STIMULI-RESPONSIVE RELEASE OF DOXORUBICIN FROM LAYER-BY-LAYER FILMS OF POLY(2-ISOPROPYL-2-OXAZOLINE) AND TANNIC ACID

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ABSTRACT

STIMULI-RESPONSIVE RELEASE OF DOXORUBICIN FROM LAYER-BY-LAYER FILMS OF POLY(2-ISOPROPYL-2-OXAZOLINE) AND TANNIC ACID

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Stimuli responsive polymers are promising materials for biomedical applications due to change in their properties in response to changes in environmental conditions. Among all the stimuli, pH and temperature are the most extensively studied ones in biomedical applications. pH is an internal trigger. pH changes at different regions of the body. Besides, pH is more acidic than the pH at tumor tissues or at an infected site in the body. Temperature can behave as both an internal and an external trigger. Increase in temperature during a disease state is an example of an internal trigger. Applying heat externally to increase the temperature of a specific part in the body during hyperthermia treatment is an example of an external trigger.

Temperature responsive polymers change their solubility with changing temperature. Thermoresponsive polymers are classified into two: i) polymers exhibiting lower critical solution temperature (LCST) in aqueous solution and ii) polymers exhibiting upper critical solution temperature (UCST) in aqueous solution. If the polymer solution shows phase separation with increasing temperature, this polymer solution has lower critical solution temperature (LCST). If the polymer solution shows phase separation upon cooling, the polymer solution has an upper critical solution temperature (UCST). Recently, poly(2-alkly-2-oxazoline)s which show LCST-type

behavior in aqueous solution have been of interest as an alternative to poly(N-isopropylacrylamide) (PNIPAM), a commonly used polymer exhibiting LCST-type behavior in aqueous solution in biomedical applications.

Layer-by-layer (LbL) self-assembly technique is an efficient and a practical method for preparation of ultra-thin multilayer films. By using stimuli responsive polymers as building blocks during multilayer assembly, the resulting LbL films can be made responsive to changes in environmental conditions. This feature specifically makes LbL films promising polymer platforms for controlled release applications from surfaces.

The study presented in this thesis reports on the fabrication of anti-cancer drug, Doxorubicin (DOX) containing multilayers of poly(2-isopropyl-2-oxazoline) (PIPOX) and Tannic acid (TA) and release of DOX from the multilayers at moderately acidic conditions. Moreover, the effect of temperature on the pH-induced release of DOX from the surface and correlated the results with the LCST behavior of PIPOX were investigated. First, PIPOX was synthesized via cationic ring opening polymerization. Prior to film construction, water soluble complexes of TA and DOX (TA-DOX) were prepared. PIPOX and TA-DOX were deposited on the surface using LbL technique through hydrogen bonding interactions at pH 6.5. Minimal amount of DOX was released at physiological pH. In contrast, pH-induced release of DOX was observed at moderately acidic conditions due to protonation of TA as the acidity increased and loss of electrostatic interactions among TA and DOX. Moreover, it is observed that raising the temperature from 25 °C to 37.5 °C increased the amount of DOX released from the surface due to conformational changes within the multilayers correlated with the lower critical solution temperature (LCST) behavior of PIPOX.

This study is the first one reporting the pH- and/or temperature-induced release of DOX from poly(2-alkyl-2-oxazoline) based hydrogen-bonded multilayers. Considering the temperature-responsive behavior of PIPOX and important biological properties of PIPOX and TA, combined with the acidic nature of tumor tissues, these multilayers which release DOX at moderately acidic conditions, can be promising drug carriers for controlled release of DOX from surfaces.

Keywords: Temperature responsive polymers, Poly(2-alkyl-2-oxazoline), Layer-by-Layer technique, Hydrogen-bonded multilayer films, Doxorubicin

ÖZ

POLİ(2-İSOPROPİL-2-OKSAZOLİN) VE TANİK ASİT KATMAN KATMAN FİLMLERİNDEN DOKSORUBİSİN'İN ÇEVRE KOŞULLARINA DUYARLI SALINIMI

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Çevreye duyarlı polimerler değişen çevre koşullarına karşı özelliklerini değiştirebilmesinden dolayı biyomedikal uygulamalar için umut vadeden malzemelerdir. pH ve sıcaklık biyomedikal uygulamalarda en fazla çalışılan iki uyarıcıdır. pH bir iç uyarıcıdır. Vücudun farklı bölgelerinde değişkenlik gösterir. Bunun yanısıra, kanserli dokularda ve vücudun enfekte olan bölgelerinde de pH normal hücrelere göre daha asidiktir. Sıcaklık hem iç hem dış uyarıcı olarak davranabilir. Vücut sıcaklığının hastalık esnasında yükselmesi sıcaklığın iç uyarıcı olarak davranmasına örnektir. Hipertermi tedavisinde vücudun belli bölgelerinde sıcaklık artışının sağlanması için harici olarak ısı uygulanması ise sıcaklığın dış uyarıcı olarak uygulamasına örnektir.

Sıcaklığa duyarlı polimerler sıcaklık değişimi ile çözünme özelliklerini değiştirirler. Sıcaklığa duyarlı polimerler iki kategoriye ayrılır : i) sulu çözeltisi alt kritik çözünme sıcaklığı (LCST) davranışı gösteren polimerler; ii) sulu çözeltisi üst kritik çözünme sıcaklığı (UCST) davranışı gösteren polimeler. Eğer polimer çözeltisi sıcaklık artışı ile faz ayrımı gösteriyor ise, bu polimer LCST davranışı göstermektedir. Eğer polimer çözeltisi sıcaklık düşmesi birlikte faz ayrımı gösteriyor ise, bu polimer

UCST davranışı göstermektedir. Son yıllarda, biyomedikal uygulamalarda çok fazla tercih edilen sulu çözelti içerisinde LCST davranışına sahip poli(N-isopropilakrilamid)'e alternatif olarak yine sulu çözelti içerisinde LCST davranışı gösteren poli(2-alkil-2-oksazolin)'ler önemli biyolojik özelliklerinden ötürü araştırmacıların ilgisini çekmektedir.

Katman-katman (LbL) kendiliğinden yapılanma tekniği, ultra ince filmler hazırlamak için etkili ve pratik bir yöntemdir. Çevreye duyarlı polimerler kullanarak hazırlanan LbL filmler çevresel değişimlere karşı duyarlı özellik gösterirler. Bu özellik, LbL filmleri yüzeyden kontrollü ilaç salımı uygulamaları için umut vadedici malzemeler haline getirmektedir.

Bu tez çalışması, hidrojen bağlı poli(2-isopropil-2-oksazolin) (PIPOX) ve Tanik asit (TA) içeren LbL filmlerden kanser tedavisinde kullanılan bir ilaç olan Doksorubisin'in (DOX) pH tetiklemesiyle salımını içermektedir. Ayrıca PIPOX'un LCST tipi davranış özelliğinin yüzeyden pH tetiklemesi ile DOX salımına etkisi irdelenmiştir.

İlk olarak, PIPOX katyonik halka açılma polimerizasyonu yöntemiyle sentezlenmiştir. LbL filmlerin üretimi öncesinde, TA ve DOX'un suda çözünür kompleksleri (TA-DOX) hazırlanmıştır. PIPOX ve TA-DOX katmanlar arası oluşturulan hidrojen bağları sayesinde LbL yöntemiyle pH 6.5'de yüzeyde biriktirilmiştir. Fizyolojik koşullarda en düşük miktarda DOX salımı gerçekleştiren PIPOX ve TA-DOX'dan oluşmuş LbL filmler, orta-asidik pH değerlerinde en yüksek miktarda DOX salımını gerçekleştirmiştir. Bunun nedeni, asitliğin artması ile TA'nın protonlanan hidroksil grupları ile pozitif yüklü DOX arasındaki etkileşimin bozulmasıdır. Sıcaklığın arttırılması ile birlikte, yüzeyden salınan DOX miktarı karşılaştırıldığında, 37.5 °C'de daha yüksek miktarda DOX salındığı gözlemlendi. Bu sonuç, 37.5 °C'de PIPOX'un LCST değerinin (36 °C) üzerinde film yapısında oluşturduğu konformasyonal değişimler ile ilişkilendirildi.

Bu çalışma poli(2-alkil-2-oksazolin) bazlı hidrojen bağlı filmlerden hem pH hem de sıcaklık tetiklemeleri ile DOX salımını gösteren ilk çalışmadır. PIPOX'un sıcaklığa duyarlı özelliği, PIPOX ve TA'nın önemli biyolojik özellikleri ve tümör dokularının

asidik özellikleri düşünüldüğünde, orta-asidik koşullarda DOX salımı gerçekleştirebilen bu filmler umut vaad eden ilaç taşıyıcılardır.

Anahtar kelimeler: Sıcaklığa duyarlı polimerler, Poli(2-alkil-2-oksazolin), Katman-katman kendiliğinden yapılanma tekniği, Hidrojen bağlı çok katmalı filmler, Doksorubisin

To My Precious Family…

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LCST	Lower critical solution temperature
UCST	Upper critical solution temperature
PNIPAM	Poly(N-isopropylacrylamide)
POX	Poly(2-alkyl-2-oxazoline)
PAA	Poly(acrylic aicd)
PMAA	Poly(methacarylic acid)
PDMAEMA	Poly(*N*,*N*-dimethylaminoethly-methacrylate)
PDMAEMA	Poly(*N*,*N*-diethyl aminoethly-methacrylate)
CROP	Cationic ring opening polymerization
PETOX	Poly(ethyl-2-oxazoline)
PIPOX	Poly(2-isopropyl-2-oxazoline)
PcPrOX	Poly(c-propyl-2-oxazoline)
PnPOX	Poly(n-propyl-2-oxazoline)
PMEOX	Poly(2-methy-2-oxazoline)
PEG	Poly(ethylene glycol)
LbL	Layer-by-layer
PVP	Poly(vinyl pyrrolidone)
PVA	Poly(vinyl alcohol)

PAAm	Poly(acrylamide)
PEO	Poly(ethylene oxide)
DOX	Doxorubicin
PVPON-*b*-PNIPAM	Poly(N-vinyl pyrrolidone-*b*-poly(N-isopropyl acryl amide)
MB	Methylene Blue
PAH	Poly(allyamine hydrochloride)
PVS	Poly(vinyl sulfate)
DS	Dextran sulfate
PEO-*b*-PHMEA	Poly(ethylene oxide)-*block*-poly(2-hydroxylethyl methacrylate)
HA	Hyloplasm Acid
R6G	Rhodamine 6G
PMEMA-*b*-PDPA	Poly[2-(N-morpholino)ethyl methacrylate-block-2 (diisopropylamino)ethyl methacrylate]
PSS	Poly(styrene sulfonate)
α-CD	Cyclodextrin
PB	Prussian Blue
PDAC	Poly(diallyldimethyl ammonium chloride)
BPEI	Branched poly (ethylene imine)
PBS	Phosphate Buffer Saline

TA	Tannic Acid
NMR	Nuclear Magnetic Resonance
GPC	Gel Permeation Chromatography
AFM	Atomic Force Microscopy

CHAPTER 1

INTRODUCTION

1.1. Stimuli Responsive Polymers

Stimuli responsive polymers change their properties in response to changes in environmental conditions. They have been widely investigated for several different applications, e.g. controlled drug release systems, tissue engineering applications, gene delivery systems [1–4]. These polymers are also named as ‘smart’ [5], ‘environmental sensitive’ [6] or ‘intelligent’ [7] polymers because of their fascinating properties, i.e. ability of rapid change in their microstructural forms with a slide change in environmental conditions [5]. This change can be triggered by either externally or internally. pH and temperature are internal triggers. The change in pH in the body can occur at certain organs or disease states. The increase in temperature in the body may occur in the presence of pyrogens, specific enzymes or antigens [8]. External triggers include light, magnetic field, electric field, and ultrasound [8]. Of note, temperature increase can be modulated externally in biomedical applications, e.g. heat-triggered subdermal implants [8]. The response of these smart polymers are mostly reversible and can be observed in the following ways: - alteration in shape, physical state, change in optical, electrical or mechanical features, surface characters, hydrophilic/hydrophobic balance as well as solubility behaviors [3, 9]. The examples of several different stimuli and the corresponding responses are given in Figure 1 [10]. The most frequently applied stimuli in biomedical applications are pH, temperature, light, magnetic field, and electric field [10]. Among all, pH and temperature are the most extensively studied stimuli in biomedical applications.

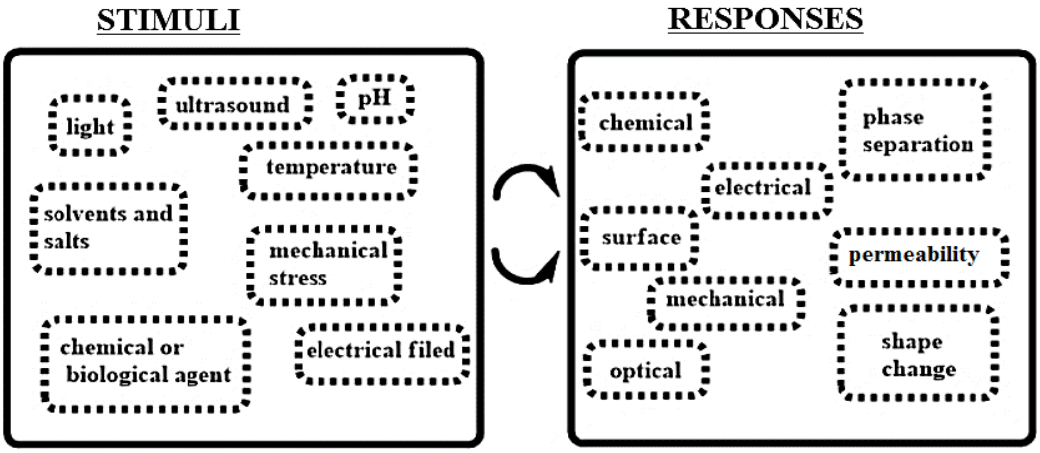


Figure 1. Examples of stimuli and responses [10]. (Modified from Schmaljohann *Adv. Drug Deliv. Rev*., 2006)

1.1.1. Temperature Responsive Polymers

Temperature is one of the most explored stimulus for biomedical applications because small changes in temperature can be easily and safely applied [8,11-12]. Temperature change in the body can occur both internally and externally. Heat can be applied to the body externally or the body temperature may arise in a disease state [8]. Temperature responsive polymers, also named as thermoresponsive polymers, go into a phase transition and a significant change in their solubility is observed at a critical temperature [8,10,12]. The phase transition in temperature responsive polymers is represented by phase diagrams which provide information about the state of the polymer solutions at a specific temperature and concentration [4,11]. A typical phase diagram is represented in Fig. 2. The lowest and the highest point of the binodal are named as lower critical solution temperature (LCST) and upper critical solution temperature (UCST), respectively [4,13]. If the polymer solution shows phase separation with increasing temperature, this polymer solution has lower critical solution temperature (LCST). If the polymer solution shows phase separation upon cooling, the polymer solution has an upper critical solution temperature (UCST) [14]. Among these two different types of thermoresponsive polymers, the polymers with LCST attract more attention than the polymers exhibiting UCST for biomedical applications. This is because most of the polymers with UCST are soluble in organic

solvents and requires high temperatures for dissolution [4]. For this reason, the main focus of interest for biomedical applications is directed to LCST type polymers [15–18].

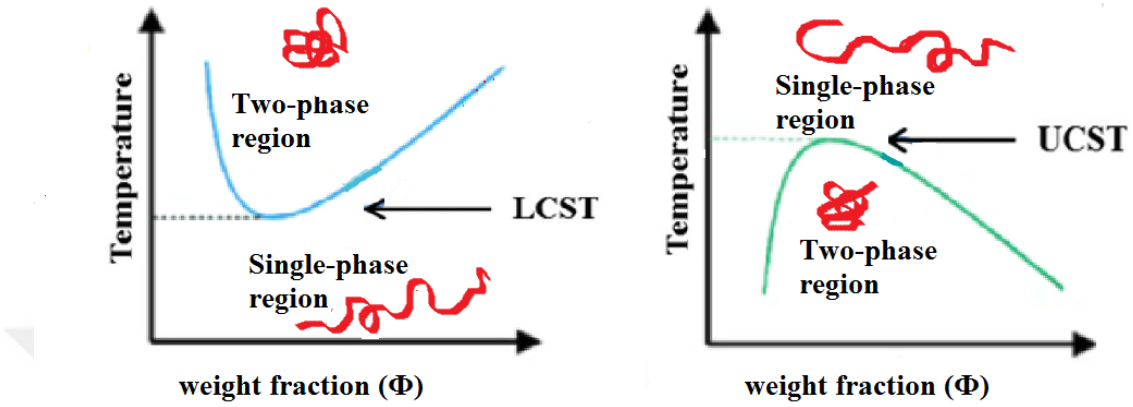


Figure 2. Typical phase diagrams (temperature vs. composition) of the polymer solutions with LCST or UCST type phase behaviours. Colored lines represent the phase separation boundaries [4]. (Modified from Hruby´ et al. *Eur. Polym. J.*, 2015)

1.1.1.1. Nature and Mechanism of LCST Behavior

The reversible phase transition of thermoresponsive polymers makes them attractive candidates for biomedical applications, specifically for controlled delivery applications [14, 19]. As discussed in the previous section, in contrast to polymers with UCST which mostly dissolve in organic solvents, polymers with LCST type behavior are preferred in biomedical applications due to their solubility in aqueous environment at low temperatures [4]. The phase separation in polymer solutions with LCST type behavior is induced by disruption of the hydrogen bonds between the polymer chains and water molecules as the temperature rises. This results in contraction of the polymer chains at the critical temperature due to enhanced intra- and inter-molecular forces contained within the polymers, resulting in a transformation of the polymer chains from the extended coil to globular state (coil-to-globule transition) (Fig. 3.) [4, 10].

Cloud point detection method is used to determine the phase boundaries in polymer solutions. When the phase separation occurs, the solution becomes cloudy. This method is qualitative since the detection is made visually. Besides, the data obtained depends on the heating rate. A more precise way to determine the cloud point is to measure the light transmission using turbidimetry. The cloud point is determined by the sharp decrease in the intensity of transmitted light [14].

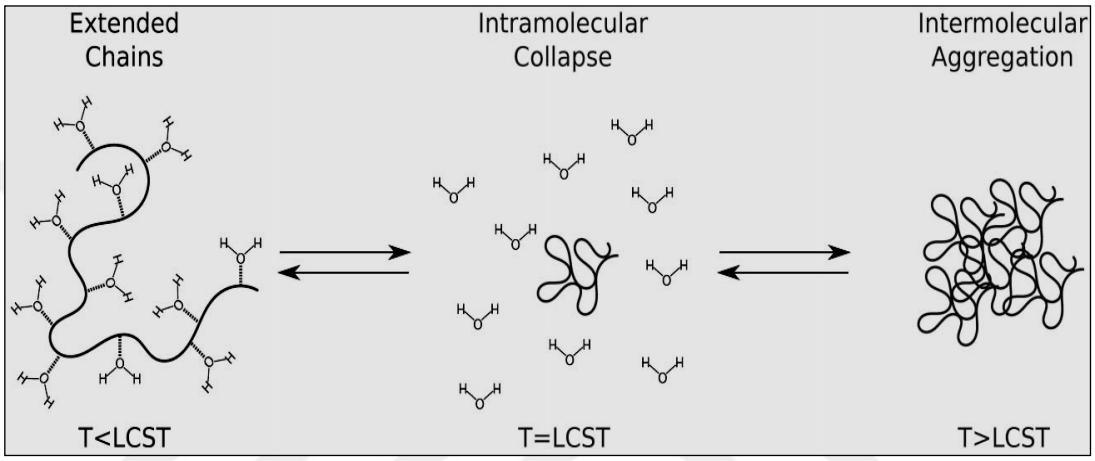


Figure 3. Schematic representation of the mechanism of phase separation in polymer solutions exhibiting LCST behavior [12].

The thermodynamic aspects of LCST type behavior can be explained by the Gibbs Equation, $\Delta G = \Delta H - T\Delta S$. Phase separation in polymer solutions is an entropy-driven process [13]. At low temperatures, the high number of hydrogen bonds among the polymer chains and water molecules assure the dissolution of polymer in aqueous environment. The enthalpy of dissolution is favorable (ΔH), whereas the high number of hydrogen bonds among the polymer chains and water molecules lead to more ordered structure and result in loss of entropy (ΔS) [12, 20]. The sign of free energy changes from negative to positive as the temperature increases [20]. The hydrogen bonds between the water molecules and polymer chains disrupt with increasing temperature and this results in a decrease in enthalpic contribution. $T\Delta S$

part of the equation dominates and ΔG becomes positive, resulting in phase separation [8, 12, 20].

There are many examples of LCST type polymers such as polyamides, polyvinylethers, poly[oligo (ethylene oxide) methacrylates] and poly(2-alkyl-2-oxazoline)s [19]. Specifically, poly (N-isopropylacrylamide) (PNIPAM) has been of interest due to its LCST of 32 °C [21] which is close to body temperature, making this polymer attractive for biomedical applications. As an alternative to PNIPAM, poly(2-alkyl-2-oxazoline)s with short alkyl chain on the pendant groups have drawn considerable attention for biomedical applications due to their solubility in aqueous solutions and temperature responsive behaviors [22, 23].

1.1.2. pH Responsive Polymers

pH is one of the most important and also well-studied stimulus among the other responsive systems due to the local pH changes in human body (e.g. stomach (pH 1.0-3.0), lysosomes (pH 4.5-5.0), colon (pH 7.0-7.5), blood (pH 7.35-7.45), and tumor cells (pH 6.5-7.2) [8, 10]. The tumor tissues are more acidic than the normal body cells (pH 7.4) due to high hydrostatic pressure inside the tumor cells leading to low levels of oxygen and high levels of lactic acid in the tumor cells [4, 25]. Therefore, pH responsive systems are promising site-specific delivery systems.

pH-responsive polymers are weak polyelectrolytes bearing pendant acid or base groups and are capable of either accepting or donating protons with a change in pH of the surrounding environment [1, 3, 6]. In this context, polyelectrolytes can be classified into two groups, i.e. anionic polyelectrolytes (polyanions) and cationic polyelectrolytes (polycations). Anionic pH responsive polymers are weak polyacids bearing carboxylic acid or sulphonic acid pendant groups which release protons and become ionized at pH above their pK_a values [11]. Among the all polyacids, poly(acrylic acid) (PAA) and its derivative poly(methacrylic acid) (PMAA) are most widely used in research studies [3, 11]. Cationic pH responsive polymers are named as polybases and contain amino or amine groups which accept protons and become ionized below the pK_a of the polycations [11]. Poly(ethylene imine)(PEI), poly(*N*,*N*-

dimethylaminoethly-methacrylate) (PDMAEMA) and poly(*N*,*N*-diethyl aminoethly-methacrylate) (PDEAEMA) are commonly used polybases in biomedical applications [10, 11].

Hydrodynamic volume, configuration, conformation and solubility of polyelectrolytes can be altered by changing pH [25]. For example, weak polyelectrolytes transform from extended to contracted conformation when the net charge on the polyelectrolyte chain is decreased by changing pH and the electrostatic repulsion among the repeating units is decreased [3].

1.1.3. Magnetic Field Responsive Polymers

The use of magnetic field in biomedical applications is a developing approach to design novel drug carrier systems. Polymers and magnetic nanoparticles are combined in the same polymer matrix to prepare magnetic field responsive polymers. Ferrite, cobalt and carbonyl iron based magnetic nanoparticles are mostly preferred due to their biocompatibility, non-toxicity and non-immunogenicity. In addition to control the movement and release of drugs, these magnetic nanoparticles can also serve as visualization agents or [2] can be used to generate heat for hyperthermia or tissue ablation treatments [26–28]. Polymeric gels doped with magnetic nanoparticles [29] or encapsulation of magnetic nanoparticles by polymers [30] or liposomes [31] have been exploited for visualization and controlled drug delivery applications. Although a lot of research has been performed on magnetic field responsive polymer systems, magnetic guiding to the target site in the body is the major drawbacks of magnetic field responsive polymer carriers in practical use [8,32].

1.1.4. Electric Field Responsive Polymers

Electric field responsive polymers change their properties in response to changes in electric current. Electric field, which is an external stimulus provides accurate control over the system by controlling the magnitude of current, duration of electric pulses and the internal between the pulses by the help of an equipment [33]. Electric field responsive polymers are polyelectrolytes with ionisable groups on the backbone. It has been shown that ionization of these groups could be controlled by changing the electric current [33]. This feature has been used to tune the swelling, shrinking or bending of hydrogels, paving the way for using these hydrogels in drug release, artificial muscle or biomimetic actuators [8]. The major concern in using electric field responsive polymers in drug delivery applications is the difficulty in adjusting the dose of electric current in order not to harm the nerve endings in the surrounding tissues [3, 34].

1.1.5. Light Responsive Polymers

A light responsive polymer goes a rapid change in structure when exposed to light. Light is a cheap, easily controlled external stimulus which does not require any physical contact or a mechanical apparatus [3]. Light responsive polymers are mostly modified by the photo-sensitizers such as azobenzene, spiropyran and triphenylmethane [35]. These photosensitive molecules provide conformational changes in the polymer chains upon exposure to light, inducing either a change in the geometry (cis-trans isomerism) or polarity [36]. Light responsive polymers are grouped into two: UV-sensitive and visible-sensitive polymers. The former ones are safe, cheap and easily applicable, therefore they are widely preferred in biomedical applications [3]. The slow rate of the conformational change is the important limitation of using light responsive polymers in biomedical applications [3].

1.2. Poly(2-Oxazoline)s

1.2.1. Polymerization of 2-substitued-2-oxazolines

The mechanism of the polymerization of 2-substituted-2-oxazolines via cationic ring opening polymerization has been described since its discovery over the last 50 years [37–40]. The living nature of cationic ring opening polymerization (CROP) allows control over the molecular weight and provides polymers with narrow molecular weight distribution (PDI=Mw/Mn~ 1.01-1.3) [41]. By using multi-functional initiators [42] or controlling end-group functionality via initiation and termination steps [43], it is possible to synthesize polymers with a wide variety of architectures [44]. In addition, the living nature of CROP suppresses the side reactions, e.g. chain transfer or chain termination reactions [45].

CROP starts with the addition of electrophilic initiators such as alkyl triflates, tosylates, alkylhalides or nonylates to a 2-oxazoline monomer [42]. After addition of the electrophile to a 2-oxazoline monomer, cationic oxozolinium propagating specie is formed. The C-O bond of cationic oxozolinium propagating specie undergoes a nucleophilic attack by the incoming 2-oxazoline monomer inducing the ring opening [43]. Importantly, 2-oxazoline monomer can only be added to the active specie from the nitrogen atom due to regioselectivity of the monomer [46]. Polymerization is terminated by the addition of a nucleophile, e.g. OH^-, NH^-, COO^- or S^- to the chain end [47]. For example, methanolic sodium hydroxide or sodium carbonate solution can be used to form hydroxyl end group, while primary (e.g. aniline, ethylenediamine), secondary (e.g. piperidine, piperazine) or tertiary amine derivatives (e.g. pyrrole, pyridine) can be used to functionalize the polymer with an amine end-group. Sodium thiolates produce sulfur end groups whereas carboxylic acid derivatives such as acrylic acid, maleic acid or cinnamic acid can be used as the terminating agents to obtain ester groups [48].

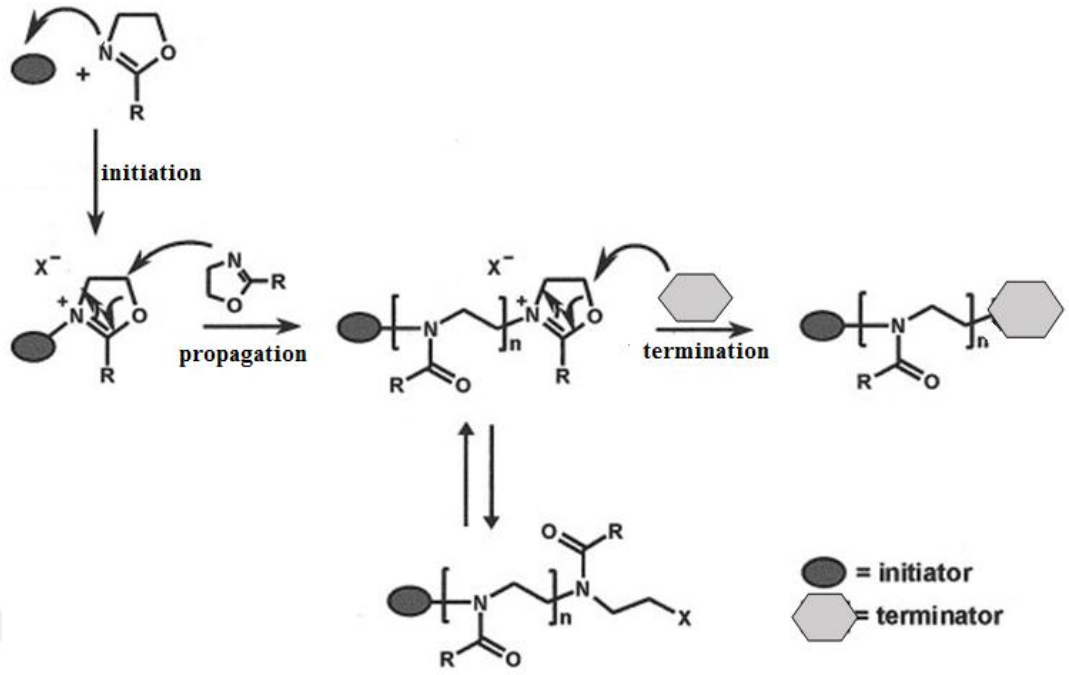


Figure 4. Schematic representation of the mechanism of living cationic ring opening polymerization of a 2-substituted-2-oxazoline [49]. Modified from Hoogenboom et al. *J. Polym. Sci., Part A: Polym. Chem.*, 2004)

1.2.2. Properties of Poly(2-alkyl-2-oxazoline)s

Poly(2-alkyl-2-oxazoline)s (POX)s with short alkyl chain on the pendant groups have been of interest for biomedical applications [22, 23] due to their solubility in aqueous environment and temperature responsive behavior [42]. LCST of POXs decrease as the pendant alkyl groups get more hydrophobic [42].

As seen in the Figure 5, poly(ethyl-2-oxazoline) (PETOX), poly(2-isopropyl-2-oxazoline) (PIPOX), poly(c-propyl-2-oxazoline) (PcPrOX), poly(n-prpyl-2-oxazoline) (PnPrOX) have LCST values around 60 °C, 36 °C [50], 30 °C and 25 °C [51]. Polymers with either short or long alkyl chain pendant groups [42] e.g. poly(2-methyl-2-oxazoline) (PMEOX) or poly(butyl-2-oxazoline) do not show LCST type behavior .

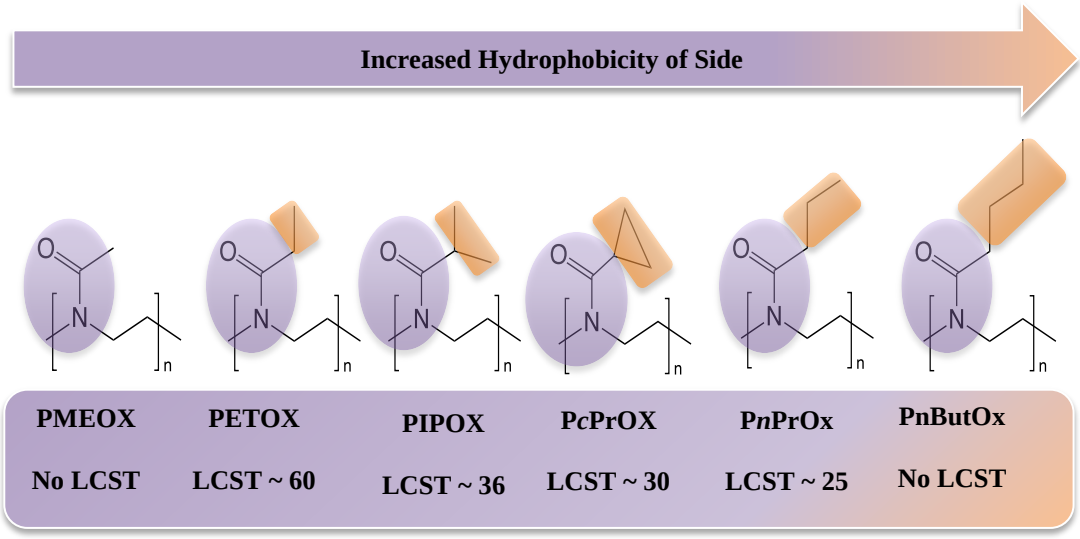


Figure 5. Examples of poly(oxazoline)s with estimated LCST values [52]. (Modified from *Encycl. Polym. Sci. Technol*, 2014).

Poly(2-alkyl-2-oxazoline)s, also called as pseudo-peptides due to their structural similarity to polypeptides, show important biological properties, e.g. biocompatibility, nontoxicity, anti-fouling [53, 54]. Importantly, POXs show higher stability than polypeptides due to tertiary amine groups in their backbones which provides invisibility and protects POXs from being detected and hydrolyzed by the enzymes [22]. In addition, similar to poly (ethylene glycol) (PEG), poly(2-alkyl-2-oxazoline)s cannot be detected easily by the immune system, so called 'stealth behavior' [47, 55]. Moreover, they show higher chemical stability than PEG due to lower polarization of N-vicinal C-H bond than that of O vicinal C-H bond in PEG structure [56].

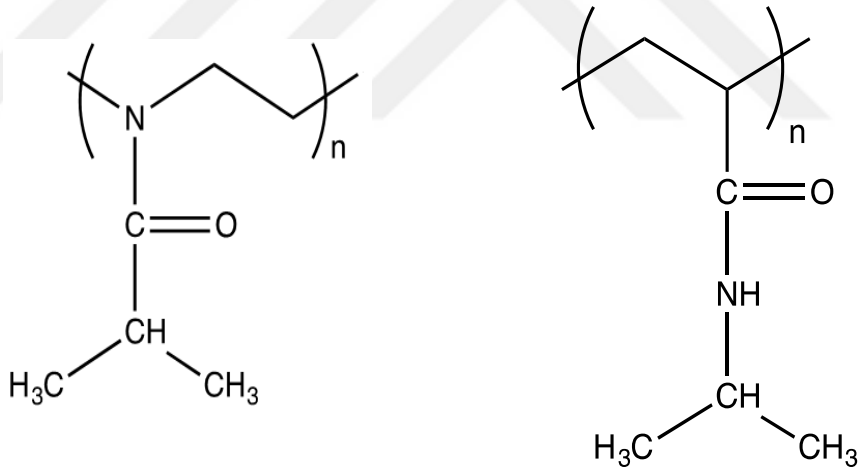
1.2.3. Poly (2-isopropyl-2-oxazoline) (PIPOX)

Among poly(2-alkyl-2-oxazoline)s, poly (2-isopropyl-2-oxazoline) (PIPOX), structural isomer of PNIPAM, comes into prominence due to its LCST of 36 °C [50]. It has later been reported that LCST of PIPOX was affected by the molecular weight

and varies between 45 °C and 63 °C within a molecular weight range of 1900 to 5700 g/mol [57].

Van Mele and co-workers reported that the minimum point in the phase diagram of PIPOX shifted to lower temperature and lower concentration as the molecular weight of PIPOX increased [58]. Importantly, in contrast to PNIPAM showing reversible phase transition with a hysteresis due to formation of intermolecular hydrogen bonds in the globule state [59], PIPOX does not show hysteresis in phase transition due to lack of hydrogen donating groups [60].

Jordon et al. demonstrated that LCST of PIPOX could be tuned by the polymer end-groups. They reported that hydrophilic end-groups increased the LCST of PIPOX, whereas hydrophobic end-groups showed an opposite effect [61]. The effects of copolymerization [62–66] and salt on the LCST of PIPOX [67] have also been reported.



PIPOX **PNIPAM**

Figure 6. Structural representation of PIPOX and PNIPAM.

1.3. Layer-by-Layer Films

Layer-by-layer (LbL) technique is a powerful method for preparation of ultra-thin multilayer films. LbL films are constructed by alternatingly immersing a substrate into solutions of interacting polymers, resulting in LbL deposition of the polymers at the surface. The driving force for film growth is generally non-covalent interactions among the polymer layers including electrostatic, hydrogen bonding, charge-transfer, hydrophobic interactions and metal ligand coordination [68]. It does not require complicated chemical reactions. LbL technique was first demonstrated by Iller in 1966 by depositing positively and negatively charged silica particles onto glass substrate [69]. Then, Decher and Hong applied this technique to polyelectrolytes and reported the first LbL films of anionic and cationic polyelectrolytes [70]. Figure 7 shows schematic representation of electrostatic LbL process [71].

H-bonded multilayers are also prepared in a similar way except that the substrate is immersed alternatingly into solutions of hydrogen-donor and hydrogen-acceptor polymers. Hydrogen bonding self-assembly allows using neutral polymers as building blocks. This is important due to the fact that polycations are more toxic than the neutral polymers [72]. Hydrogen-bonded multilayers were first reported by Rubner and Stockton [73]. They demonstrated that polyaniline could be LbL assembled at the surface using several different neutral polymers, e.g. (poly(vinylpyrrolidone) (PVPON), poly(vinyl alcohol) (PVA), polyacrylamide (PAAm), and poly (ethyelene oxide) (PEO) [73]. Hydrogen-bonded self-assembly in organic solvents has been reported by Wang et. al. [74]. This discovery paved the way for using a wide variety of polymers as building blocks during film fabrication. Later, Sukhishvili and Granick demonstrated the first example of hydrogen-bonded multilayers of neutral polymers and polycarboxylic acids [75, 76]. Those films, which were deposited at low pH, could be erased from the surface with increasing pH values. These films are called "erasable films" and are promising carriers for the fast release of biological molecules at increasing pH values [75].

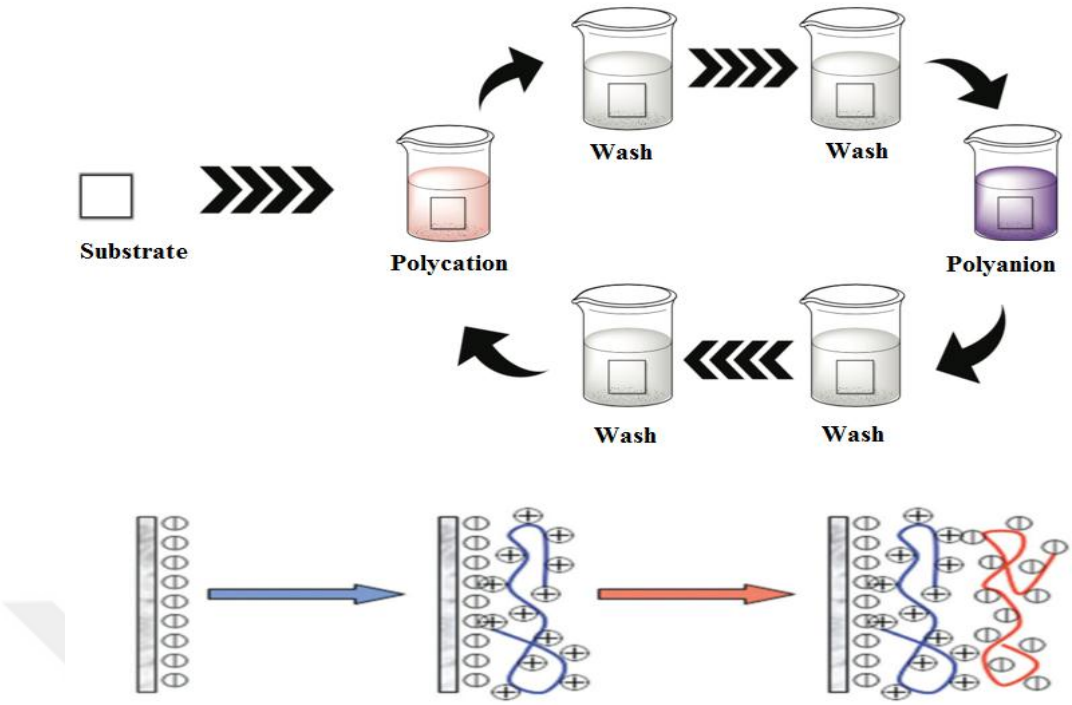


Figure 7. Schematic representation of layer by layer assembly of polyelectrolytes [71]. Modified from Keeney et al. *J. Mater. Chem. B*, 2015.

LbL is an easy and economical way to fabricate ultra-thin films. It does not require complicated equipments. There is no limitation in the size and shape of the substrate for LbL assembly [77]. Multilayers can be deposited not only at planar surfaces, e.g. glass, quartz, silicon wafer or mica [78] but also at 3D substrates, e.g. capsules, nanotubes [79]. LbL technique allows precise control in film thickness. In addition, control over the composition and morphology of the films can be achieved by tuning the assembly and post-assembly conditions [79]. Importantly, LbL technique allows incorporation of functional molecules (e.g. drug, DNA, dyes or magnetic nanoparticles) within the multilayers [80]. By using stimuli responsive polymers, the resulting LbL films can be made responsive to changes in environmental conditions. This feature specifically makes LbL films promising polymer platforms for controlled release applications from surfaces [71, 81]. In this context, controlled release of drug molecules from LbL films by changing environmental conditions, e.g. pH, temperature, ionic strength, light, has been extensively investigated [82–84]. In addition to drug delivery applications, stimuli responsive LbL films can be used in

many different areas such as preparation of sensors, gene delivery platforms or organic electronic devices [68].

Hydrogen-bonded multilayer films is of specific interest among many examples of stimuli responsive LbL films due to relatively low toxicity of neutral polymers than polycations and response of the multilayers at mild pH conditions.

1.3.1. Temperature Responsive LbL Films

LbL films of homopolymers with LCST are mostly constructed via hydrogen bonding interactions among the polymer pairs. Many research groups have previously reported on hydrogen-bonded multilayers of temperature-responsive homopolymers including PNIPAM [85–93]. A study by Quinn and Caruso demonstrated the effect of LbL deposition temperature on the thickness and internal structure of LbL films of PNIPAM and PAA. They found that when LbL films were constructed at 30 °C, which is very close LCST of PNIPAM (~32 °C [21]), multilayers were thicker and rougher than multilayers prepared at 10 °C or 21 °C. Additionally, they showed the effect of temperature on the loading and release properties of the multilayers [94]. Sukhishvili reported similar findings using poly(vinyl methyl ether) (PVME) with a LCST of 36 °C. They showed that film thickness increased with increasing deposition temperature [95].

In another study, Sukhishvili and co-workers reported on the release of the anti-cancer drug, Doxorubicin (DOX) from the multilayers of poly(N-vinyl pyrrolidone-*b*-poly(N-isopropyl acryl amide) (PVPON-*b*-PNIPAM) micelles with temperature-responsive PNIPAM-cores. Multilayers which were constructed above the LCST of PNIPAM were capable releasing DOX when the temperature was lowered below LCST of PNIPAM due to disintegration of the micellar cores [96].

The studies concerning multilayers of poly(2-alkyl-2-oxazoline)s are limited. The first example of LbL films of poly(2-alkyl-2-oxazoline)s using PIPOX and TA as building blocks was reported by Erel et al. This study contrasted the pH-stability of PNIPAM containing multilayers to PIPOX containing ones [97]. De Geest and

Hoogenboom and their co-workers demonstrated the LbL deposition of poly(n-propyl oxazoline) and TA at temperatures below or above the LCST of poly(n-propyl oxazoline) and found that growth mechanism differs depending on the assembly temperature [98]. In another study, same researchers presented a detailed investigation of the thermodynamics of the multilayer assembly of poly(2-alkyl-2-oxazoline)s and TA [72]. Caruso and co-workers prepared low-fouling LbL capsules using either linear or brush-like poly(2-oxazoline)s and poly(methacarylic acid) (PMA) [99]. Same group also reported on the intracellular degradability and redox-responsive properties of multilayers composed of thiol containing poly(2-ethyl-2-oxazoline) brushes and PMA [100] .

1.3.2. pH Responsive LbL Films

pH is one of the most extensively studied stimuli in controlled delivery applications due to local pH changes in the body. The properties of LbL films can be tuned by changing pH due to change in net charge on the weak polyelectrolytes. This feature can be used to induce drug release from the surface of multilayers by changing pH. For example, Chung and Rubner demonstrated pH-controlled release of methylene blue (MB) from the surface of electrostatic multilayers. In this study, they deposited poly(allyamine hydrochloride) (PAH) and PAA at pH 2.5 where PAH (pK_a~8-9) was fully positively charged and PAA (pK_a 5.5) was partially negatively charged. MB was loaded at pH 7 when PAA was further ionized via electrostatic interactions among positively charged MB and negatively charged PAA. MB was released from the surface as the pH was decreased due to protonation of PAA and loss of electrostatic interactions among PAA and MB [101].

In another study, insulin (isoelectric point, pI = 5.4) was LbL deposited using different polyanions, such as poly(vinyl sulfate) (PVS), PAA, or dextran sulfate (DS) at acidic conditions. Insulin was released from the surface at moderately acidic conditions and neutral conditions due to disruption of electrostatic interactions among insulin and the polyanions as the pH was increased due to charge reversal of insulin from positive to negative [102].

LbL films can be made pH-responsive by including hydrolytically degradable polymers within the multilayers. For example, Wood et al. demonstrated that Heparin can be released from the multilayers of Heparin and poly(β-aminoester) as the poly(β-aminoester) degrades hydrolytically and layers are disassembled [103]. Moreover, drug molecules can be conjugated to polymers via pH-responsive covalent bonds, e.g. hydrazone [104] and carbamate [105] bonds. Such drug coupled polymers can be used as building blocks in the LbL self-assembly process and the release of the drugs from the surface can be induced by a pH trigger. For example, Hammond and co-workers also constructed multilayers at neutral pH and released DOX at acidic conditions. In that study, they conjugated DOX to PHEMA-core blocks of poly(ethylene oxide)-*block*-poly(2-hydroxylethyl methacrylate) (PEO-*b*-PHEMA) micelles via carbamate linkage and used such micelles as building blocks to construct hydrogen bonded multilayers. The release of DOX from the surface was induced via carbamate cleavage at acidic conditions [105].

In another study, Cao and He showed that multilayers of a glucocorticoid drug, Prednisolone conjugated poly(vinyl pyrrolidone) (PVP) and PAA which was constructed at acidic conditions (pH 3) could release Prednisolone from the surface specifically at moderately acidic pH (pH 5) due to cleavage of the hydrazone bonds [106] .

It is also possible to release drug molecules from the multilayers by changing ionic strength. Microgels, produced from crosslinking of PAH and Dextrane (PAH-D) was LbL deposited at the surface using Hyaloplasm Acid (HA) at pH 7.4. Ibuprofen was loaded into the multilayers at pH 7.4 via electrostatic interactions among the protonated amino groups of PAH-D and carboxylate groups of Ibuprofen. The release of Ibuprofen was triggered by exposing the film to 0.9 % normal saline [107].

Hydrogen-bonded multilayers are of specific interest for pH-induced release of functional molecules from the surface due to response of multilayers at mild pH conditions.

Sukhishvili and co-workers prepared hydrogen-bonded multilayers of poly(ethylene oxide) and (PEO) poly(methacrylic acid) (PMA) at strongly acidic conditions (pH 2). They loaded positively charged Rhodamine 6G (R6G) by creating excess negative

charge within the multilayers at pH 4.2. Release of R6G was induced either by dissolving the multilayers at high pH values or by exposing the films to acidic environment, resulting in protonation of PMA and loss of electrostatic interactions among PMA and R6G [108].

In another study, Erel at al. demonstrated release of pyrene from hydrogen-bonded multilayers of TA and block copolymer micelles of poly[2-(N-morpholino)ethyl methacrylate-*block*-2-(diisopropylamino)ethyl methacrylate] (PMEMA-*b*-PDPA). Multilayers were constructed at neutral pH when PMEMA-*b*-PDPA was in the micellar form. When the pH was decreased below the pKa of PDPA block, PMEMA-*b*-PDPA micelles disintegrated and pyrene was released from the surface [109].

1.3.3. Magnetic Field Responsive LbL Films

Magnetic nanoparticle containing LbL films are promising carriers for side-specific drug delivery due to the fact that magnetic nanoparticles, together with the multilayers can be directed to the target site by applying magnetic field [83]. For example, Lu et al. prepared LbL films with dual responsive properties, i.e. pH- and magnetic field- responsive. They deposited LbL films of oligochitosan and sodium alginate onto oil-in-water type hybrid emulsion droplets containing magnetic nanoparticles and Dipyridamole drug molecules. They showed that the direction of the capsules could be modulated by applying magnetic field, whereas the drug release from the surface could be induced by a pH change [110].

Incorporating magnetic nanoparticles into LbL films also makes such films to be used for imaging purposes [111]. In addition, magnetic nanoparticles could be used to create local heating within the multilayers, which may be interesting for hyperthermia application. For example, it was demonstrated that DOX loaded magnetic alginate microspheres coated with LbL films of electrostatic PAH and poly(styrene sulfate) (PSS) were capable of releasing DOX by applying high frequency magnetic field. The applied magnetic field provided heating of the magnetic nanoparticles and increased the diffusion rate of the loaded drug. Moreover, it was reported that application of magnetic field induced energy could

result in oscillation and vibration of the magnetic nanoparticles which might have affected the wall permeability of the polymer coating [112].

1.3.4. Electrically Responsive LbL Films

The net charge on electrically responsive polymers with ionizable groups on their backbone can be modulated by applying electric current [113]. This feature can be used to control the stability of electrostatic LbL films and release of functional molecules from the surface [113]. For example, Iwate and co-workers demonstrated that electrostatic multilayers of PEI and plasmid DNA which were stable at physiological conditions were capable of releasing plasmid DNA upon application of an electric pulse. They found that both increasing number of layers and increasing electric field strength resulted in an increase in the amount of plasmid DNA released from the surface [114].

In another study, Hammond and co-workers fabricated electrostatic LbL films of non-toxic, electroactive Prussian blue (PB) nanoparticles and hydrophilic antibiotic Gentamicin. Negatively charged PB transforms into neutral state when oxidized upon application of electric field. This results in loss of electrostatic interactions among Gentamicin and PB, followed by the disintegration of multilayers and release of Gentamicin [115]. Same researchers previously reported on the voltage-controlled release of dextrane sulfate from a hybrid multilayer film containing LPEI, PB, LPEI and radiolabeled ^{14}C-dextran sulfate DS. They found that release could be triggered only at + 1.25 V [116].

1.3.5. Light Responsive LbL Films

Light trigger was also used to release drug molecules from LbL films. For example, Cao and co-workers demonstrated that azobenzene modified PAA could be loaded into Rhodamine B conjugated cyclodextrin (α-CD) via host-guest interactions. Then, these azobenzene modified PAA loaded α-CD-RhB was LbL deposited at the surface using poly(diallyldimethyl ammonium chloride) (PDAC) as the counter polymer.

Exposing multilayer films to UV-light resulted in loss of host-guest interactions among azobenzene modified PAA and α-CD-RhB, followed by release of α-CD-RhB from the surface [117].

Light-induced release of fluorescein isothiocyanate (FITC)-labeled dextran molecules was also examined from the surface of LbL films blended with gold nanoparticles. Application of light in the near IR provided heating of gold nanoparticles and resulted in rupture of the LbL capsule walls which then induced the release of FITC-labeled dextran from the surface [118].

1.4. Aim of Thesis

The aim of this thesis is to produce multilayers which release minimal amount of DOX at physiological conditions but release DOX at moderately acidic conditions. Moreover, the effect of temperature on the pH-induced release of DOX from the surface and its correlation with the LCST behavior of PIPOX in aqueous environment was investigated.

In general, hydrogen-bonded multilayers are of specific interest in biomedical applications due to lower toxicity of neutral polymers than the polycations and pH-response of the films at mild conditions. However, most of the hydrogen-bonded films are stable at acidic conditions and are capable of releasing positively charged functional molecules with increasing pH. Therefore, it is challenging to prepare hydrogen-bonded multilayers which provide minimal release at physiological conditions but trigger release of positively charged drug molecules, e.g. DOX, at acidic environment. In contrast to pH-induced DOX releasing electrostatic LbL films [119–123] , there are only few studies reporting the pH- or temperature-induced release of DOX from hydrogen bonded multilayers [96, 105, 124, 125]. Among these, only Hammond and co-workers presented preparation of multilayers which were stable at neutral pH and released DOX at acidic conditions. In that study, they conjugated DOX to PHEMA-core blocks of poly(ethylene oxide)-*block*-poly(2-hydroxylethyl methacrylate) (PEO-*b*-PHEMA) micelles via carbamate linkage and used such micelles as building blocks to construct hydrogen bonded multilayers. The

release of DOX from the surface was induced via carbamate cleavage at acidic conditions [126]. Different from this study, this thesis presents a simple strategy, i.e. preparation of TA-DOX and using them as building blocks during LbL assembly, to include DOX into hydrogen-bonded multilayers which release DOX at moderately acidic conditions, while show minimal release at physiological conditions. Importantly, this study is also the first reporting the pH- and/or temperature-induced release of DOX from poly(2-alkyl-2-oxazoline) based hydrogen-bonded multilayers. Considering the temperature-responsive and important biological properties of poly(2-alkyl-2-oxazoline)s combined with the acidic nature of tumor tissues, these multilayers which release DOX at moderately acidic conditions, can be promising drug carriers for controlled release of DOX from surfaces.

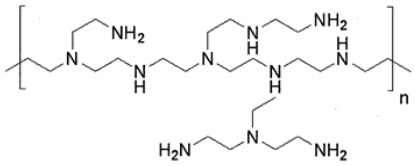
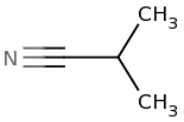
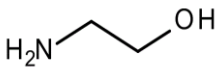
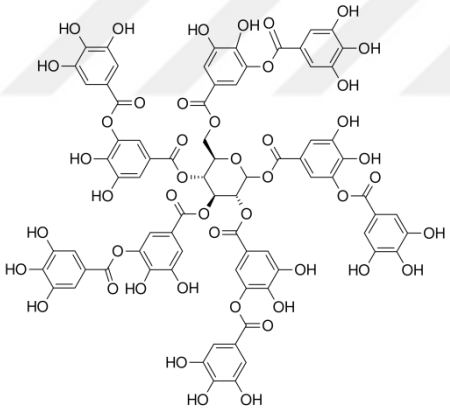
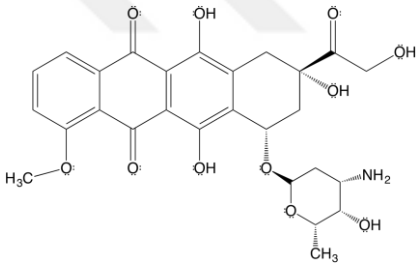
CHAPTER 2

EXPERIMENTAL PART

2.1. Materials

Phosphate buffer saline (PBS); branched poly(ethylenimine) (BPEI; Mw: 25,000); dibasic sodium phosphate; sodium hydroxide, hydrochloric acid, ethanolamine (>99%), cadmium acetate dihydrate (98%), methyl p-toluenesulfonate (98%), acetonitrile (>99.9%), 2-butanol (>99%) were purchased from Sigma-Aldrich Chemical Co. Tannic acid (TA; Mw 1701.20), monobasic sodium phosphate, sulfuric acid (98%) and isobutyronitrile (>98%) were purchased from Merck Chemicals. Doxorubicin hydrochloride was purchased from Alfa Aesar. The deionized (DI) H_2O was purified by passage through a Milli-Q system (Millipore) at 18.2 MΩ. All materials and chemicals were used as received without any further purification.

Table 1 . Structures of Polymers and Chemicals

Branched Poly(etyhlenimine)  Sigma Aldrich Chemicals	**Isobutrylnitrile & Ethanolamine**   Merck Chemicals Sigma-Aldrich Chem.
Tannic Acid  Merck Chemicals	**Doxorubicin**  Alfa Aesar

2.2 Methods and Instrumentation

Nuclear Magnetic Resonance (NMR): ^{1}H-NMR measurements were carried out at room temperature using Brucker Spectrospin Avance DPX-400 Ultra shield instrument operating at 400 MHz.

Gel Permeation Chromatography (GPC): Gel permeating chromatography measurements were carried out in Agilent instrument (Model 1100) consisting of Refractive Index (RI) detectors and three Macherey-Nagel columns which are packed with a highly cross-linked macroporous, spherical polystyrene-divinyl-benzene polymers matrix (PS/DVB) (Columns 300 × 7.7 mm, particles 5μm). 0.01 M LiBr/DMF was used as an eluent at a flow rate of 0.7 mL/min at 50 °C. The calibration was performed using poly(methyl methacrylate) (PMMA) standards (Polymer Laboratories).

Dynamic Light Scattering (DLS) and Zeta-potential Measurements: Hydrodynamic size and zeta-potential measurements were performed using Zetasizer Nano-ZS equipment (Malvern Instruments Ltd., U.K.).

Ellipsometry: Film thickness measurements were performed using a spectroscopic ellipsometer of Optosense, USA (OPT-S6000).

Fluorescence Spectroscopy: Release studies were conducted using a Perkin Elmer LS55 Fluorescence Spectrometer.

Atomic Force Microscopy (AFM): AFM imaging of the films was performed using an NT-MDT Solver P47 AFM in tapping mode using Si cantilevers. Roughness values were obtained from images with 2 x 2 μm scan size.

pH Meter: During the experiments, Starter 3000 bench pH meter was used for adjusting pH of solutions. Calibration of pH meter was performed using three standard buffer solutions at pH 3, pH 7 and pH 10.

2.3. Synthesis of 2-isopropryl-2-oxazoline

2-isopropyl-2-oxazoline was synthesized as described previously with a slight modification [63]. Briefly, ethanolamine (0.052 mol, 3.52 mL) and isobutyronitrile (0.043 mol, 3.9 mL) were mixed with cadmium acetate dihydrate (1.08 mmol, 0.29 g) under argon atmosphere. The reaction was stirred under reflux at 130°C for 48 hours. The crude product was distilled at 50°C under reduced pressure and dried over CaH_2.

^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) = 4.21 (t, J= 9.02 Hz, 2 H), 3.82 (t, J= 9.26 Hz, 2 H), 2.57 (m, J= 7.27 Hz, 1 H), 1.2 (d, J= 6.90 Hz, 6 H).

2.4. Synthesis of Poly(2-isopropryl-2-oxazoline) (PIPOX)

PIPOX was synthesized by cationic ring opening polymerization as described previously by Meyer and Schlaad with a slight modification [65]. Briefly, acetonitrile (10.0 mL) and 2-isopropyl-2-oxazoline (41.0 mmol, 4.9 mL) were added into an argon purged reaction flask capped with a condenser. Then, p-toluene sulfonate (0.4 mmol) was added to the flask and the reaction was stirred in preheated oil bath at 80 °C for 48 hours. The mixture was cooled to room temperature and quenched with 2-Butanol (1.2 mmol). After quenching, the reaction was stirred for additional 3 days at 80 °C. PIPOX solution was concentrated under reduced pressure, dialyzed against deionized water for 2 days (SpectroPor7 regenerated cellulose dialysis membrane, molecular weight cutoff: 3.5 kDa) and the product was isolated by freeze-drying.

^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) = 3.46 (br, 4 H), 2.41-2.30 (br, 2 H), 1.12 (br, 6 H). GPC (eluent: dimethyl formamide, Mn = 15 kDa, PDI= 1.30)

2.5. Preparation of TA-DOX Complexes

0.01 M phosphate buffer was prepared at pH 6.5. DOX and TA solutions were prepared using phosphate buffer solutions. 0.01 mg/mL DOX solution at pH 6.5 was gradually added onto 0.2 mg/mL TA solution at pH 6.5 with a volume ratio of 1:4

(DOX:TA) and magnetically stirred for 30 minutes. Complexation time was specifically limited to 30 minutes to avoid degradation of TA with time.

2.6. Deposition of Multilayers

Silicon wafers were cleaned with concentrated sulfuric acid for 85 min and rinsed with DI water. After drying under nitrogen flow, silicon wafers immersed into 0.25 M NaOH solution for 10 minutes. The substrates were then thoroughly rinsed with DI water and dried under nitrogen flow. Wafers are immersed into branched polyethylene imine (BPEI) and tannic acid (TA) solutions for 30 min and 15 min at pH 5.5 as precursor layers prior to multilayer film deposition. The concentration of BPEI and TA solutions were 0.5 mg/mL and 0.2 mg/mL, respectively. Multilayers were self-assembled at the surface by immersing the BPEI/TA coated silicon wafers alternatingly into 0.2 mg/mL solutions of PIPOX and TA-DOX complexes at pH 6.5 for 15 minutes each with 2 intermediate rinsing steps with 0.01 M phosphate buffer solutions at pH 6.5) in between. For control experiments, PIPOX/TA multilayers were prepared using the same procedure except that pure TA was used rather than TA-DOX complexes during film construction. Of note, TA solution was refreshed every 1 hour during film fabrication to avoid deposition of the degradation products at the surface.

2.7. pH-stability of the Multilayers

pH-stability was examined by exposing the multilayers to either 0.01 M phosphate buffer solutions or PBS solutions (prepared as dissolving of 1 tablet into 200 mL DI water. The resulting solution was composed of 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer solution) at different pH values for 30 minutes. pH of the solutions were adjusted by addition of 0.1 M HCl or 0.1 M NaOH into solutions. After exposure to buffer solutions, multilayer coated substrates were dried under nitrogen flow and the film thickness was measured using an ellipsometer. pH-stability experiments were performed at 25°C and 37.5°C. For long-term release experiments, multilayers were exposed to PBS solutions at 25°C or 37.5°C for 7 hours at different pH values. Figures concerning pH-stability of the multilayers are

represented as fraction retained at the surface as a function of pH. Fraction retained at the surface is calculated by dividing the film thickness at a certain pH by the initial thickness of the film.

2.8. DOX Release from Multilayers

20-bilayer films of PIPOX and TA-DOX were constructed on both sides of a glass slides. In order to minimize the quenching and self-diffusion of DOX from the multilayers during film construction, deposition time for each polymer layer was kept 5 minutes for release experiments. Of note, the film thickness decreased only by ~ 20 % when the deposition time was kept 5 minutes for each layer. Multilayers were immersed into PBS solution at pH 6.5 or pH 5.5 or pH 7.5. Samples were taken from the solution to monitor the release of DOX from the multilayers. DOX was excited at 490 nm and the intensity at 588 nm was monitored as a function of time using a fluorescence spectrophotometer. DOX release was followed until the change in intensity at 588 nm becomes insignificant.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Synthesis of 2-isopropyl-2-oxazoline

2-subsitituted-2-oxazolines can be synthesized from nitriles, carboxylic acids, aldehydes or 2-methyl-2-oxazoline [42]. In general, starting from carboxylic acids or nitriles, known as Wenker Method and Witte-Seeliger Reaction, respectively are preferred to synthesize 2-substituted-2-oxazolines [127]. Despite the ammonia forming during the Witte-Seeliger Reaction, both simplicity of one pot reactions and the wide variety of nitriles as starting materials make this method advantageous for synthesis of 2-subsitituted-2-oxazolines with diverse chemical structures [128].

In this study, the synthesis of 2-isopropyl-2-oxazoline was carried out successfully via Witte Seeliger reaction. Isobutrylnitrile was converted to 2-isopropyl-2-oxazoline upon reacting with ethanolamine in the presence of a Lewis-acid catalyst, cadmium acetate. The reaction was performed at 130 °C for 48 hours under argon flow to replace ammonia produced during the reaction. The product was distilled at 50°C under reduced pressure (Fig. 8). Figure 9 shows the NMR spectrum of 2-isopropyl-2-oxazoline.

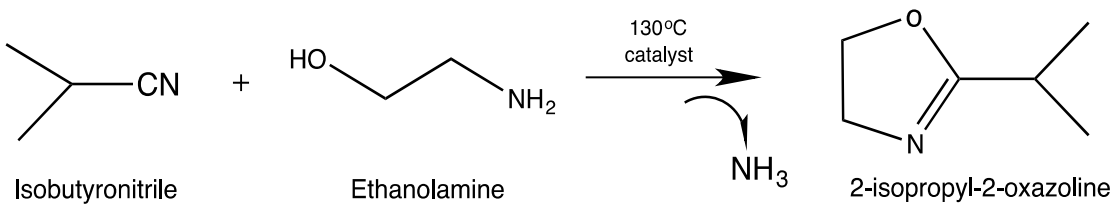


Figure 8. Synthesis reaction of 2-isopropyl-2-oxazoline.

^{1}H-NMR spectrum of 2-isoprpoyl-2-oxazoline showed that methyl protons (d) at 1.2 ppm (d, 6H, C**H**$_3$), the methine proton (c) at 2.57 ppm (br, 1H, -C**H**-), the methylene protons neighbor to nitrogen (b) at 3.82 ppm (t, 2H, -NC**H**$_2$CH$_2$ -) and the methylene protons neighbor to oxygen (a) at 4.21 ppm (t, 2H, -CH$_2$C**H**$_2$O-).

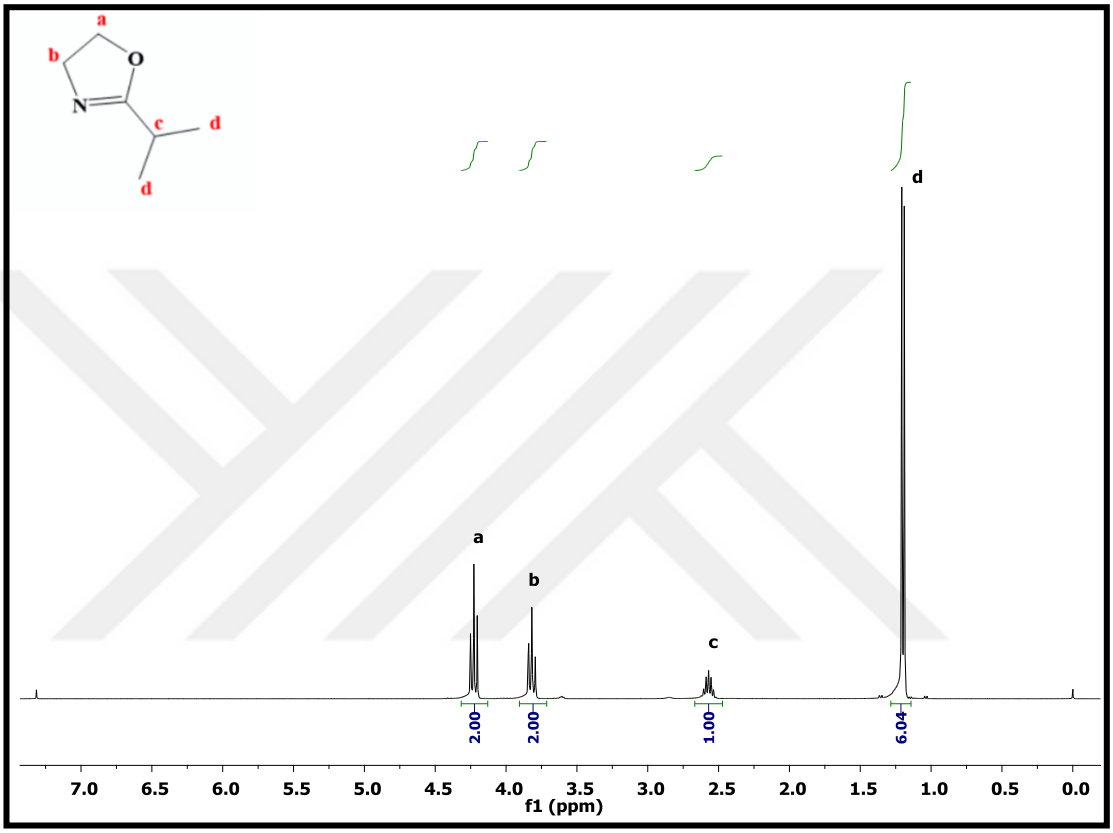


Figure 9. ^{1}H NMR spectrum of 2-isopropyl-2-oxazoline.

3.2. Synthesis of Poly(2-isopropyl-2-oxazoline) (PIPOX)

The synthesis of PIPOX was performed via cationic ring opening polymerization. This technique allows synthesis of poly(2-alkyl-2-oxazoline)s with a wide variety of architectures. The polymerization is initiated by different initiators including alkyl tosylates, triflates or halides [48].

Synthesis of poly(2-isopropyl-2-oxazoline) (PIPOX) via cationic ring opening polymerization as carried out in acetonitrile at 80°C for 48 hours, using methyl p-

tosylayte as the initiator. The reaction is initiated by the electrophilic attack of methyl p-tosylayte to nitrogen of cyclic 2-isopropyl-2-oxazoline ring leading to formation of oxozolinium species. The C-O bond of cationic oxozolinium propagating specie undergoes a nucleophilic attack by the incoming 2-oxazoline monomer inducing the ring opening. The polymerization was continued until the desired molecular weight was obtained. The reaction was terminated by 2-butanol (Fig. 10)

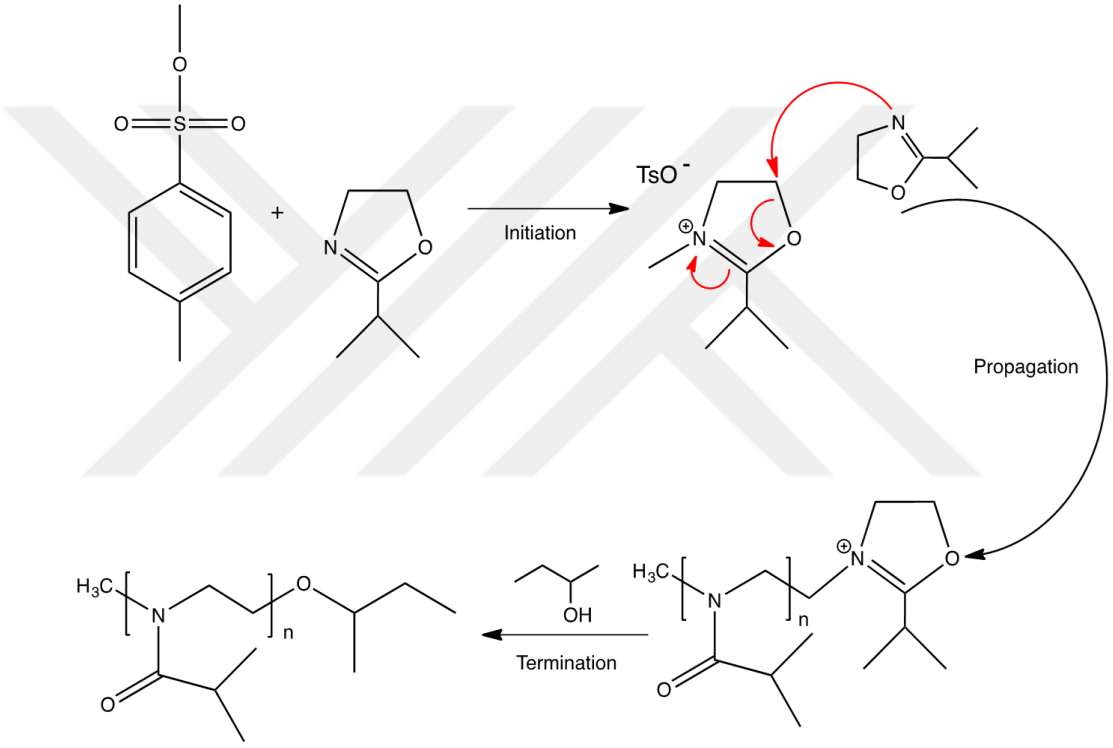


Figure 10. Schematic representation of mechanism of synthesis of PIPOX**.**

^{1}H-NMR spectrum of PIPOX showed that methyl protons (c) at 1.12 ppm (br, 6H, -CH(C**H**$_3$)$_2$), the methine proton (b) between 2.41-2.30 ppm (br, 1H, -C**H**CO-) and the methylene protons on the backbone (a) at 3.46 ppm (br, 4H, -NC**H**$_2$C**H**$_2$-).

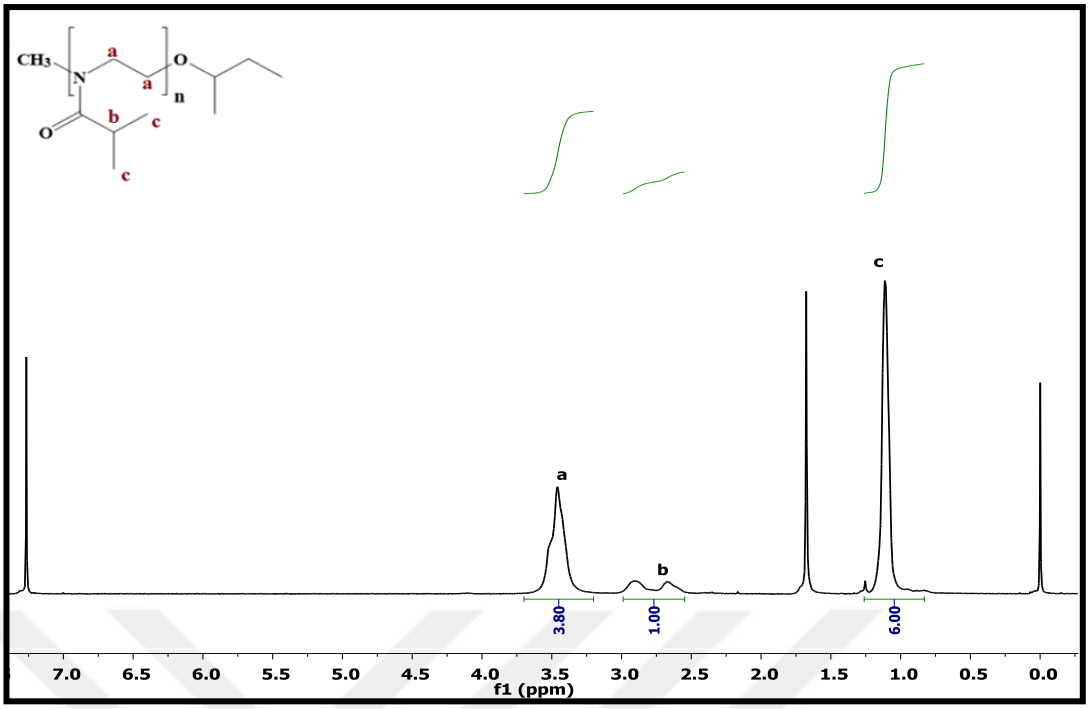


Figure 11. ^{1}H NMR spectrum of poly(2-isopropyl-2-oxazoline) (PIPOX).

The molecular weight of PIPOX was determined by GPC analysis (Mn=1.5x10^{4} g/mol); Mw= 1.9 X10^{4}) polydispersity index, PDI=1.30) (Figure 12).

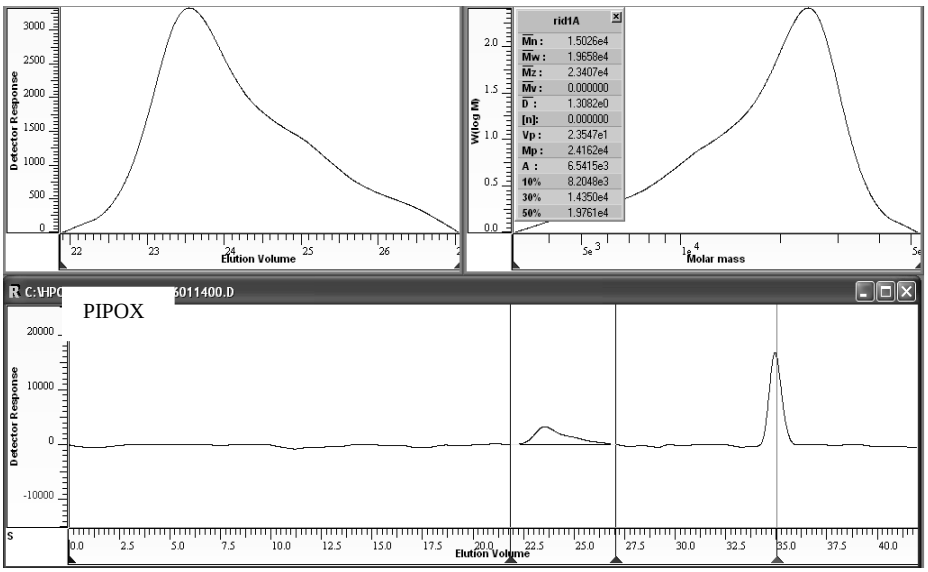


Figure 12. GPC traces of PIPOX (PMMA standard, eluent: 0.01 M LiBr/DMF, flow rate: 0.7 mL/min, temperature: 50 °C, RI detection).

3.3. Determination of Cloud Point Temperature in PIPOX Solution

The cloud point of PIPOX was determined in aqueous environment by monitoring the change in hydrodynamic size with increasing temperature using dynamic light scattering technique. Figure 13 shows the hydrodynamic size values plotted as a function of temperature. The shift in the number average size distribution curves to higher values with increasing temperature was displayed in Appendix. The results showed that the cloud point of PIPOX used in this study was approximately ~ 40 °C. Precipitation was observed at temperatures above 40 °C.

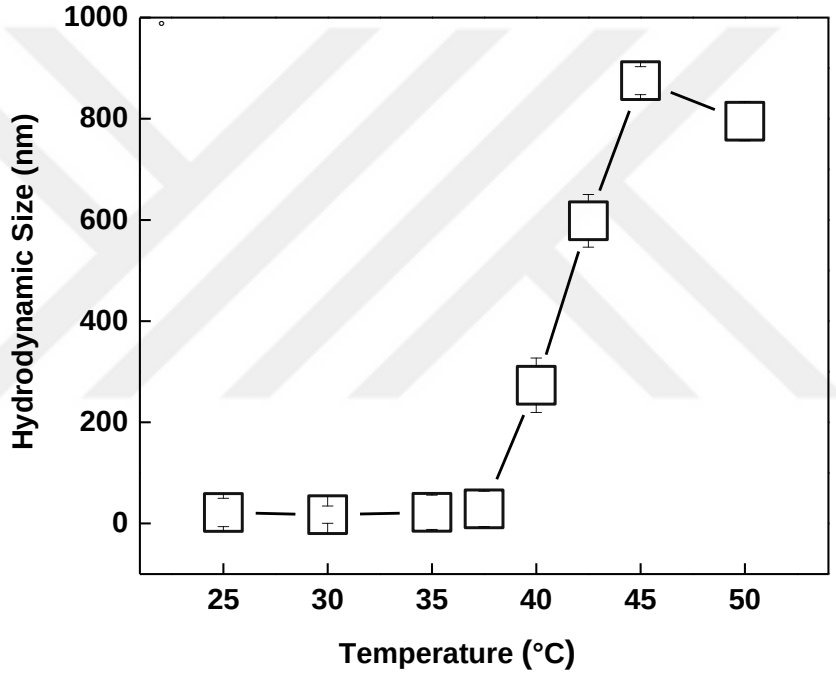
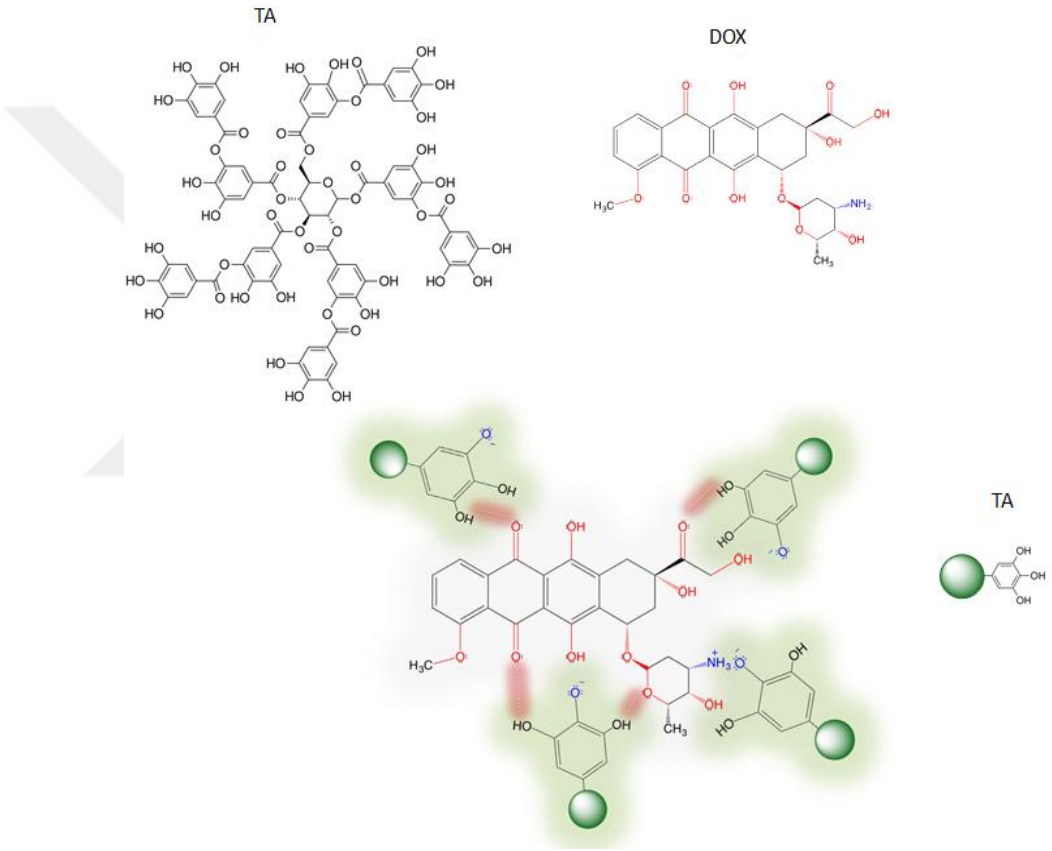


Figure 13. Change in hydrodynamic size of PIPOX as a function of temperature.

3.4. TA-DOX Complexes in Aqueous Solution

Prior to multilayer assembly, water soluble complexes of TA and DOX were prepared. 500 μL of 0.01 mg/mL DOX solution was added onto 2 mL of 0.2 mg/mL TA solution at pH 6.5 when TA is partially ionized (pK_a of TA ~ 8.5 [129]) and bears

both hydroxyl and phenolate groups. Thus, phenolate groups of TA could associate with protonated amino groups of DOX (pK_a of DOX ~ 8.25 [130]), whereas free hydroxyl groups of TA could later participate in hydrogen bonding driven multilayer self-assembly. Of note, in addition to electrostatic interactions, TA and DOX may also associate through H-bonding interactions among the hydroxyl groups (in the protonated form) of TA and carbonyl, ether and hydroxyl groups of DOX. Scheme 1 shows the chemical structures of TA and DOX and the association among TA and DOX molecules (Scheme 1).



Scheme 1. Chemical structures of TA and DOX and the association among TA and DOX molecules at pH 6.5.

As seen in Figure 14, a shift in the size distribution curve to higher values was monitored upon addition of DOX into TA solution. A slight decrease in zeta potential (from -36.2 ± 2.1 mV to -32.8± 2.2 mV) was recorded due to compensation

of some of the negative charges on TA molecules by the protonated amino groups of DOX. The reason to keep TA:DOX molar ratio quite high (27:1) in TA-DOX was to avoid the precipitation of TA-DOX during film fabrication process. Of note, TA-DOX with lower TA:DOX molar ratio was also prepared. The size distribution curves shifted further to higher values and the zeta potential values became gradually less negative as the amount of DOX in the complexes increased. However, precipitation of TA-DOX speeded up as the TA:DOX ratio decreased. Figures including the size and zeta potential values of TA-DOX complexes with different TA:DOX ratios as well as the images showing the increase in turbidity of the solutions with increasing amount of DOX in the complexes can be found in Appendix. It is worth to note that another reason to keep the TA:DOX ratio quite high was to leave enough free hydroxyl groups on TA after complexation for a robust LbL film growth.

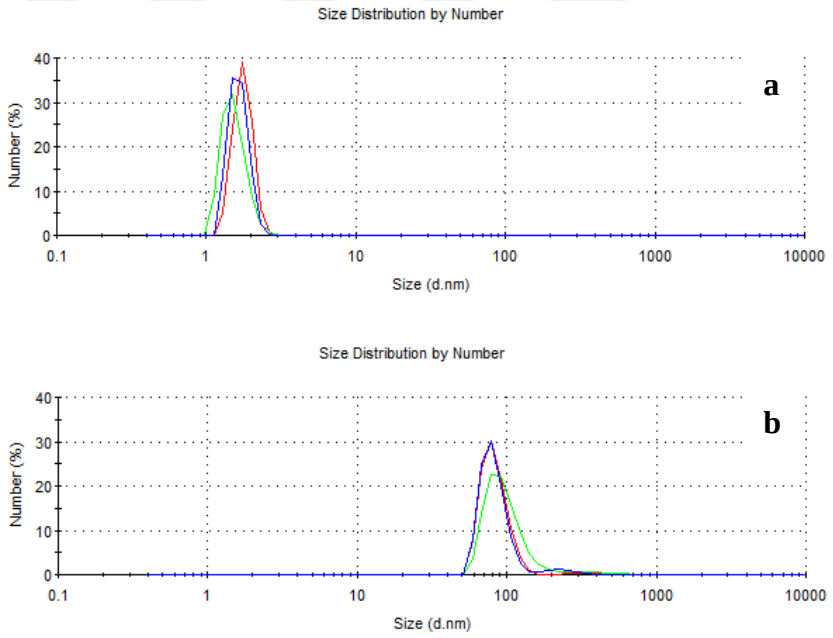
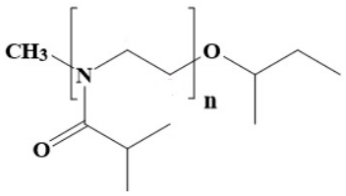


Figure 14. The size distribution curves of pure TA (a) and TA-DOX complexes (b).

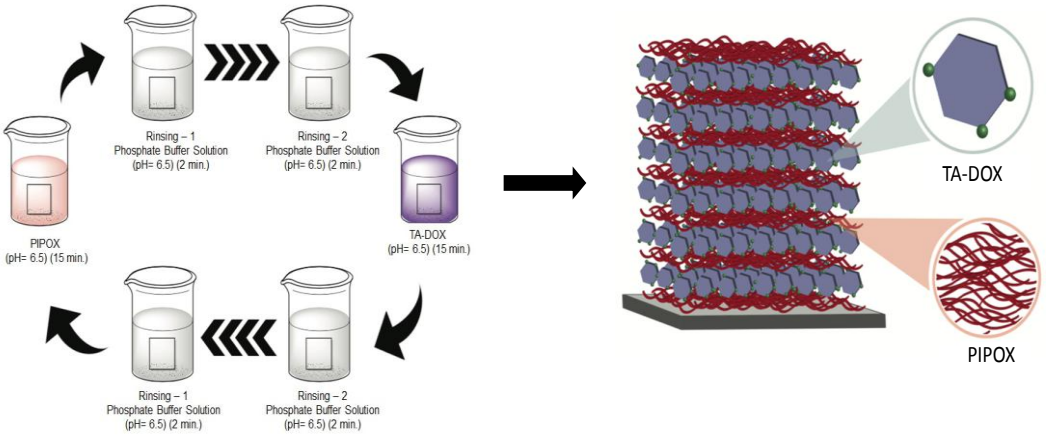
3.5. Multilayers of PIPOX and TA-DOX Complexes

DOX containing films were prepared by LbL depositing PIPOX and TA-DOX onto a substrate at pH 6.5. The driving force for multilayer growth was the hydrogen

bonding interactions among the carbonyl groups in the amide group of backbone of PIPOX as proton acceptor and hydroxyl groups of TA as the proton donor. As previously mentioned, TA, with a pK_a of ~ 8.5, is partially ionized at pH 6.5. Thus, while the phenolate groups of TA can electrostatically bind to protonated amino groups of DOX at pH 6.5, protonated hydroxyl groups of TA can form hydrogen bonds with the carbonyl groups of PIPOX. In order to ensure deposition of multilayers onto substrate, precursor layers (BPEI as 0.5 mg/mL at pH 5.5 and TA as 0.2 mg/mL at pH 5.5) were deposited at first, then multilayers were fabricated by dipping into solutions of the building blocks sequentially. Scheme 2 and Scheme 3 illustrate the chemical structure of PIPOX and multilayer formation via LbL deposition process.



Scheme 2. Chemical structure of PIPOX.



Scheme 3. Schematic representations of LbL film deposition cycle and the multilayers of PIPOX and TA-DOX.

Multilayers of PIPOX and TA-DOX showed a linear growth profile with ~ 2.5 nm increment per bilayer. For comparison, multilayers of PIPOX and bare TA molecules were also prepared (Fig. 15). Multilayers of PIPOX and TA-DOX were thinner than that of PIPOX and TA due to lower number of free hydroxyl groups remained on TA upon complexation with DOX, resulting in a decrease in the extent of association among the hydrogen bonding polymer pairs at the surface. Of note, the dissociation of TA should have increased in the presence of positively charged DOX molecules. Therefore, the number of free hydroxyl groups on TA was expected to be lower than that on bare TA under the same conditions.

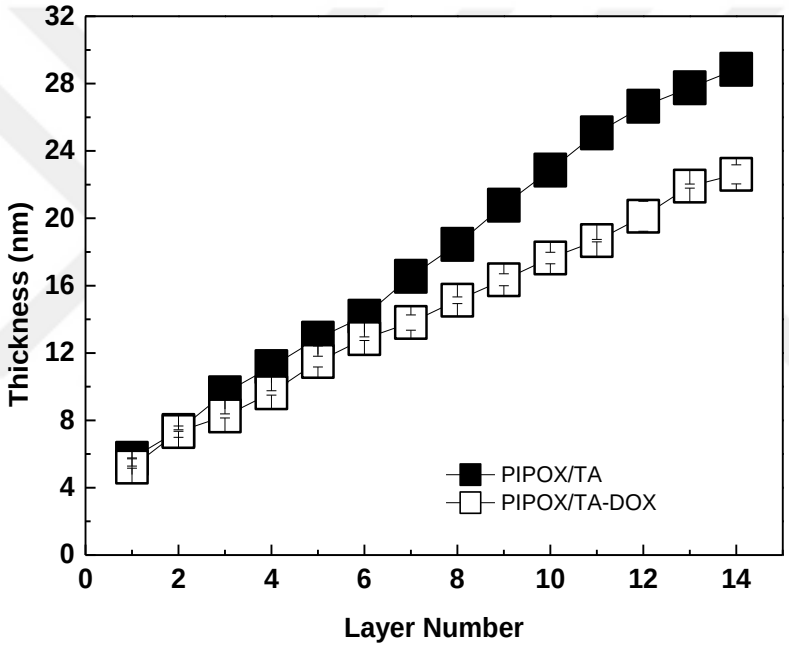


Figure 15. Multilayer growth of PIPOX/TA and PIPOX/TA-DOX at pH 6.5. Multilayers were grown onto a precursor film with a thickness of 5.5 nm. The thickness values on the graph include the precursor layer thickness.

The first study on poly(oxazoline) containing LbL films was reported by Erel and Sukhishvili [97]. They showed that LbL films of PIPOX and TA could be grown at strongly acidic conditions and those multilayers showed response only at basic conditions [97]. In this present study, the construction of multilayers near below the

physiological pH, providing DOX being included within the multilayers during film assembly which could then be released at moderately acidic conditions was investigated. Caruso and co-workers reported on the construction of multilayers of linear or brush poly(2-oxazoline)s with poly(methacrylic acid) (PMAA) and found that film fabrication condition was limited to pH [99, 100]. By taking advantage of the high pK_a of TA, the LbL deposition pH was extended to pH 6.5 which allowed incorporation of positively charged DOX into the multilayers during film assembly. In addition, Hoogenboom and de Geest and their co-workers have also reported multilayers of poly(oxazoline)s and TA. The thermodynamics of the LbL assembly of TA with 2-methyl, 2-ethyl or 2-n-propyl substituted poly(oxazoline)s were scrutinized [72] and also investigated the effect of temperature on the growth mechanism of the multilayers [98].

Deposition of multilayers onto surface was investigated with Atomic Force Microscopy method. AFM imaging studies showed that PIPOX/TA-DOX films had higher surface roughness than that of PIPOX/TA film by comparing 4 and 7- bilayers of PIPOX/TA-DOX and PIPOX/TA films (Fig. 16). Irregular packing of TA-DOX was contribute to higher surface roughness of the PIPOX/TA-DOX films was displayed in Fig 16. In addition, the lower number of binding points among PIPOX and TA-DOX than that among PIPOX and TA might have led to higher number loops within the multilayers resulting in increased surface roughness. Rubner and Sukhishvili have previously reported on the correlation between the number of binding points among the layers and surface roughness for electrostatic [131] and hydrogen-bonded [95] multilayers, respectively. When the number of layer was increased, the difference in roughness got more significant. The higher surface roughness of PIPOX/TA-DOX films can be correlated with the incomplete and irregular packing of the TA-DOX at the surface. When PIPOX meets with the surface coated with TA-DOX, the adsorption on the TA-DOX, in other words, the higher parts of the surface are more likely than being adsorbed on the incomplete parts, resulting in an increase in surface roughness with increasing number of layers.

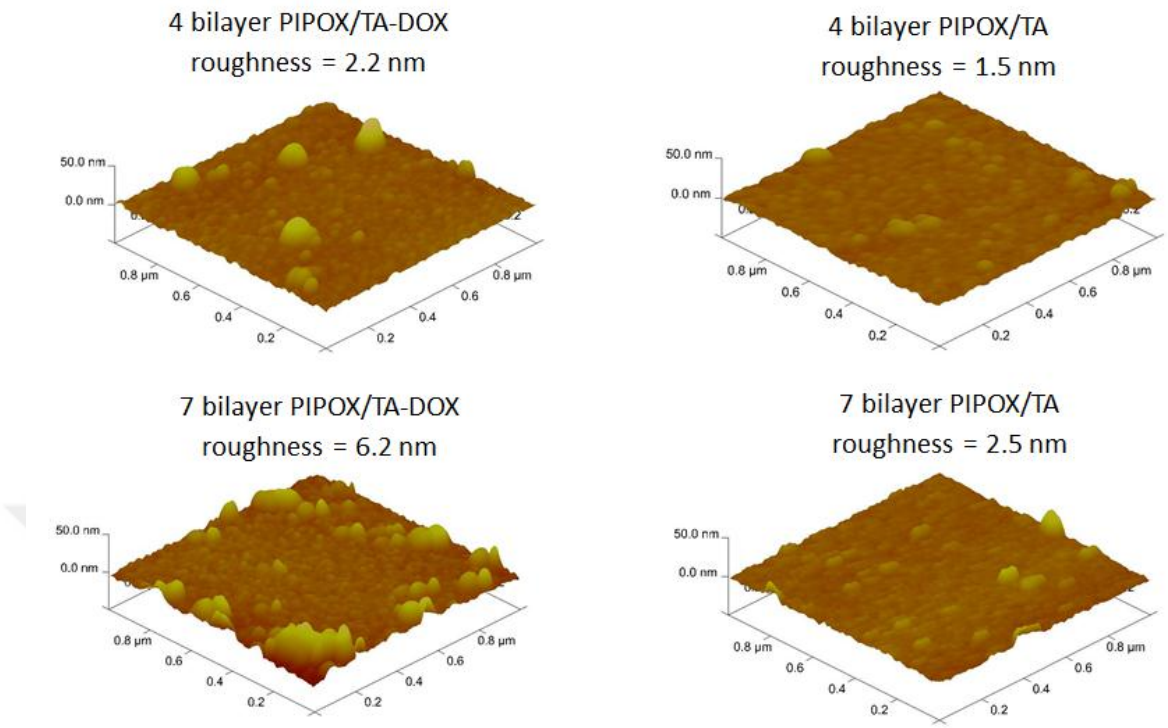


Figure 16. AFM images and rms roughness values of 4- and 7- bilayers of PIPOX/TA-DOX and PIPOX/TA films. The average surface roughness values were estimated over 2 µm x 2 µm areas on three different randomly selected places of the sample surface**.**

3.6. pH-stability of Multilayers

pH-stability of the LbL films were examined prior to DOX release studies. Deposited multilayers of PIPOX and TA-DOX at pH 6.5, were exposed to 0.01 M phosphate buffer solutions of decreasing pH for 30 minutes. For comparison, PIPOX/TA films which were prepared at pH 6.5 and exposed to similar conditions were also examined and represented in Figure 17.

As clearly seen in the Figure 17, film thickness remained almost constant for both films in the acidic region. This can be explained by the protonation of hydroxyl

groups of TA with decreasing pH, enhancing the H-bonding interactions among PIPOX and TA and keeping the multilayers intact. The same process was applied for the control of pH stability of multilayers with increasing pH. When multilayers were exposed to basic conditions, both films were stable until pH 8.5. PIPOX/TA films showed a sharp decrease in film thickness and removed almost completely from the surface at pH 9 due to ionization of TA and loss of H-bonding interactions among PIPOX and TA layers. Different from PIPOX/TA films, multilayers composed of PIPOX and TA-DOX showed gradual decrease in film thickness between pH 8.5 and pH 10.5. The difference in the pH-stability profiles can be explained by the electrostatic screening of negative charge on TA by DOX molecules within the multilayers. The negative charge arising from the ionization of hydroxyl groups of TA with increasing pH was screened by the positively charged DOX molecules. This leads to both lower electrostatic repulsions among the TA molecules and lower increase in osmotic pressure than that in PIPOX/TA multilayers, thus higher stability at basic pH. The effect of electrostatic screening and compensation of the excess charge within the multilayers by multivalent salt ions on the post-assembly stability of hydrogen-bonded multilayers has been reported earlier [131, 132].

Erel et al. has previously reported that PIPOX/TA films which were constructed at pH 2, eroded completely at pH 10 when exposed to buffer solutions of increasing pH for 30 minutes [97]. Here, the same polymer pair was constructed at pH 6.5, multilayers completely dissolved at pH 9. At pH 2, TA has higher number of protonated hydroxyl groups, therefore PIPOX/TA films have higher number of binding points among the layers which led to greater stability at the basic region.

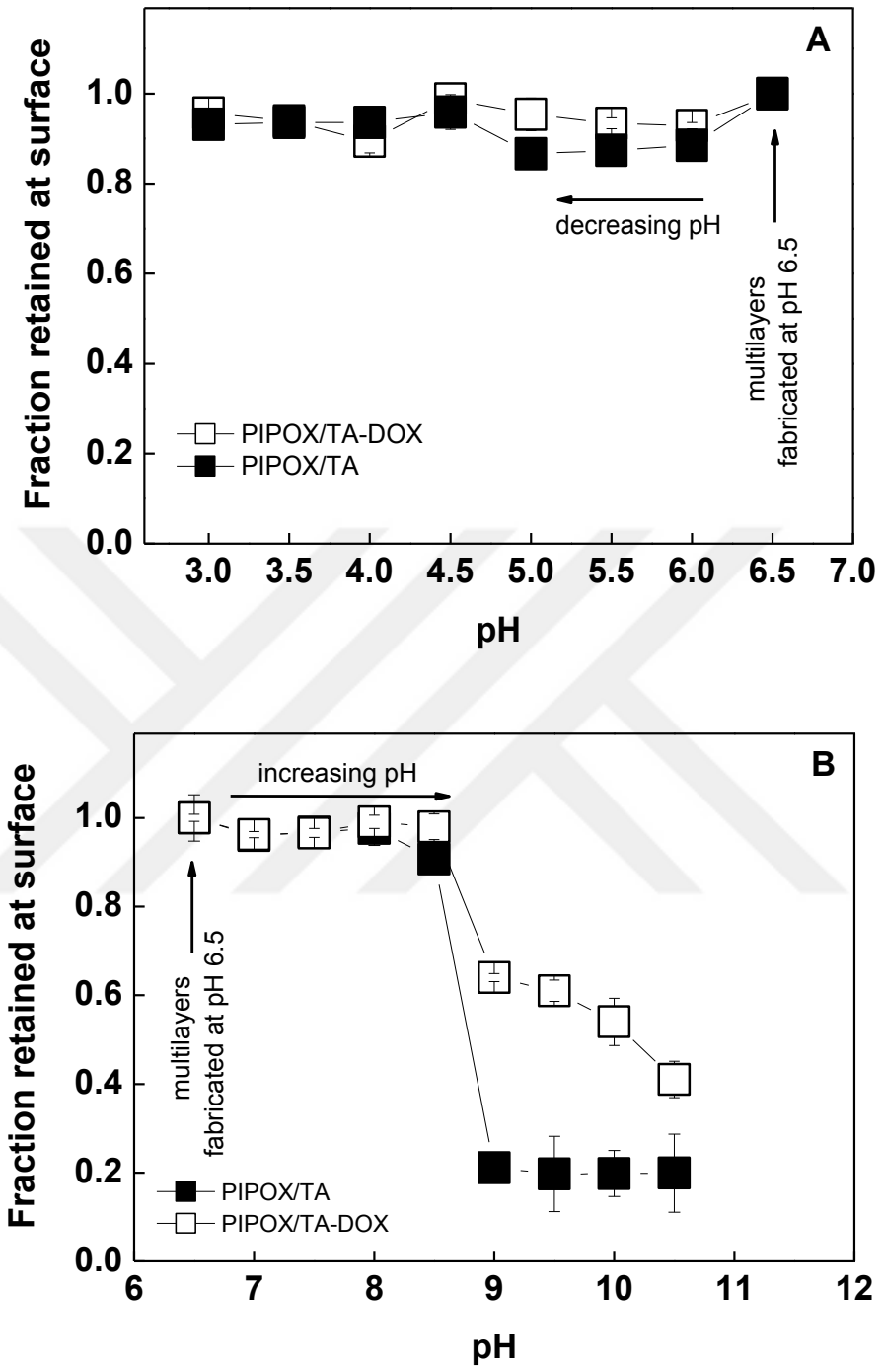


Figure 17. pH stability of multilayers of PIPOX and TA-DOX at acidic (Panel A) and basic (Panel B) pH conditions. pH stability of PIPOX/TA films are plotted for comparison.

3.7. pH stability in PBS Solution

The aim of this study was to develop a pH-responsive model nano-carrier in which the drug release could be triggered by the acidic nature of the tumor environment. For this reason, multilayers should release no or minimal amount of DOX at physiological conditions but release DOX at moderately acidic conditions. To mimic the biological conditions, the stability of DOX containing multilayers in PBS at acidic and neutral conditions were performed prior to release studies.

The thickness increased by ~ 20 % when the films were exposed to PBS solutions at acidic or neutral conditions. The comparison of the fraction retained at the surface of PIPOX/TA-DOX films at low and high ionic strength at acidic and neutral conditions was shown at Figure 18. The difference in the mass adsorbed at the surface between low and high ionic strength conditions can be correlated with the absorption of salt ions from the PBS solution by the multilayers. On the other hand, additional water molecules were expected to be entrapped within the film structure due to enhanced hydrophilicity of the polyelectrolytes when paired with the salt ions [134]. Furthermore, the pH-stability of multilayers in PBS solution were contrasted at 25 °C and 37.5 °C. As seen in Figure 19, there was no a significant difference between the behavior of the films at 25 °C and 37.5 °C when the multilayers were exposed to PBS solutions of different pH only for 30 minutes.

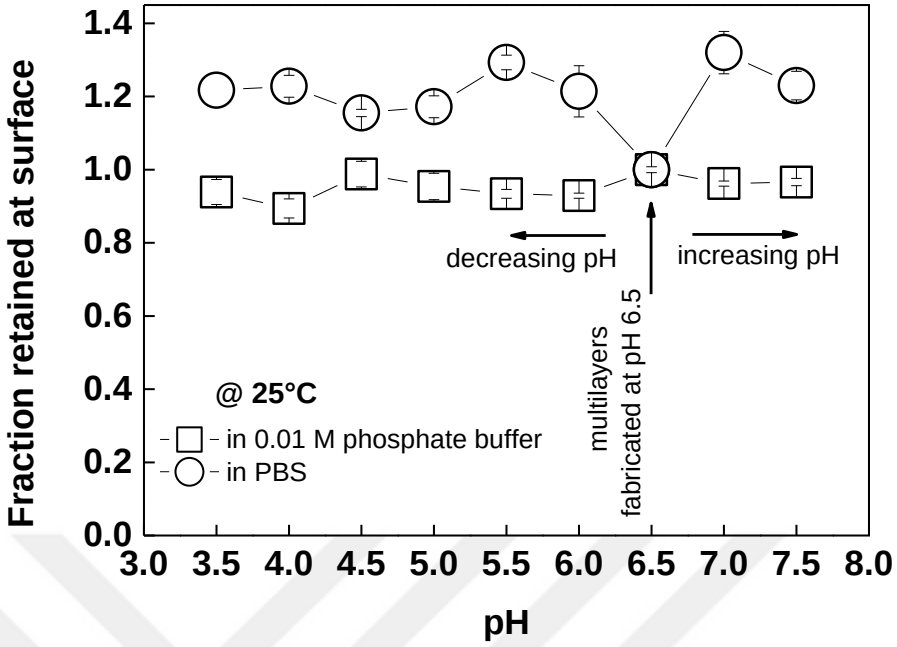


Figure 18. pH-stability of multilayers of PIPOX and TA-DOX at low and high ionic strength at acidic and neutral conditions at 25 °C**.**

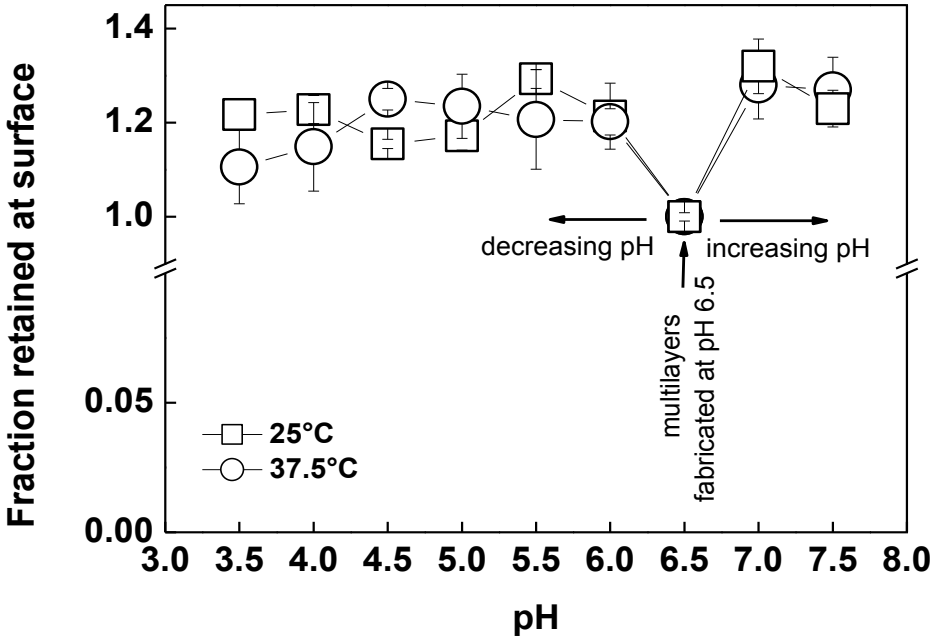


Figure 19. Comparison of the pH-stability of the multilayers in PBS at 25 °C and 37.5 °C.

3.8. Long-term pH-stability in PBS Solution

Prior to release studies, long-term film stability in PBS was also examined. Multilayers were exposed to PBS solution at pH 7.5, pH 6.5 or pH 5.5 at 25 °C or 37.5 °C for 7 hours. Figure 20 shows the fraction retained at surface after exposure to PBS solutions for 7 hours at 25 °C or 37.5 °C. As discussed in the previous section in detail, the mass adsorbed at the surface increased both at 25 °C and 37.5 °C. However different from the results obtained with short term exposure to PBS solutions, long-term exposure to the same conditions at 37.5 °C resulted in greater amount of mass adsorbed at the surface than that at 25 °C. This difference can be correlated with the LCST behavior of PIPOX. PIPOX, which has a cloud point of ~ 40 °C as determined by hydrodynamic size measurements in water using DLS technique represented in Section 3.3, is expected to transform from extended coil to phase separated globular conformation in long term at 37.5 °C. This conformational change possibly resulted in formation of void-like structures which might have retained greater amount of salt ions and water molecules within the multilayers.

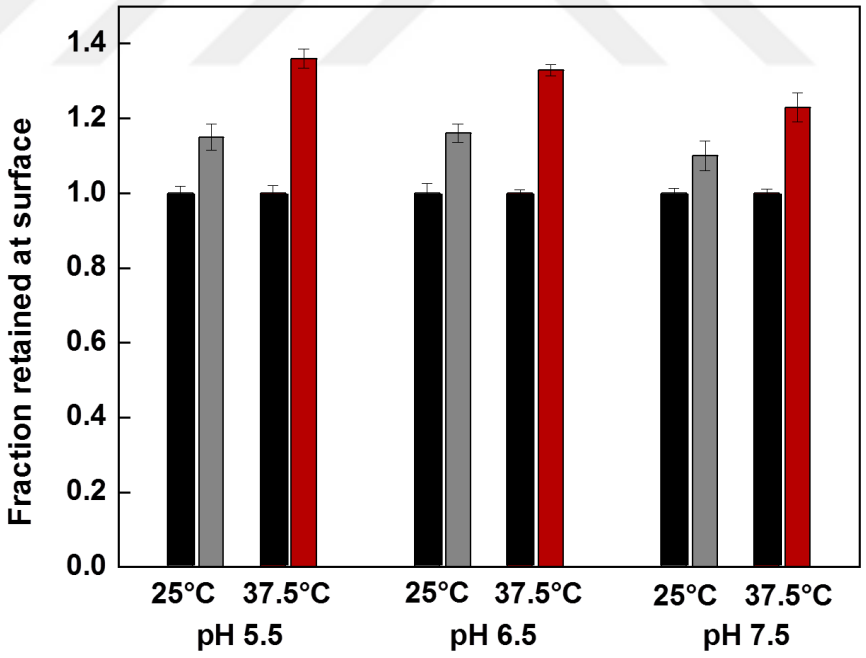


Figure 20. pH-stability of DOX containing multilayers in PBS at pH 5.5, pH 6.5 and pH 7.5 at 25 °C (grey columns) and 37.5 °C (red columns) for seven hours. Black columns represent the multilayers before immersion into PBS solution.

The AFM images of the multilayer films after exposure to 37.5 °C for 7 hours also support the post-assembly morphological changes within the multilayers. As seen in Figure 21, an increase in the lateral dimensions of the surface aggregates was observed when the multilayers were exposed to 37.5 °C both at pH 7.5 and pH 5.5, possibly indicating the swelling of the films due to greater amount of water molecules entrapped within the multilayers.

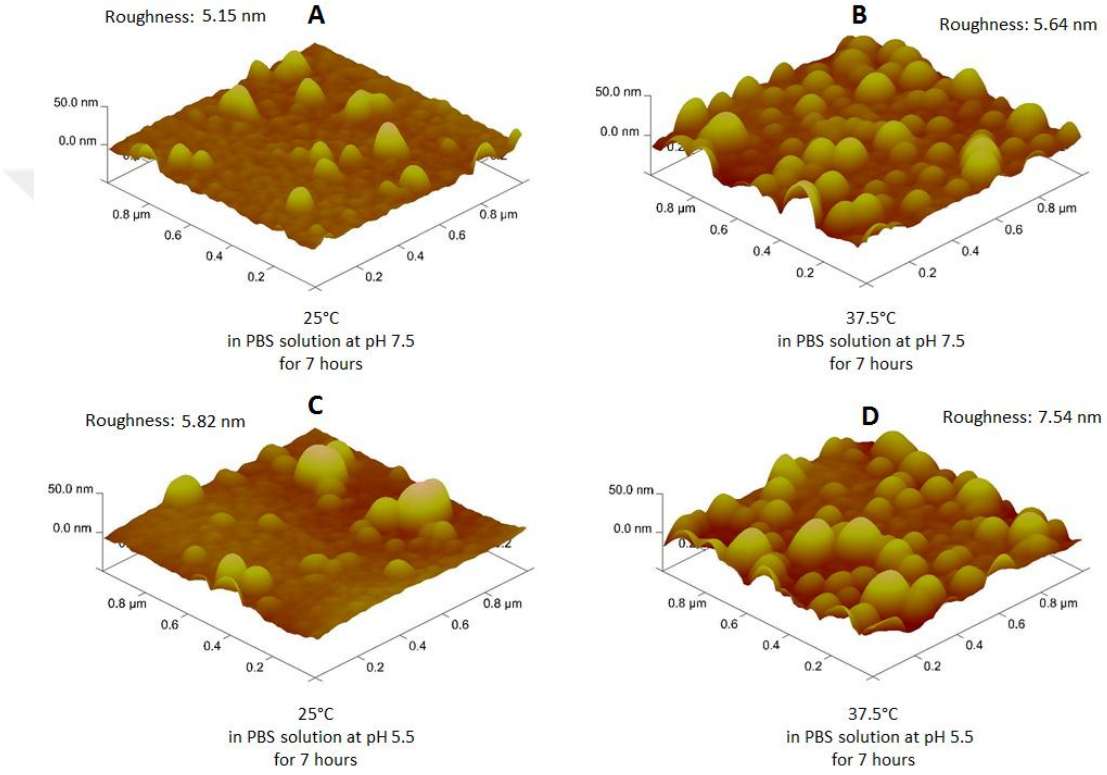


Figure 21. AFM images of multilayers composed of PIPOX and TA-DOX after exposure to PBS solution for 7 hours at pH 7.5 at 25 °C (A); at pH 7.5 at 37.5 °C (B); at pH 5 at 25 °C (C) and at pH 5 at 37.5 °C (D). The average surface roughness values were estimated over 1 µm x 1 µm areas on three different randomly selected places of the sample surface**.**

3.9. DOX Release from the Multilayers

As the aim of study, fabricated multilayer DOX carriers should show no or minimal amount of DOX release at physiological conditions but release DOX at a moderately acidic environment. For this reason, DOX release experiments at pH 7.5 and pH 5.5 in PBS solution were performed. Additionally, as control experiment, the release of DOX was performed at pH 6.5, the pH at which the multilayers were constructed.

Multilayers were exposed to PBS solution at pH 7.5, pH 6.5 or pH 5.5. Samples were taken from the solution every 30 minutes and the fluorescence intensity at 588 nm was followed as a function of time until no significant change in the intensity was recorded. The DOX release at pH 7.5, pH 6.5 and pH 5.5 at 25 °C and 37.5 °C are shown in Figure 22.

The amount of DOX released from the multilayers at pH 7.5 was lower than that at pH 6.5 at both 25 °C and 37.5 °C. This can be explained by further ionization of TA with increasing pH and enhanced electrostatic interactions among TA and DOX molecules, providing minimal amount of DOX release from the surface at pH 7.5. DOX released from the multilayers at pH 6.5 was via self-diffusion mechanism. In contrast to release at pH 7.5, the release at pH 5.5 was significantly higher than that at pH 6.5 at both 25 °C and 37.5 °C. The release at pH 5.5 is based on a pH-responsive mechanism, DOX release is triggered by the protonation of the hydroxyl groups of TA as the acidity increased, resulting in partial loss of electrostatic interactions among TA and DOX molecules and release of DOX from the surface (Fig. 23). The significant difference in the amount of DOX released from the multilayers at pH 6.5 and pH 5.5 assures the pH-responsive release of DOX from the surface. Scheme 4 illustrates the DOX release from the multilayers at pH 7.5 and pH 5.5.

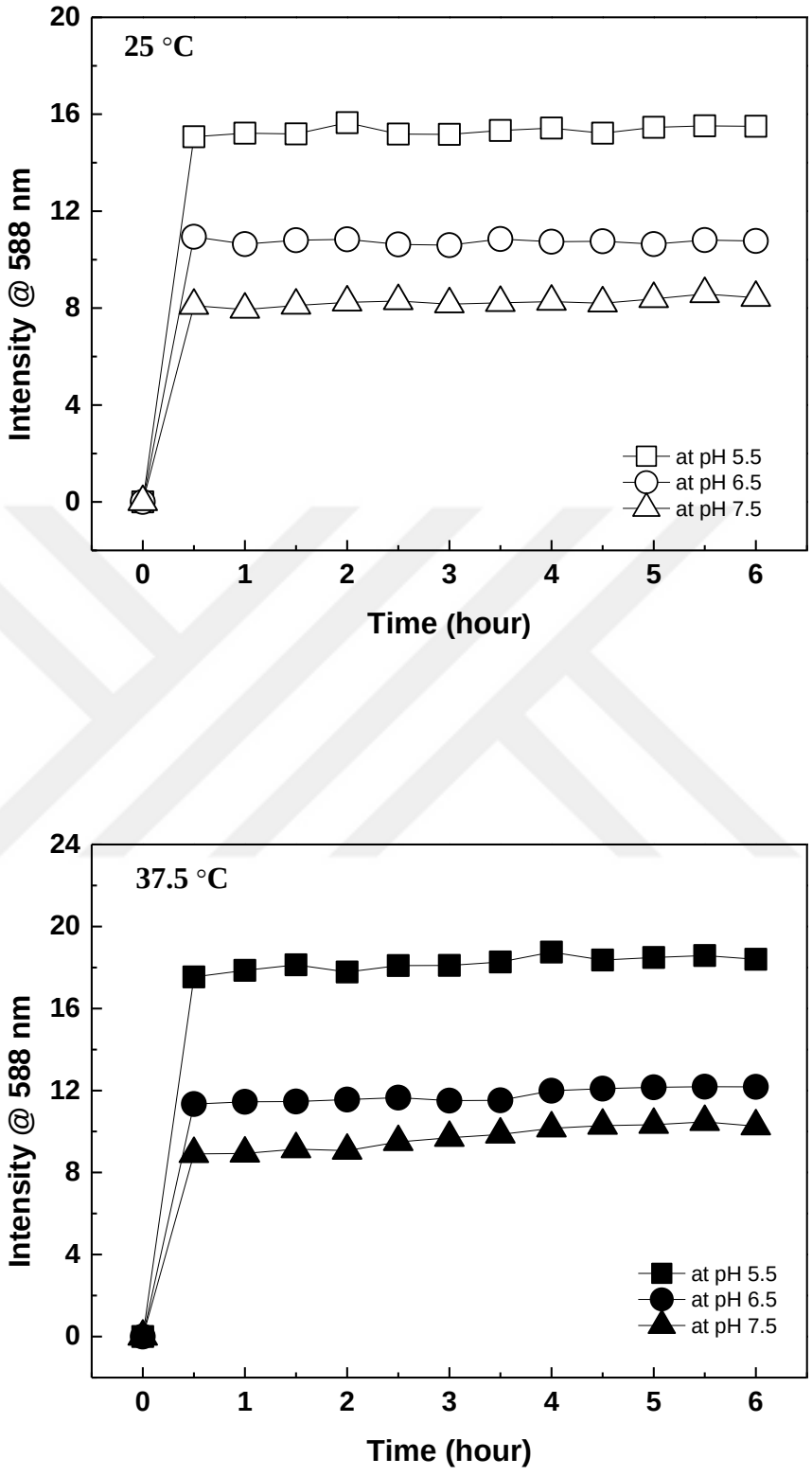
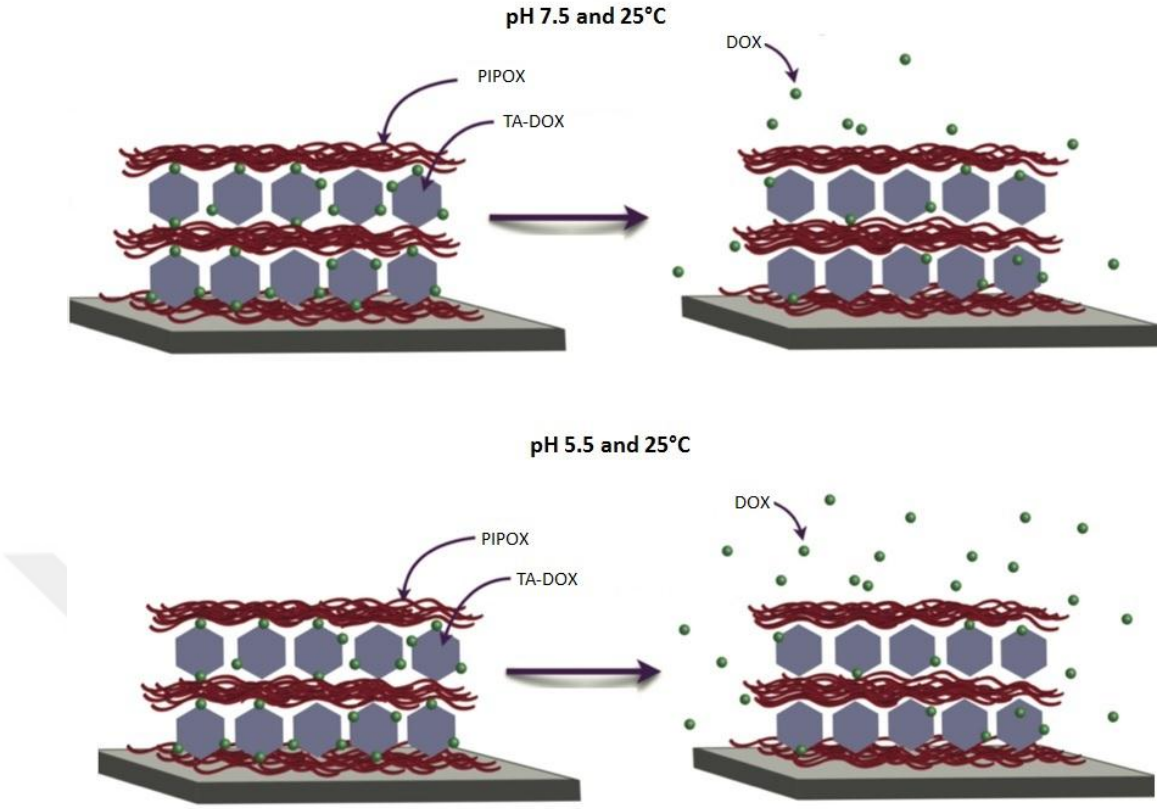


Figure 22. DOX release at pH 5.5, pH 6.5 and pH 7.5 at 25 °C and at 37.5 °C from the multilayers of PIPOX and TA-DOX.



Scheme 4. Schematic representation of DOX release from the multilayers of PIPOX and TA-DOX at pH 7.5 and pH 5.5 at 25 °C.

Of note, the emission spectrum of 10^{-7} M DOX showed that the intensity at 588 nm at pH 7.5 was higher than that pH 6.5 and the intensity at pH 6.5 was higher than that pH 5.5 (intensity at 588 nm at pH 7.5=18.29; intensity at 588 nm at pH 6.5=16.3; intensity at nm at pH 5.5=13.61). Therefore, for further analysis, a calibration curve will be prepared and the amount of DOX release at pH 7.5, pH 6.5 and pH 5.5 will be compared in more detail. Considering the differences in the intensity at 588 nm at different pH values, the difference in the amount of DOX released from the multilayers at pH 7.5 and pH 5.5 should increase. This detailed analysis is expected to demonstrate more clearly the pH-induced release of DOX at moderately acidic conditions.

A comparison in the amount of DOX released at 25 °C and 37.5 °C showed that DOX release increased at 37.5 °C. For example, as seen in Figure 23, the amount of DOX released at pH 5.5 at 37.5 °C was ~ 20 % higher than that at pH 5.5 at 25 °C. As discussed in Section 3.3, the cloud point of PIPOX was found to be ~ 40 °C in water. PIPOX is expected to adopt globular conformation close to its LCST resulting in formation of void-like structures within the multilayers. These voids possibly facilitated the release of DOX from the surface at 37.5 °C. Although the difference was smaller than that observed at pH 5.5, DOX release at pH 6.5 and pH 7.5 was also higher at 37.5 °C than that at 25 °C (Fig. 22). Scheme 5 illustrates the DOX release at pH 5.5 at 25 °C and 37.5 °C. Sukhishvili and co-workers reported on the enhanced permeability of thymol blue from multilayers containing polymers with a LCST [87]. Erel et al. has also reported on the effect of temperature on the pH-responsive release of pyrene from multilayers containing micelles of poly[2-(*N*-morpholino)ethyl methacrylate-*block*-2-(diisopropylamino) ethyl methacrylate] (PMEMA-*b*-PDPA) with PMEMA coronal chains exhibiting LCST behavior [109].

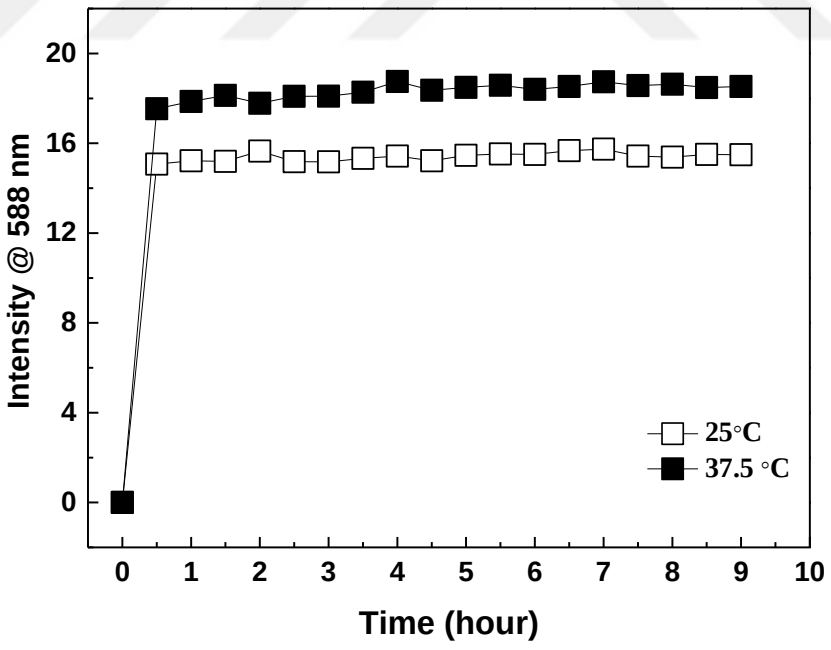
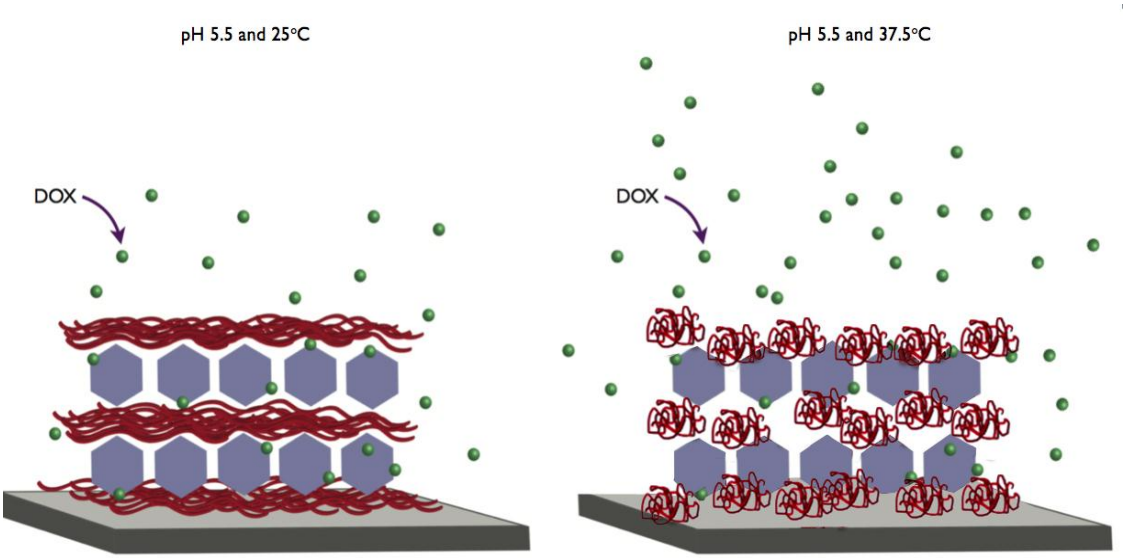


Figure 23. Comparison of the DOX release at 25 °C and 37.5 °C at pH 5.5.



Scheme 5. Schematic illustration of DOX release at 25 °C and 37.5 °C at pH 5.5.

Hammond and co-workers showed pH-triggered release of DOX at acidic conditions from hydrogen-bonded multilayers of block copolymer micelles. In that study, DOX was conjugated to the micellar core blocks via carbamate linkage and DOX release from the surface was induced via carbamate cleavage at acidic conditions [126]. Our study presents a simple approach to load and release DOX from/into the multilayers at moderately acidic conditions. DOX release from hydrogen-bonded multilayers has also been reported by other research groups. Kharlampieva and co-workers used DOX loaded silica nanoparticles as templates for LbL deposition of PVPON and TA at moderately acidic conditions. DOX release from the coatings was then examined at increasing pH values [124]. Sukhishvili and co-workers reported on hydrogen-bonded multilayers of block copolymer micelles with temperature-responsive PNIPAM cores and demonstrated the release of DOX at a temperature below the LCST of PNIPAM [96]

CHAPTER 4

CONCLUSIONS AND OUTLOOK

In this thesis, a simple strategy to incorporate DOX into hydrogen-bonded multilayers which showed minimal amount of DOX release at physiological pH but exhibit pH-induced release of DOX at acidic conditions was demonstrated. LbL films of PIPOX and water soluble complexes of oppositely charged TA and DOX were prepared at pH 6.5. The minimal DOX release at physiological pH can be correlated with the further ionization of TA with increasing pH and enhanced electrostatic interactions among phenolate groups of TA and primary amino groups of DOX. In contrast, pH-induced release at acidic conditions can be correlated with the protonation of TA as the acidity increased and with the loss of electrostatic interactions among DOX and TA. The amount of DOX released from the multilayers increased when the temperature was raised from 25 °C to 37.5 °C. This can be correlated to the conformational changes within the multilayers at a temperature close to LCST of PIPOX. The transition of PIPOX chains from extended coil to globular conformation was expected to result in formation of voids within the multilayers which possibly facilitated the release of DOX from the surface.

Hydrogen-bonded LbL films are of interest for biomedical applications due to lower 1toxicity of neutral polymers than the polycations as well as pH-response of the films at mild pH. However, most of the hydrogen-bonded films are stable at acidic conditions and are capable of releasing positively charged functional molecules with increasing pH. Therefore, it is challenging to prepare hydrogen-bonded multilayers which provide minimal release at physiological conditions but trigger release of positively charged drug molecules, e.g. DOX, at acidic environment. The only example of hydrogen-bonded multilayers which are stable at physiological pH but

release DOX at acidic conditions was demonstrated by Hammond and co-workers [126]. In that study, DOX was conjugated to micellar core blocks of block copolymer micelles via carbamate linkage which was cleaved at acidic conditions, resulting in release of DOX molecules from the surface.

The work presented in this thesis is a simpler approach to induce DOX release from hydrogen-bonded multilayers at acidic conditions by taking advantage of the high pK_a of TA which ensured incorporation of DOX into the multilayers and at the same time enabled multilayer deposition at near-neutral pH. This study contributes to fundamental understanding of structure-property relationship in stimuli responsive hydrogen-bonded multilayers. Considering the acidic nature of tumor tissues together with the important biological properties of PIPOX and TA, such dual responsive multilayers can be promising for controlled delivery applications from surfaces.

The future work on this study will include the following parts:

1. Biological activity of multilayers will be examined by fabrication of PIPOX/TA-DOX multilayers on silica nanoparticles and exposing them to different cancer cells such as colorectal or cervical cancer cells.

2. Superparamagnetic iron oxide nanoparticles will be incorporated within the multilayers and release of DOX will be induced via application of AC magnetic field.

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APPENDIX

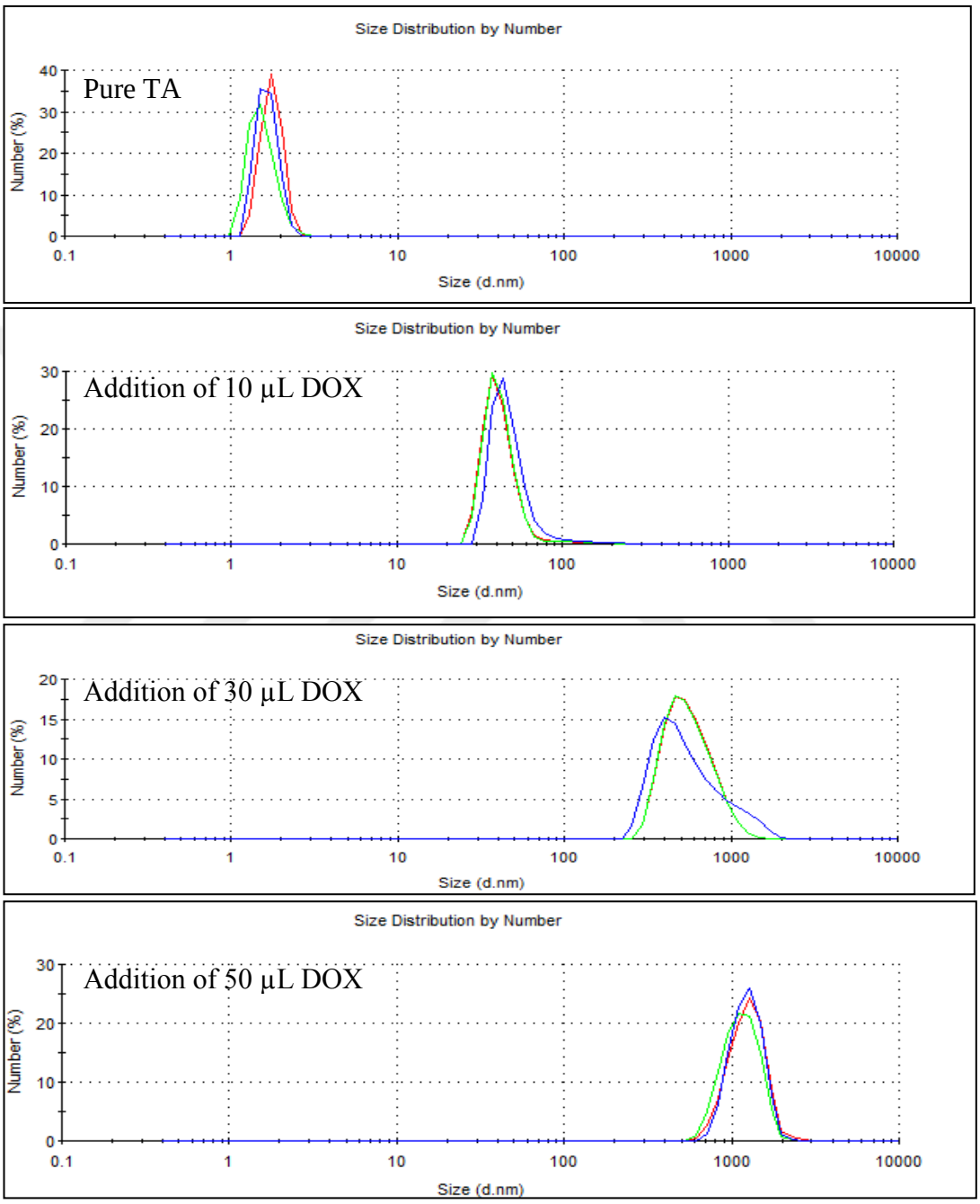

Figure A. 4. The change in the particle size distribution values with increasing amount of DOX in the TA-DOX.

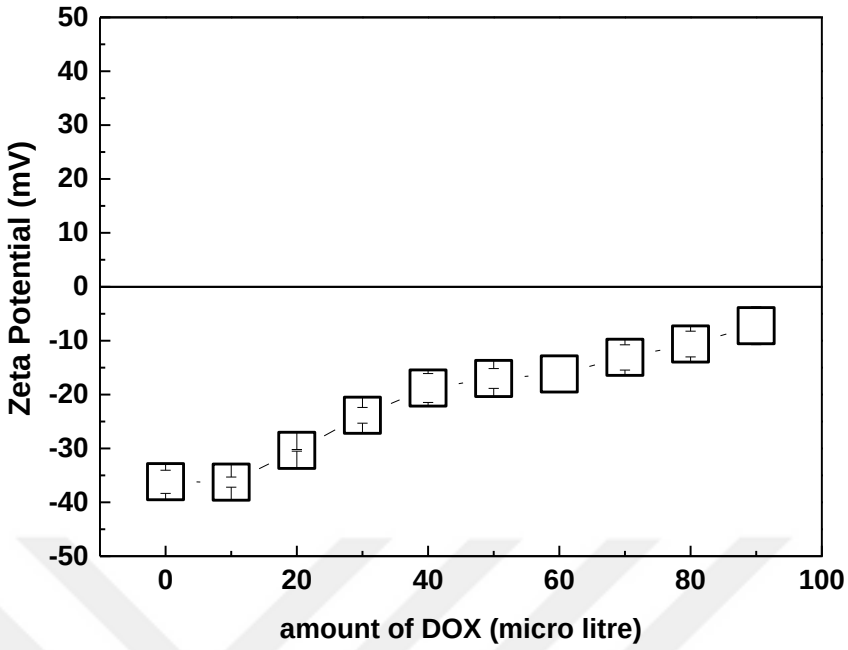

Figure A. 5. The change in zeta-potential values with increasing amount of DOX in the TA-DOX.

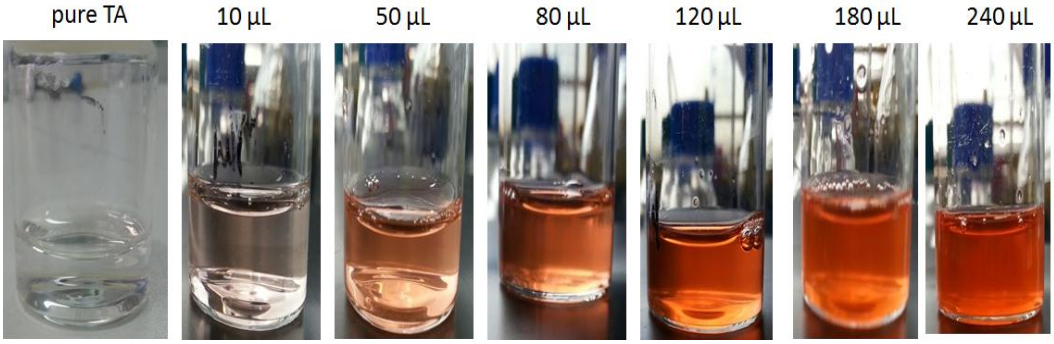

Figure A. 6. The images demonstrate the increase in the turbidity of the solutions with increasing amount of DOX addition.

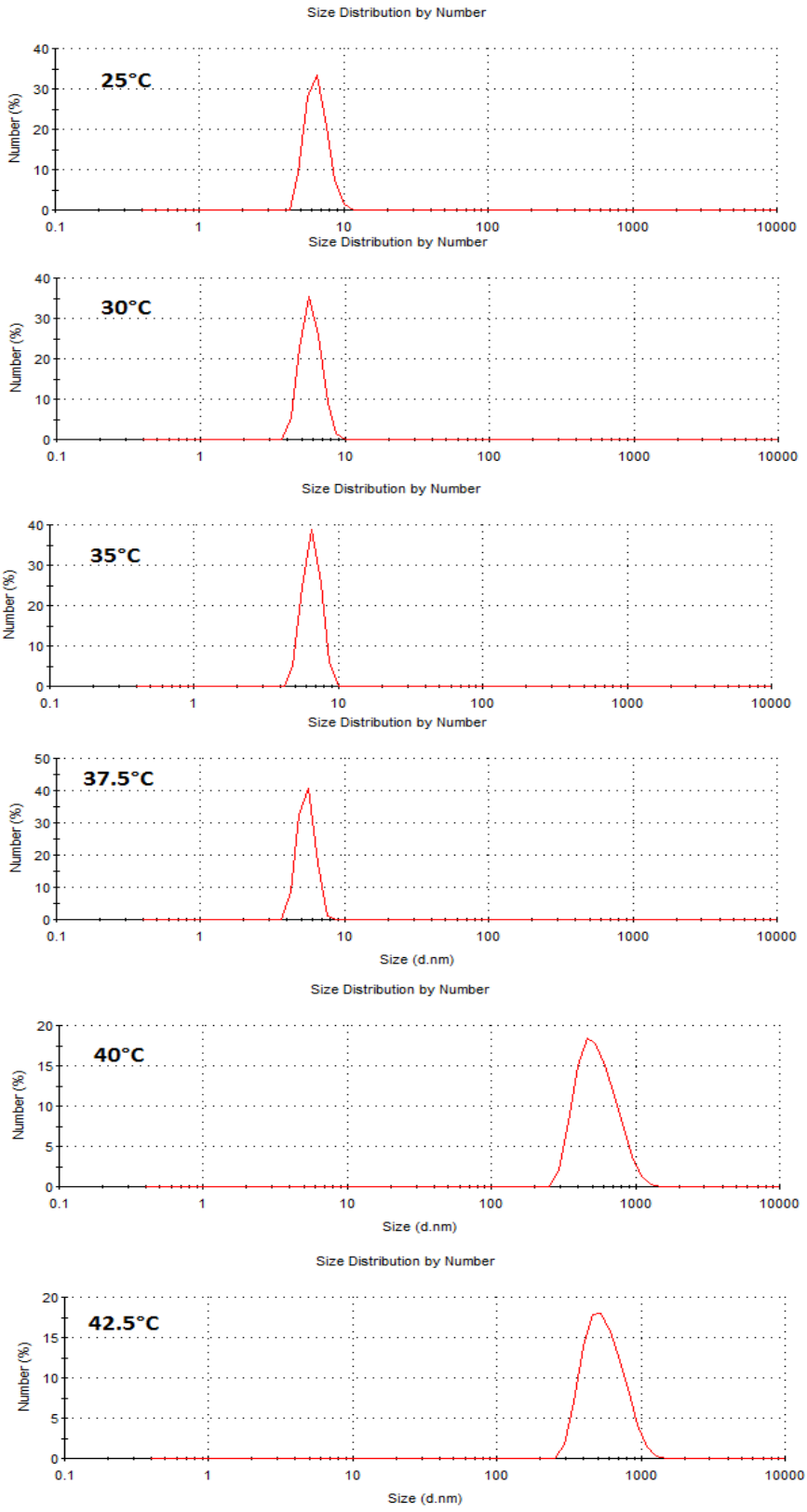

Figure A. 4. The size distribution by number curves of PIPOX at different temperature.