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Layer by layer deposition of chitosan/xanthan thin films studied *in situ* by QCM

S. A. Yaneva[1*], G. E. Alexieva[2], T. S. Velinov[2]

[1]*University of Chemical Technology and Metallurgy, Department of Fundamentals of Chemical Technology, 8, Kliment Ohridksi Blvd, 1756 Sofia, Bulgaria.*

[2]*Sofia University, Department of Solid State Physics and Microelectronics, 5, James Bourchier Blvd, 1164 Sofia, Bulgaria*

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The layer by layer (LbL) technique was used to obtain a layered film consisting of two natural polyelectrolytes: chitosan (CHI) and xanthan (X). The buildup of the film was monitored by a quartz crystal microbalance (QCM). The obtained films showed exponential growth and, besides, some of the accumulated mass was washed away by the buffer. This was attributed to the "free" CHI molecules which penetrate the entire film because of the initial difference of the chemical potential in and out the film. When glutaraldehyde was added as a crosslinking agent substantial differences in the film growth were observed. First, the crosslinked CHI layers become impermeable for "free" molecules and thus each layer interacts only with its two adjacent layers. This leads to a linear growth of the polyelectrolyte films. Second, crosslinking makes the outer layers stable enough to resist to dissolution during deposition of subsequent layers.

Keywords: QCM, chitosan, xanthane, thin films, drugs release.

INTRODUCTION

The Layer-by-layer self-assembly technique is a powerful tool for production of polyelectrolyte multilayer thin films that are widely used in drug delivery [1], biosensors [2], food science [3] and tissue engineering [4]. Among the polyelectrolytes, the natural-based polymers received many applications due to their excellent biological performance, similarity with the extracellular matrix and good chemical flexibility [5]. Most of natural polyelectrolytes are polysaccharides such as hyaluronan, chitosan and xanthan [6-8]. CHI is a biodegradable, non-toxic and biocompatible material which seems to be the only important polysaccharide that charge positively in a suitable water based solution. Due to its porous structure and amine groups, chitosan is well-suited for attachment of biomolecules and the chitosan films are commonly used to elaborate selective coatings for biosensors [9]. Another successful medical application of CHI is its use for delivery container where various vesicles could be filled with drugs for controlled release after its degradation under low pH [9].

Many of CHI properties are explained by the presence of primary amine groups in its molecule. At pH lower than 6.5, this amines are positively charged and chitosan is soluble. At pH higher than 6.5 the amines are more deprotonated, and CHI becomes insoluble [9]. Thus, the deposited CHI films must be kept at a pH above 6.5 to prevent them from dissolving. The ionic strength and pH of the solution also influence the charge density of the adsorbed and adsorbing layers which in turn plays an important role in the LBL processing of the weak polyelectrolytes.

Xanthan gum is a commercially important polysaccharide that has various applications in diverse fields of food and pharmaceutical industries. It is a polysaccharide produced as a secondary metabolite in biotechnological fermentation process based on cultivation under aerobic conditions of the microorganism *Xanthomonas campestris*. Many microorganisms are able to metabolize high molecular polysaccharides, but X gum is the only bacterial polysaccharide obtained in larger industrial conditions. X has gelling nature which determines its ability in retarding drug release and potential of entrapping the drug within the gel [10].

Despite of several publications concerning properties of CHI/X gel complexes or focused on the controlled drug release potential of the system [12-14], data about building the CHI/X multilayer system using a QCM microfluidic system appear as not yet reported. Tablets of CHI/X were prepared by direct compression method [15]. The samples were made by mixing solutions of the two electrolytes and then stirring [12, 16]. The precipitates are separated either by ultracentrifugation or by filtration. The same method is used to obtain a gel for enzyme immobilization [17, 18]. The rheological and structural characteristics of a hydrogel spheres

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* To whom all correspondence should be sent:
E-mail: sp_yaneva@uctm.edu

composed of CHI and X, and obtained by adding small drops of xanthan in chitosan solution were studied [19]. The same method was used for studying the influence of the pH solutions and the polymer concentration on the crosslinking between CHI and X, and to obtain a matrix for controlled drug released [8, 14]. A LbL method combined with dipping technique was used to obtain multilayered CHI/X films and the influence of pH and ionic strength on the films formation were studied by optical methods [20].

A film consisting of alternating layers of these two natural polyelectrolytes seems a good candidate for delivery and retarded release of drugs. CHI will be the carrier while the gelling nature of X layers will make the drug diffusion and/or film degradation slower. Luckily, the two polyelectrolytes charge oppositely in water based solutions and thus electrostatic attraction can be used to perform the LbL deposition technique. QCM is a sensitive technique for measuring mass changes on the quartz resonator [11] and when it is incorporated in a microfluidic platform it allows the building of the multilayer system to be monitored in real time. The aim of this research was to study the influence of different factors as pH of the rinsing buffer solution, air flow and crosslinking reagent on the formation of CHI/X structure obtained by LbL technique. The deposition of multilayer structures was performed by using a fluidic system to deliver the solutions to a quartz crystal microbalance.

EXPERIMENTAL

Reagents

Chitosan with medium molecule weight and 75-85% degree of deacetylation, Xanthan (from *Xanthomonas campestris*), NaOH p.a. (99%), Glutaraldehyde, and Acetic acid were obtained from Sigma-Aldrich. $NaH_2PO_4*2H_2O$, p.a. was purchased from Valerus Co.

LbL assembly of the multilayered structure

Solutions of 0.1% w/v chitosan and 0.05 % w/v xanthan in 0.1 M sodium acetate buffer (pH 4.0) were made. In order to remove the excess polymers after each deposition of polysaccharides a rinsing procedure was performed with 0.1 M sodium phosphate buffers having different pH: pH 7.0 and pH 7.5. A 2.5 % v/v glutaraldehyde in 0.1 M phosphate buffer with pH=7.0 was used as a crosslinking reagent. The first deposited layer in our experiments was always CHI. Often, in order to improve the attachment of the CHI molecules to gold, the latter is first modified, for example with cysteamine [21]. To keep things as simple as possible, and also, in view of future medical applications we didn't modify the gold surface. Deionized water was first introduced in the fluidic system until the QCM data stabilized and then the CHI solution was applied directly.

LbL characterization

Scanning electron microscopy

The morphology of the obtained structures was observed with a dual beam scanning electron/ focused ion beam system LYRA I XMU, TESCAN after being coated with a conductive thin gold film.

QCM experiments

A quartz crystal microbalance with a nominal resonance frequency of 10 MHz was used for in situ monitoring the formation of polyelectrolyte layers. Sensor chips from Attana A100 (Attana AB, Sweden) were used. The cell volume was approximately 4 μl and the electrode surface – 16 mm^2. Multilayer films were fabricated on the gold electrode of quartz AT-cut resonators. These resonators oscillate in a thickness-shear mode with a displacement vector parallel to the plate surface. According to Sauerbrey equation [22] there is a linear relationship between the deposited onto the quartz plate mass Δm and the frequency change Δf: $\Delta m = - C\Delta f$ provided that this additional mass is less than 2% of the quartz plate mass. In our case, the constant C is 0.7 ng/Hz. The frequency was recorded every second from the beginning of the introduction of the liquid flow in the cell by a frequency counter and then stored in a computer and displayed on the computer screen for convenience. All of the reported experiments were repeated three times and the repeatability was good.

RESULTS AND DISCUSSION

With regard to future medical applications of the CHI/X films we first varied the pH of the buffer but in all cases it was kept near that of pure water. Then, in another set of experiments, an air was introduced in the fluidic system in order to make the layers more compact and resilient to the shear forces that fluids exert on the attached to the QCM layers. Finally, the influence of a cross-linker, namely, glutaraldehyde, on the film growth was investigated.

The surface of obtained LbL thin films was investigated by SEM (Fig. 1). The SEM images confirmed that there is CHI/X films formed onto the quartz resonators. Fig. 1a shows the surface of a film without added glutaraldehyde while in Fig. 1b a film with glutaraldehyde added as a cross-linker is presented. Both films show uneven surfaces. However, a difference can be seen – the film without glutaraldehyde has a flake like structure whilst the surface of the film in Fig. 1b appears to have continuous and smoother structure. This difference in the structures may be in the origin of the obtained results which are discussed later in the text.

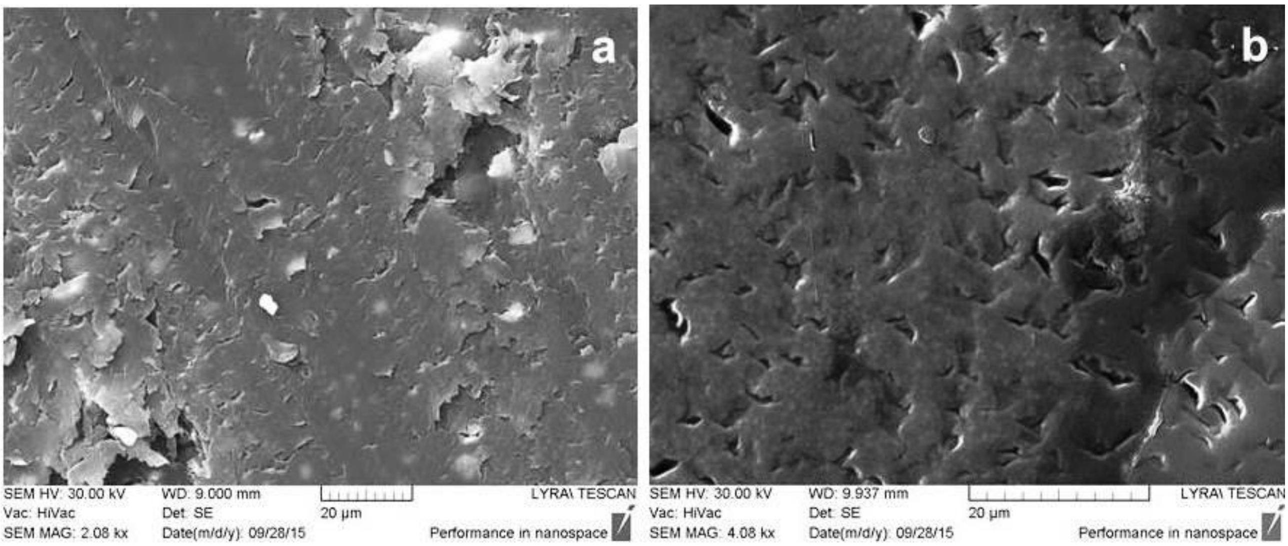

Fig. 1. SEM images of a sample without glutaraldehyde (a) and with glutaraldehyde (b) added as a crosslinking agent.

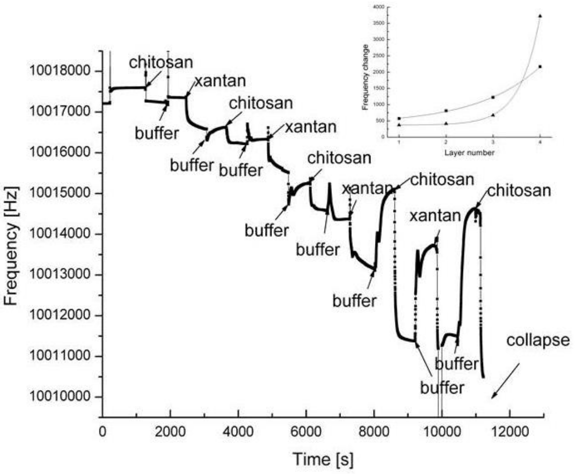

Fig. 2. Raw signal obtained from CHI/X layers at pH=7.0. Arrows show polyelectrolyte or buffer injection. In the inset the frequency shift after each CHI layer (■) and X layer (▲). Lines are an exponential fit to the corresponding symbols.

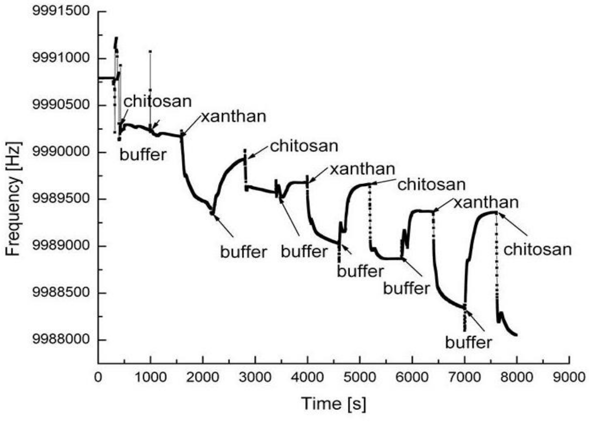

Fig. 3. Raw signal obtained from CHI/X layers at pH=7.5. Arrows show polyelectrolyte or buffer injection.

The QCM results of the use of 0.1 M phosphate rinsing buffers with different pH are presented in Figure 2 and 3. The order of the stages of each cycle of depositions was chitosan - buffer - xanthan - buffer. It is seen that the frequency shifts associated with depositions of polymers increases after each cycle but at the same time more mass is washed away by the phosphate buffer from the layers with the increment of the cycle number.

In the first experiment, (Fig. 2) four complete cycles were performed before the resonator stopped oscillating. The final frequency shift (Δf) was about 6,900 kHz. In the second procedure, shown in Fig. 3, three complete cycles of depositions were realized and then the oscillator stopped. The final frequency shift (Δf) was about 2,250 kHz meaning that the increased pH of the buffer resulted in about three times less polyelectrolyte mass deposited onto the substrate. This could be attributed to a reduction of the positive charges in CHI molecules as a result of the higher alkalinity of the buffer. Furthermore, the higher the effective charge density of X chains is the more the free COO^- groups along the X chains repel each other to make the chains with more extended conformations, which does not favor the X adsorption.

In both experiments, however, the frequency shift was unusually high compared to other structures with polysaccharides. For example the formation of chitosan – hyaluronan was studied by QCM and the frequency change was about 400 Hz at the fundamental frequency after deposition of four bilayers using high molecular weight CHI [23]. This change corresponds to about 1600 Hz in our case because the fundamental frequency of the

QCM we use is 10 MHz compared to 5 MHz [23]. The high frequency change can be attributed to the non-homogeneous structure of our layers which, in case of films without glutaraldehyde has a flake-like structure as can be seen from the scanning electron microscopy image (Fig. 1a). Such a structure favors the entrapment of water because CHI is hygroscopic and forms hydrogen bonds with water. This bonded water becomes a part of the film and contributes to the frequency change. Also, apart from CHI molecules bound electrostatically to the X chains in the process of the film building, another type of CHI chains, called "free chains" can diffuse from the surface into the volume [23, 24]. Their existence is explained by the different initial chemical potential of CHI molecules in the solution and the film. As a result, these molecules diffuse into the film until the chemical potentials in and out the film equalize or the electric potential barrier becomes high enough to prevent "free chains" crossing the interface between the solution and the film. In the next stage, however, when the structure is washed by a buffer these "free" molecules diffuse back in the solution. In principle, same arguments may hold true for the polyanion molecules but in our case the X molecules are much less mobile. Such an explanation of the rapid increase of the mass deposited onto the resonator is also consistent with the results obtained when glutaraldehyde is introduced in the system as a crosslinking agent. These results are discussed later in the paper together with the details on the film growth.

In order to optimize the deposition conditions for the multilayered system and stabilize the polymer layers, in another set of experiments we introduced air into the flow system after each deposition of CHI (results not shown). Usually, when polyelectrolyte films are exposed to air they become more compact and rigid, and we hoped that this would reduce the mass decrement during washing steps. Unfortunately, the results were not very different from the ones shown in Fig.2 and Fig. 3. Crucially, the tendency for washing away some material from the film after rinsing with phosphate buffer remained.

Many polyelectrolyte films can be crosslinked with reagents such as phenols, DTBP or glutaraldehyde making them more chemically resistant and physically harder [9]. In our next experiments we used glutaraldehyde as a crosslinking agent. The aldehydes groups of glutaraldehyde molecule bind covalently to CHI amine groups of different CHI molecules (or the same one), therefore establishing a strong link between them and forming polymer network. In our case, the crosslinking stages were carried out with using a glutaraldehyde water solution. The same buffer but without glutaraldehyde was used for rinsing. The sequence of deposition was as follows: chitosan solution - glutaraldehyde solution - buffer solution - xanthan solution - buffer solution. The time for each stage was 5 min. The results are presented on Fig. 4. The total frequency shift here is about 6.3 kHz, and, as can be seen from Fig. 4, the tendency of washing away the last layer after introducing buffer in the fluidic system isn't observed. We can draw the conclusion that the stable decrease of the resonant frequency of the QCM after the introduction of glutaraldehyde reflects the improvement of the stability of the polymer films.

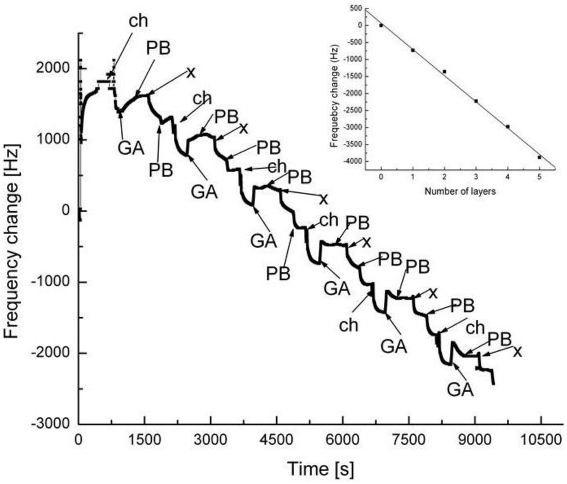

Fig. 4. Raw signal obtained from CHI/X layers at pH=7.01 with glutaraldehyde added after each CHI layer. Arrows show chitosan (ch), xanthan (x) buffer with glutaraldehyde (GA) or pure phosphate buffer (PB) injection. In the inset data for the frequency change after each double layer (CHI/X) deposition are shown together with a linear fit.

Another striking difference is the frequency change after every cycle with and without added glutaraldehyde. In the inset of Fig. 2 the change of the resonant frequency before introducing the buffer into the fluidic system is presented for CHI and X layers separately. It is seen that this change is exponential, presumably reflecting the exponential growth of the layers. Such an exponential growth isn't surprising since most of the polysaccharides films are known to grow exponentially when LbL technique is used [23]. This behavior is attributed to the mobile CHI chains (in this case) that can penetrate not only into the adjacent layers but the whole system. Thus, such type of growth relies on the diffusion of "free" chains in and out of the whole structure during each deposition step. This explanation is consistent with the decrement of the deposited mass onto the quartz resonator during the washing stage. Such a decrement is observed also in [23] for the system chitosan/hyaluronan although on a much smaller scale. In view of the large decrement of the QCM resonant frequency, it seems that in our case some dissolution of the films due to not strong enough electrostatic attraction is also a plausible explanation. The added glutaraldehyde binds the different CHI molecules of one and the same layer and this seems to have two major effects. First, the crosslinked CHI layers become impermeable for “loose” molecules and thus each layer interacts only with its two adjacent layers. This leads to a linear growth of the polyelectrolyte films [23]. In the inset of Fig. 3 this linear growth is emphasized by showing the film thickness after each deposition of a double layer CHI/X and the best linear fit with linear correlation coefficient (r) 0.998. Second, crosslinking makes the outer films stable enough to resist to any possible dissolution.

CONCLUSIONS

In this short communication, we report the results of the study of CHI/X multilayers formation on gold substrates. The recording of the QCM resonant frequency every second allows monitoring of the film formation under different conditions in real time. Without a crosslinking agent the growth of the film is exponential. Besides, after a few layers a part of film is washed away by the buffer. These two phenomena are attributed to the diffusion of “free” CHI molecules throughout the entire film which may be is the reason of partial dissolution of the last layer. However, the addition of glutaraldehyde as a crosslinker after deposition of each CHI layer seems to limit the diffusion of CHI chains to the neighboring layers. This results in linear growth of the film and, more importantly, in the formation of stable films **-** the first step towards a study of potential applications of the system consisting of chitosan / xanthan layers as a carrier of drugs and their controlled release.

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In situ ИЗСЛЕДВАНЕ НА ОТЛАГАНЕТО СЛОЙ ПО СЛОЙ НА ТЪНКИ ПОКРИТИЯ ОТ ХИТОЗАН/КСАНТАН ЧРЕЗ КВАРЦОВО КРИСТАЛНА МИКРОВЕЗНА

Сп. А. Янева[1], Г. Е. Алексиева[2], Цв. С. Велинов[2]

[1]*Химикотехнологичен и металургичен университет, Катедра "Основи на химичната технология", бул. „Св. Климент Охридски, 8, 1756, София, България.*

[2]*Софийски университет, Катедра "Физика на твърдото тяло и микроелектроника", бул. Джеймс Баучер 5, 1164, София, България.*

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(Резюме)

За получаване на многослойни филми от природните полиелектролити хитозан и ксантан е използвана техниката „слой по слой". Нарастването на филма се регистрира чрез кварцово кристална микровезна. При формиране на многослойните структури се наблюдава експоненциално нарастване като при това част от натрупаната маса се отмива от буфера. Това се обяснява с наличието на „свободни" хитозанови молекули, които проникват в целия многослоен филм, в следствие на разликата в химичния потенциал в и извън филма.

При включването на глутаров алдехид като омрежващ агент се наблюдават значителни разлики при нарастването на филма. Първо, омрежените хитозанови слоеве стават непроницаеми за „свободните" молекули. По този начин всеки слой взаимодейства само с двата му съседни слоя, а филмът нараства линейно. Също така омрежването прави филмите достатъчно стабилни, за да не бъдат отмивани при отлагането на следващите слоеве.