Original Article

Release of Doxorubicin's Active Ingredient from the Hydrogels Derived from Acrylamide and their Biological Functions

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ABSTRACT

Poly (HEMA/acrylamide/ methyl methacrylate) (PHAM) and Poly (Acrylamide/ methyl methacrylate)(PAM) polymers were synthesized for using in the release of Doxorubicin drug. Poly (HEMA/acrylamide/methyl methacrylate) (PHAM) and Poly (Acrylamide/methyl methacrylate) (PAM) composite hydrogels were prepared by a radical addition reaction in aqueous media formed by HEMA, acrylamide, and methacrylamide, with the presence of N,N'-methylenebisacrylamide. The characterization of the polymer was performed by FTIR analysis. Swelling and drug absorption properties of the polymers were analyzed in distilled water. Polymers' toxic effects were investigated by XTT assay. It has been observed that the entire drug, which was added to the solution with 1 g of polymer, was absorbed for 3.5 h. The absorption of these polymers was found quite high. PHAM polymer showed >800% swelling and PAM polymer showed >600% at 600 min. PHAM polymer was less toxic than PAM polymer. These results, this new polymer is very suitable for the release of Doxorubicin drug.

Key words: Doxorubicin, Poly(HEMA/acrylamide/methyl methacrylate), Poly(Acrylamide/methyl methacrylate), Drug release.

INTRODUCTION

Controlled release has been used in the medical field first in 1960. A drug system is called as "controlled release system" when polymers are used for controlling drug release. The synthesis of the polymer is an important part of the controlled release. Synthesized polymeric structure should transport the active ingredient to the target area at optimum conditions. This polymers should not interact with the active ingredient, should be biocompatible and biodegradable, should be produced easily and cost effectively. Controlled release systems are formed for providing these needs.[1-2]

Hydrogels are cross-linked network structures that are swellable in water. With this property, hydrogels are similar to normal tissues and they can be used in bio-medical applications. Hydrogels are quite useful on controlled drug release systems because they respond the changes on pH, temperature, ionic concentration. They have swelling capacity and control the diffusion of drug molecules. With the macro pores that they have, they show fast swelling kinetics. The capacity of absorbing the active pharmaceutical ingredient is the first step for using polymers as a drug carrier system.[3-4] Hydrogel polymers are quite good on absorbing drug molecules with the macro-porous structure. It is possible to get sufficient information about their absorption capacity by examining their swelling properties. Absorption capacity of a hydrogel polymer increases parallel to its swelling capability. The reason of preferring hydrogels in this study is their high absorption capacity.[5]

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Acrylamide is a chemical with H2C=CHCONH2 formula. It allows the occurrence of polymeric reactions easily through its C=C double bonds, which drives this chemical forward on the production of many polymers. Methacrylamide is prone to polymerization through C=C double bonds.[6] HEMA has H2C=CCH3COOCH2CH2OH formulas and it can join polymerization through double bonds. The polymers of these three chemicals exhibit very good swelling property. It is known that polymers with good swelling capability are widely used in both drug release and drug absorption.[7] In this study, new Poly (HEMA/ acrylamide/ methyl methacrylate) (PHAM) and Poly (Acrylamide/ methyl methacrylate) (PAM) polymers with doxorubicin's active ingredient were examined absorption capacity and cytotoxic effects of the polymers.

MATERIALS AND METHODS

Materials and Reagents

Acrylamide (AAm), N,N-methylene bisacrylamide (NNMBA), ammonium persulphate (APS) were obtained from Merck (Darmstadt, Germany). N,N,N',N'-Tetramethylenediamine (TEMED), 2-Hydroxyethyl methacrylate (HEMA), methyl methacrylate were obtained from Sigma (St. Louis MO, USA).

Dimethylsulfoxide (DMSO), Cell Proliferation Kit II (XTT), Dulbecco's Modified Eagle's Medium - high glucose from Sigma (St. Louis, MO, USA). Penicillin, streptomycin and trypsin from Gibco (Paisley, England), Eagle's Minimum, fetal bovine serum (FBS) from Biochrom (Berlin, Germany), phosphate buffer saline (PBS) tablet from Medicago (Uppsala, Sweden).

Synthesis of PHAM and PAM Polymers

Poly (HEMA/acrylamide/methyl methacrylate) (PHAM) and Poly (Acrylamide/methyl methacrylate) (PAM) polymers was prepared by a radical addition reaction. 5 mmol HEMA, 3 mmol acrylamide and 2 mmol methyl methacrylate were dissolved in water. 1,2 mmol N,N'- methylenebisacrylamide was added as cross binder, 0,02 mmol APS (ammoniumpersulphate) was added for starting polymerization and 0,1 mmol TEMED (N,N,N',N'-tetramethylenediamine) was added as accelerator; they were mixed for 1 min and filled into pipets; they were hold at 22°C for 24 h. Then polymers were removed from pipets, cut in pieces of 3-4 mm, washed with double distilled water and dried in the vacuum incubator.

Characterization of PHAM and PAM Polymers

Structural characterization of PHAM and PAM polymers was performed through swelling experiments and Fourier Transform Infrared (FTIR, PerkinElmer Spotlight 400).

a. Swelling

In order to determine swelling behaviors of the prepared hydrogels, each hydrogel polymer has been set to constant weigh. The swelling degree of the PAM and PHAM hydrogels were increased with time and reached a constant value. Swelling and macro porosity of the polymers were calculated using Equation 3 and Equation 4, respectively.

$$\text{Swelling S (\%)} = \left(\cos\frac{W_s - W_o}{W_o} \times 100\right) \quad (1)$$

$$\text{Macro porosity M (\%)} = \left(\cos\frac{W_{s-W\text{squeezed}}}{W\text{squeezed}}\right) \times 100 \quad (2)$$

Ws: Mass of the swelled polymer

W0: Mass of the dry polymer

Wsqueezed Mass of the polymer squeezed by applying pressure

When determining the amount of macro pores of the synthesized hydrogels, the hydrogel were added into 50 mL distilled water. Hydrogels were re-weighted after their equilibrium mass. Swelled hydrogels were put into a syringe; the water inside them was removed by applying pressure and re-weighted. The amount of macro pores were calculated using the formula below:

$$\text{The amount of macro pores (\%)} = +\left(\frac{W_d - W_{\text{squeezed}}}{W_d}\right) \times 100 \quad (5)$$

Wd: the mass of the hydrogel that reached its equilibrium mass (g).

Wsqueezed: the mass of the hydrogel that reached its equilibrium mass after squeezing (g).

a. FTIR

FTIR spectrum of PAM and PHAM composite hydrogels were recorded using KBr technique (PerkinElmer Spotlight 400). The resolution of FTIR spectrum was 4 cm-1 and the scanning wavelength was 400-4000 cm-1.

Polymer (PHAM and PAM) Doxorubicin Couples

PHAM and PAM polymers were hold with the active ingredient of doxorubicin drug in phosphate buffer (PBS) for 5 days, in an incubator at 37 °C. Absorption

amounts were calculated through the samples taken from the solution every day. The samples were measured in UV-vis at 485 nm and it was found that 95% was absorbed at the end of 5th day. Dried polymers were placed into PBS solution for release, in an incubator at 37°C. The release of samples were determined for 20

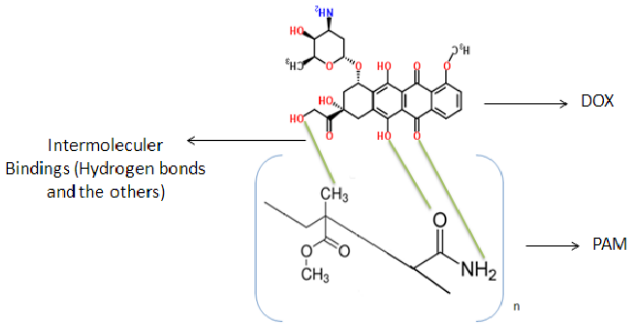

Figure 1: DOX loaded PAM

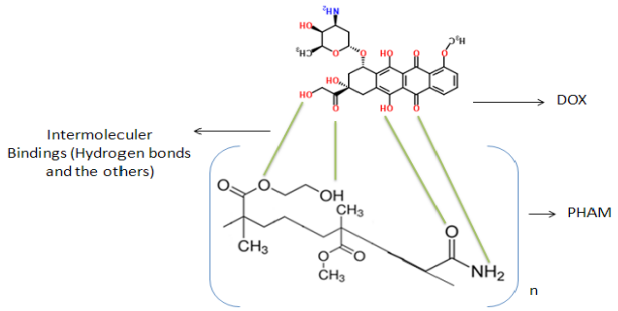

Figure 2: DOX loaded PHAM

days. The drug absorption was provided at physiological pH and body temperature 37° C (Figure 1 and Figure 2).

Adsorption and Release

Polymers were incubated with doxorubicin in phosphate buffer solution in incubator, for 5 days, at 37o C. The samples were kept at -20°C for two days. They were purified by washing with 98% ethyl alcohol and dried inside the incubator for 24 h. Dried polymers were placed into PBS for releasing. Drug release was investigated using a UV-Vis Spectrometer (PG Instruments) and the characteristic release of Doxorubicin at 485 nm was measured for 24 h. The adsorbed doxorubicin polymer was incubated in PBS solution with pH 7.4, at 37°C. The amount of released doxorubicin was measured after 24 h.

Cell culture

Medium environment that we have used in our study was DMEM medium consisting of 10% fetal bovine serum (FBS), 1% L-glutamine, 100 IU/mL penicillin and 10 mg/mL streptomycin. Cancer cell lines that were produced in DMEM medium were multiplied in the incubator at 37°C, with 95% humidity and 5% CO_2. Cytotoxicity tests were performed on Mouse Fibroblast cell line (L929) through XTT Test, where L929 have been procured from ATTC.

Cytotoxic Activity of Hydrogels

10x104 cells were plated in each well of 96 wells cell culture plate in 200. Meanwhile, for sterilization purposes polymers were hold under UV light for 4 h, in 70% alcohol for 1 h. The polymers were washed with PBS 3 times. After 24 h of incubation, polymers were removed from wells and XTT was applied. % viability was calculated.

RESULTS AND DISCUSSION

Swelling Experiments of Hydrogels

Dried PAM and PHAM hydrogels are glassy and strict, but swollen gels are soft. When swelling the hydrogels were strong and elastic enough to retain their shape. The swelling degree of the PAM and PHAM increased with time and reached a constant value after a certain point. This value may be named as an equilibrium or a maximum swelling (Seq %). The Seq % values were shown in Figure 3.

PHAM polymer showed >800% swelling and PAM polymer showed >600% at 600 min. The swelling (%) of PHAM polymer was bigger than PAM polymer. These results showed that PAM and PHAM polymers were very useful for drug release. According to literature, if a polymer show good swelling properties, it can be useful for drug release.[8-9] The maximum swelling properties of the polymers were about %800-900 (PHAM) and %600-700 (PAM) at 1600 min. Any decomposed or degradation mechanism were not observed for polymers (no discoloration, no shape loss, no reaction) and the polymers were stabilized in these experiments. Also the stability of the polymers had been continued after months.

FTIR

IR spectrum of PHAM and PAM polymers were shown in Figure 4(a)-(b) and Figure 5(a)-(b).

In Figure 4(a) the peaks belonging to hydroxyl were seen at 3027 cm-1, whereas in Figure 4(b) they were split into two at 3047 and 3010 cm-1. On the other hand, strong

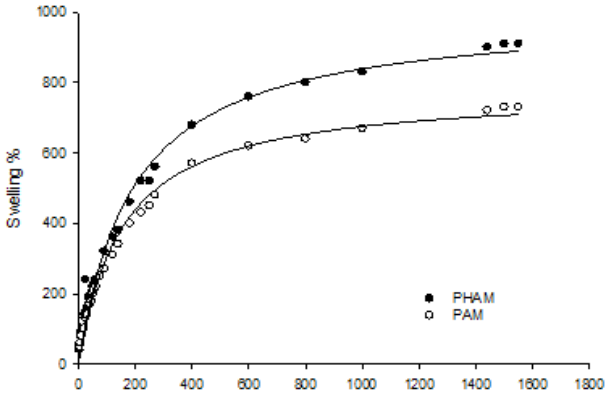

Figure 3: Swelling graph of PHAM and PAM polymers

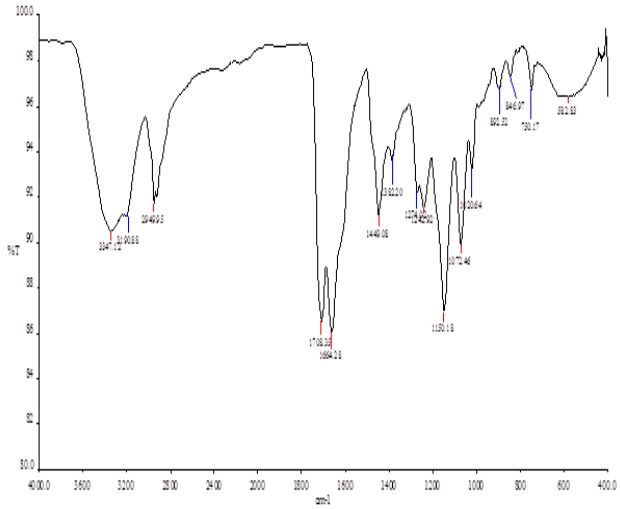

Figure 4 (a): FTIR analysis of Doxorubicin adsorbed PHAM.

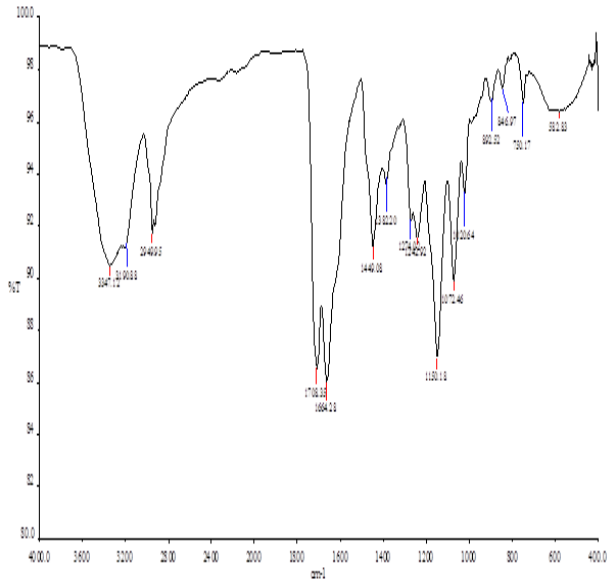

Figure 4 (b): FTIR analysis of PHAM polymer.

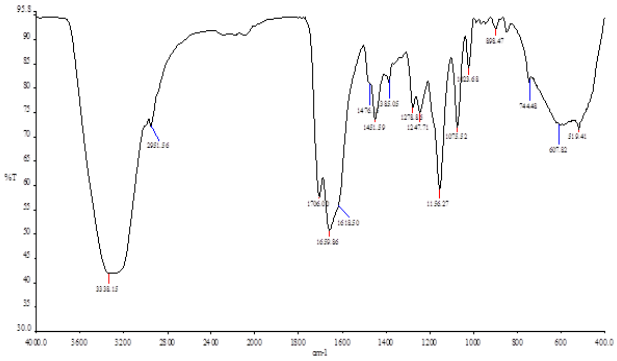

Figure 5: FTIR analysis of Doxorubicin adsorbed PAM

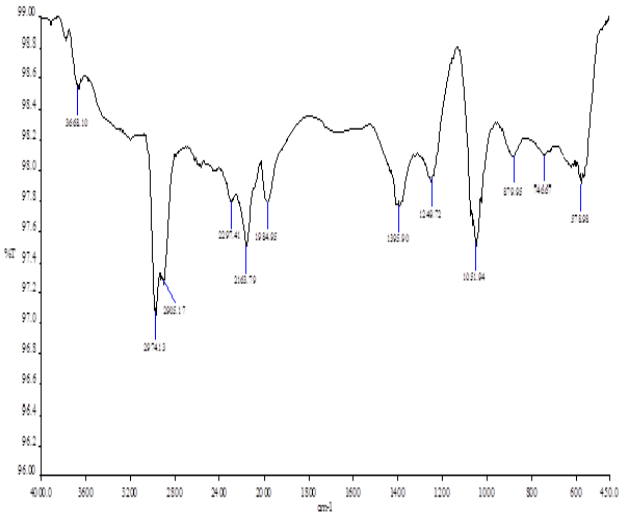

Figure 5 (b): FTIR analysis of PAM polymer.

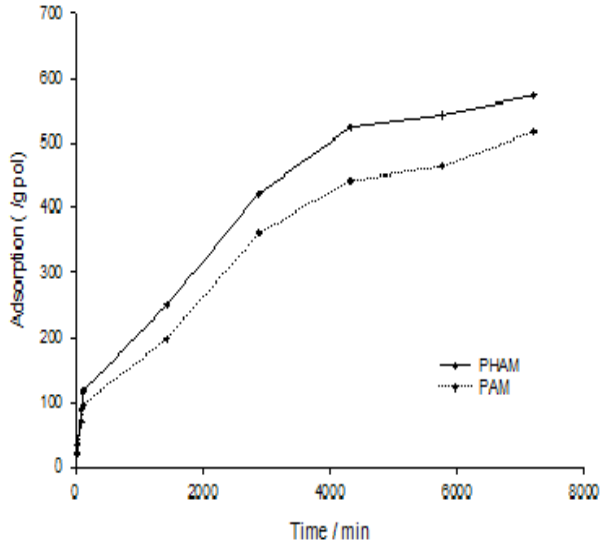

Figure 6: Adsorption of drug from PAM and PHAM

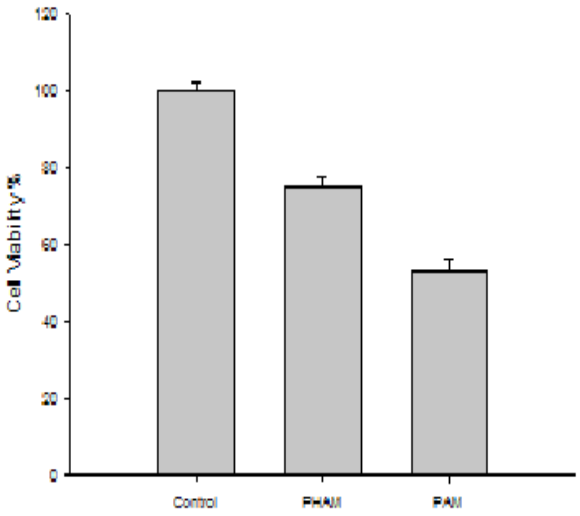

Figure 7: % Viability of the Polymer in L929 cell line. (Control (without polymer), PHAM, PAM)

carbonyl peaks were observed at 1715-1709 cm-1 in Figure 4(a) were shifted to de 1660 cm-1 in Figure 4(b). The shallow wide peak seen at 615 cm-1 in Figure 4(a) was split into two in Figure 4(b), at 742 and 582 cm^{-1}. According to FTIR analysis, it can be concluded that due to steric barrier, Doxorubicin's active ingredient was bond to the carboxyl group, which was located at the end of the polymer. The change in the out of plane C-H bending at 615 cm^{-1} support this conclusion.[10-12]

In Figure 5(a) the peaks belonging to hydroxyl were seen at 3338 cm^{-1} and the peaks belonging to alkyl group were seen net to them, at 2951 cm-1; whereas in Figure 5(b) alkyl peaks were observed at 2974 and 2906 cm-1, instead of hydroxyl peaks. The strong carbonyl and C=C peaks seen at 1706-1618 cm-1 in Figure 5(a) were not identified in Figure 5(b). The peaks of triple carbon-carbon bonds, which were observed at 2200-1900 cm-1 in Figure 5(a), were not observed in Figure 5(b). The peek belonging to C-C vibrations, which was identified at 1476-1385 cm^{-1} in Figure 5(b), were not seen in Figure 5(a). According to FTIR analysis, it can be concluded that due to steric barrier, Doxorubicin's active ingredient was bond to the carboxyl and C=C groups of the polymer, which were located at the end.[10-12]

$$\text{Adsorption} = \frac{\text{first adsorption} - \text{equilibrium adsorption}}{\text{weight of polymer (g)}} \text{X100} \qquad (6)$$

After 24 h, PHAM polymer released % 17.14 of the drug, whereas PAM polymer released % 13.12 in Figure 6. At the end of the 5th days, PHAM adsorbed the drug by %570 , whereas PAM polymer adsorbed it by % 510, Wu and Ofner have examined the adsorption of the doxorubicin on polypropylene polymer and found that the polymer adsorbed only %45 of the drug.[13] Linhardt et al. have tested the same drug, under approximately the same conditions with poly (methyl methacrylate)-chitosan- heparin covered active carbon. This polymer only adsorbed the drug by 20 mg/g.[14] On the other hand, there are other polymers that adsorb doxorubicin more than our polymer. The best example is graphene oxide that Wang et al. have used (1200-1300 mg/g).[15] But graphene oxide is not advantageous because it is quite costly.

Cytotoxic Effects

XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) is a tetrazolium salt, it is decomposed by the dehydrogenase enzyme, which is present in the mitochondria of the metabolically active cells and it is transformed to formazan, which is soluble in water. The density of the orange color arising from formazan is proportional to the number of living cells. Cell viability is determined by reading the density of the orange color, which has been formed at the end of the incubation period, in a micro plate reader, at 450-475 nm.

Many elements that are released from acrylic resin may cause cytotoxicity. This was because of the excess monomer inside the polymer, which was not polymerized and removed through purification.[16-17] The viability ratio of the PAM polymer, which has been obtained without using HEMA monomer, was lower than both control group and PHAM polymer in Figure 7. The high toxicity resulted from higher acrylic monomer content. These kinds of polymers are usually inert ones; however the human body can be seen them as foreign objects and try to protect the organisms against them. Thus, the level of cytotoxicity is desired to be as low as possible.[18] As a result, PHAM polymer was less toxic than PAM polymer.

Figure 7. % Viability of the Polymer in L929 cells. (Control (without polymer), PHAM, PAM)

CONCLUSION

The results of FTIR analysis revealed that the active pharmaceutical ingredient was bond to the C=C double bonds of the polymer. Swelling tests showed that swelling percentage of PHAM polymer was bigger than PAM polymer. Cytotoxicity test disclosed that PHAM polymer was less toxic than PAM polymer. Consequently,

these synthesized polymers have been suitable for this active pharmaceutical ingredient.

ACKNOWLEDGEMENT

None

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ABBREVIATIONS USED

XTT: 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide; **TEMED:** N,N,N',N'-Tetramethylenediamine; **FTIR:** Far infrared spectroscopy; **PHAM:** Poly (HEMA/acrylamide/methyl methacrylate); **PAM:** Poly (Acrylamide/ methyl methacrylate)

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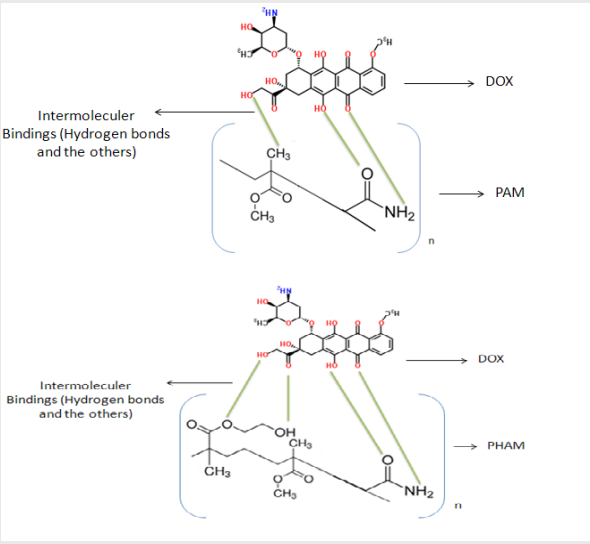
PICTORIAL ABSTRACT



SUMMARY

- Poly (HEMA/acrylamide/ methyl methacrylate) (PHAM) and Poly (Acrylamide/ methyl methacrylate)(PAM) polymers are suitable for this active pharmaceutical ingredient.

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Afsin Kariper: He is researcher at Erciyes University. He study on physical chemistry in PhD. He published thin films, material science, drugs, science education articles. He is focused on physical or physical chemistry properties of thin films, material science, drugs and influential factors of their properties. He has been study in Erciyes University.

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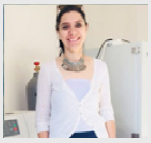

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