside the solenoid using stand. AMF apparatus was switched on for 10 minutes at 400Amps, and the temperature was recorded at every 50 seconds using Alcohol thermometer. No metal object should be place inside the solenoid so as to get the only heating effect due to the samples contained.

Figure 5. alternating magnetic field apparatus.

2.3.2. Confocal Microscopy

Confocal microscope (In Cell Analyzer 6000) is a laser line-scanning confocal imaging system based on patented CMOS camera detection technology. Unlike conventional confocal microscopy, which relies on a physical aperture

to reject out-of-focus light, IN Cell Analyzer 6000 uses the electronic rolling shutter of a CMOS camera as a confocal aperture.

For imaging the cell growth on the films and fibres normal optical microscopy can't be deployed, because the samples of films and fibres itself were opaque and was unsuitable for observing cell growth on them under the optical microscope. For this kind of samples Confocal microscopy seemed to be more fruitful, also this provide a wide range of contrast. In order to visualize cells and get the images there is need for staining the sample with appropriate florescent dyes that can help to excite the dyes at the certain wavelength of light so as to make the cells visible. For preparing cells for staining HeLa cells were primarily fixed with 400ul of 3.7% Formaldehyde for 20 minutes and then washed thrice with 1X pbs. For ensuring that the dye permeates inside the cells, 400ul of (triton™ x-100) solution was added and left for10 minutes and the washed thrice with 1X PBS solution. Then after all the non specific proteins to the dye were blocked by adding 5% FBS in 1X of pbs for one hour and then washed thrice with the 1X PBS. After the cells are ready add two drops of Phalloidin Green i.e. (Actin based stain) to the cells half an hour and then wash the cells thrice with 1X of pbs. For staining the nucles of the cells Hoechst dye was added 2ug/ml and washes four times and then dip the stained samples in 1X of PBS. Using (In Cell Analyser 6000) cell growth was observed.

2.3.3. Vibrating sample Magnetometer

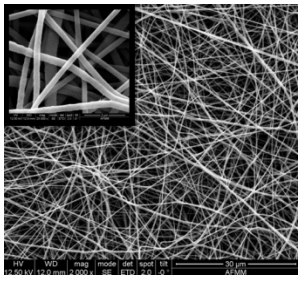
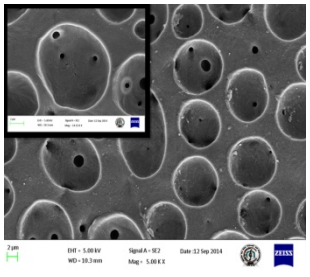
Sample is sticked to a vibrating rod with the Teflon tape in between two huge electromagnets which when applied magnetic field starts vibrating and starts generating readings. Only one sample at a time can be loaded which take approx 1hour to get the full readings.

2.4. Results and Discussion

2.4.1. Characterization of PCL-xFO microstructures

Surface roughness of the film as shown in Figure16. is due to the formation of porous structures on its surface and the average pore size measured for the different samples were, PCL film (9.9804µm), PCL10FO film (4.7955um), PCL30FO film (2.7824um). On the basis on following observation, it seems that the pore size of different samples decrease as the increase in concentration of MNP's.

Increase in surface roughness of the surface of different samples comes from the agglomerated MNP's on the surface as confirmed by the atomic force microscopy for which the average root mean square roughness was measured was, PCL film (72.4um), PCL10FO film (74.8um), PCL30FO film (80.0um). From this conclusion made is that, on increasing the MNP's concentration now the surface rough that comes into play is due to the agglomeration of the MNP's on the surface. For the context of fibre sample the average diameter as measured was PCL fibres (76.4um), PCL10FO fibres (74.8um), and PCL30FO fibres (78.5um). This shows that absence or, presence of different concentration of MNP's does not affect the fibres thickness as much. Possible reason for this might be the earlier parameters such as voltage applied, distance between the electrodes and flow rate of the solution were kept constant for the fabrication of fibres.

(a)  (b) 

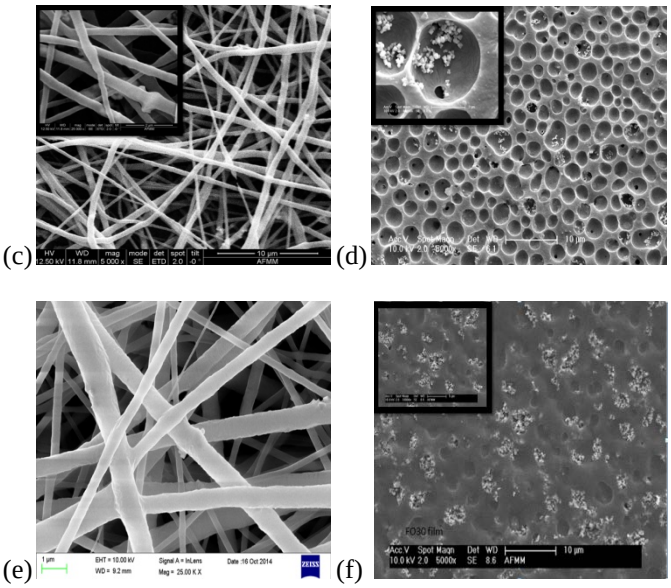


Figure 16. **(a)&(b) PCL fibres and film, (c)&(d) PCL10FO fibres and film, (e)&(f)PCL20FO fibres and PCL30FO film.**

Observing the increase and decrease in the thickness of the films and fibres a check for the Hydrophobicity and Hydrophilicity is done by using contact angle goniometry, which was performed using deionized water. The measurements were recorded for PCL film (80º), PCL10FO film (79º), and PCL30FO film (77º), from this its concluded that their is no significant difference in then contact angle, thus there is no change in the hydrophobic nature of the sample.

A graph is plotted in between the magnetic moment (emu/gm) vs. magnetic field (gauss) which confirms the presence of magnetic material inside the sample i.e. films and fibres. PCL10FO film (5emu/g), PCL30FO film (15emu/g), Alone FO(55emu/g), but alone PCL films/fibres do not respond to the magnetic field because it is non magnetic in nature. As we can observe in the Figure 11. The PCL30FO films show more magnetic moment (values) then the PCL10FO films which confirms the increase in the concentration of MNP's.

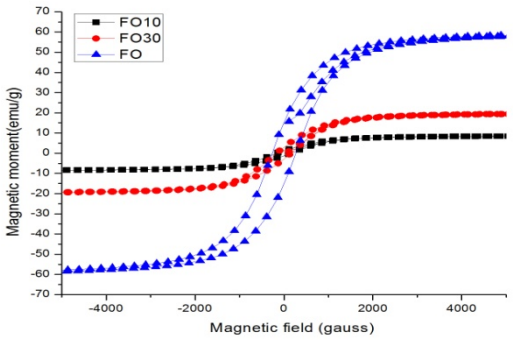


Figure 11. **Plot showing hysteresis loop of different samples analysed by VSM.**

2.4.2. X-Ray Diffraction

The both samples were set for the XRD, which confirmed the presence of Iron Oxide MNP's on comparing the both samples to their respective reference sample i.e. Iron oxide MNP's itself a represented at (311) plane where there is the spike for iron oxide sample. PCL is a semi-crystalline polymer for which we get the output spikes on the different samples itself due to the presence of PCL in each sample represented at(111) plane.

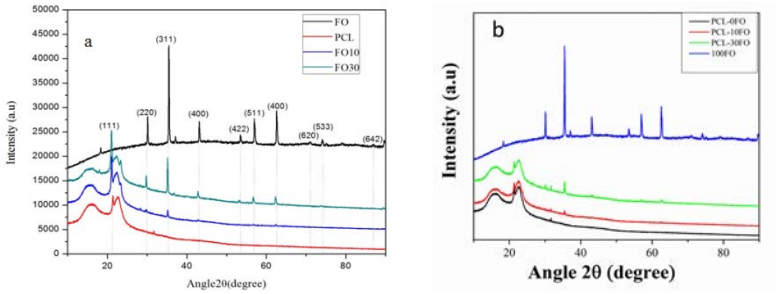


Figure 10. **XRD intensity plot for fibres and films.**

2.5. Cell viability assay (MTT)

For estimating the growth of cells on the fibres and films was done by MTT assay. MTT assay was set up by preparing the culture well plates and the desired amount of films and fibres were seeded and place into the culture wells. After the culture well plate wells are confluent with cells, then carefully media was removed. 0.5ml of MTT solution was added to the sample containing wells followed by incubation for 2 hours. After 2 hours MTT solution was removed and 0.5ml of Dimethyl sulphoxide (DMSO) was added to the culture wells. After addition of DMSO the solution was transferred into another culture well plate and the absorbance was recorded using spectrophotometer at 570nm.

Absorbance reading for both the samples were taken, then converted to percentage cell viability readings and plotted with respect to the days of incubation.

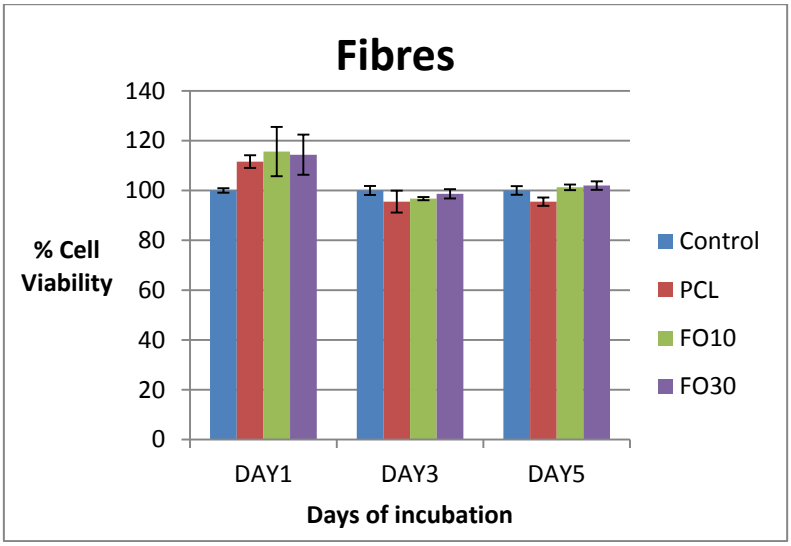


Figure 12. **Plot showing percentage cell viability on fibres.**

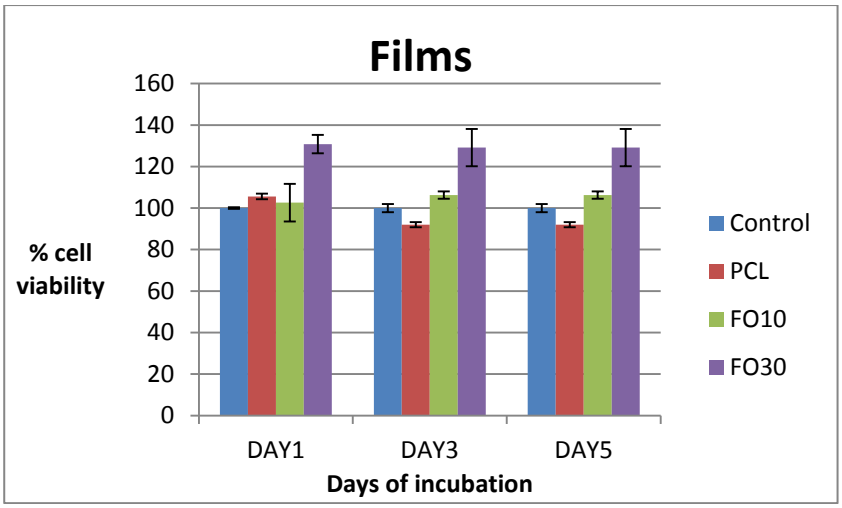
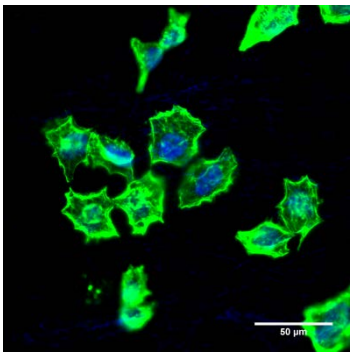
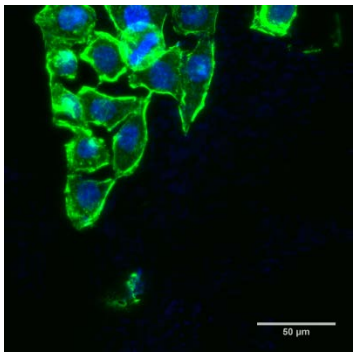
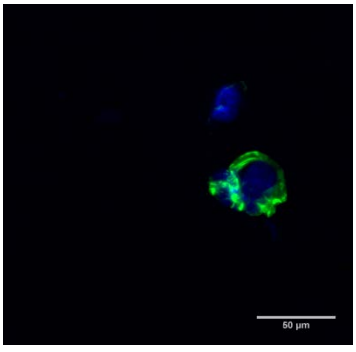
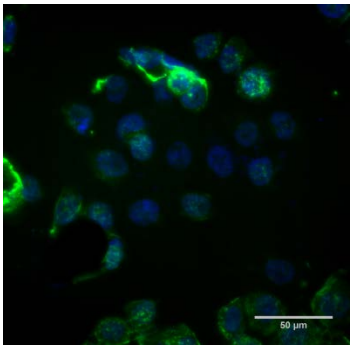


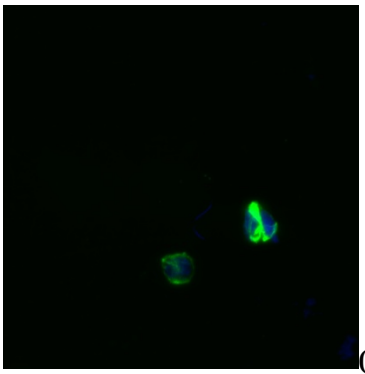
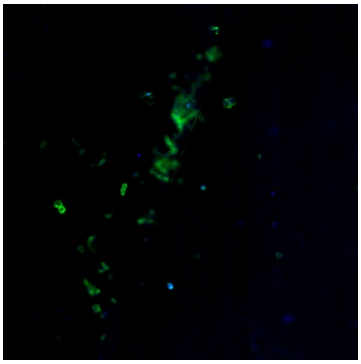
Figure 13. Plot showing percentage cell viability on films.

2.5.1. Observation of cell morphology on the different samples

As shown in Figure 15. Cell growth was observed to be more or less constant on the samples, but the interesting morphology was observed on the different samples for example growth on the PCL10FO fibres, seems to be the independent spread on the other hand on the PCL10FO films cells are grown more closer to each other.

(a).  **(b).** 

(c).  **(d).** 

(e).  (f). 

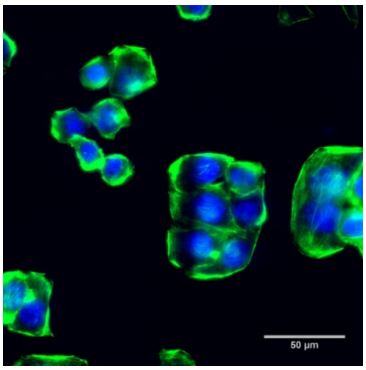
(g). 

Figure 15. **growth of hila cells on diffrent samples,(a) FO10 fibres, (b) FO10 film, (c) FO30 fibres, (d) FO30 film, (e) PCL fibres, (f) PCL film, (g) control, blue- Nucleus, green- Cytoplasm.**

2.5.2. Cellular viability are exposing them to the alternating magnetic field

By keeping the alternating magnetic field constant at 400amps for the 10 minutes we reached up to the range of 43 ºC to 45 ºC which was sufficient to kill cancerous cells. For the confirmation the cell viability test (MTT assay) was done. Following observation as shown in the Figure 16. for the PCL film, PCL10FO film, PCL30FO film a very less cell killing was reported, which might be caused due to the improper heat transfer from the film to the cells due to the improper contact of the cells at film surface. Whereas the remarkable cell growth and their killing was observed on the PCL30FO fibres.

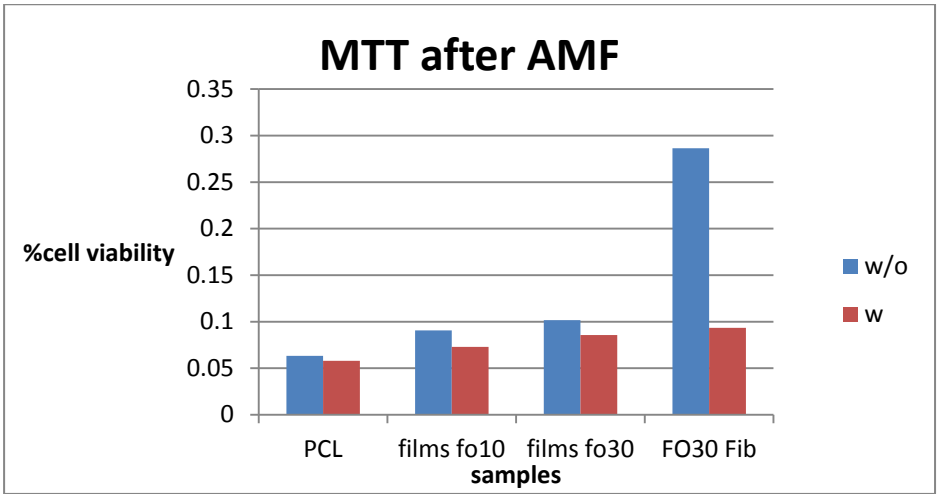


Figure 16. **Plot showing effect on cells blue for the without magnetic field and red for with magnetic field.**

3. Conclusions

As observed from the above results now it's easy to make simple concepts about the aim of the whole experiment. The PCL selected as the substrate for the cell growth seems to be suitable for the cell culture experiment as the remarkable cell growth was reported in MTT Assay without any toxic effects. Still more improvements and optimization of certain parameters like concentration of MNP's, use of more temperature sensitive apparatus to measure even the smallest change in the temperature of the films on the application of AC magnetic fields and experimenting with different cell lines like 3T3 NIH can be done in order to increase the efficiency and effectiveness of the whole assembly. These type of microstructures can be deployed for the treatment of certain types of cancer.

3.1. Future plans

Many therapeutic devices can be made out by using such kind of assemblies. As the PCL used in the experiment is biocompatible and no immuno toxic effects have been reported during experiment. Some bigger film can be fabricated in order to kill some skin cancer like squamous cell carcinoma as the procedure is highly selective only for the cancer cells normal cells are left unaffected. We can fabricate some microdevices that can be send inside the body through blood circulation for the targeted drug delivery to the tumours inside, or we coat small nanobeads with PCL iron oxide composite so as to make them biocompatible for the drug delivery applications.[3]

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