ENZYMATIC DEGRADATION OF POLY(3-HYDROXYBUTYRATE-CO-3-HYDROXYVALERATE) NANOPARTICLES LOADED WITH ACTIVE PRINCIPLES

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We describe in this paper the drug release behaviour of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) nanoparticles loaded with anticancer drugs. The present research paper shows the direct activity of two esterase enzymes within macromolecular chains with generation of new products with lower molecular weight. The drug release profile under enzymatic conditions was evaluated by UV-VIS spectroscopy and the new degradation products were characterized by FTIR-ATR.

Keywords: polyester, nanoparticles, anti-tumoural drugs, release profile, enzyme

1. Introduction

Polymeric materials have always played a key role for daily life nanomedicine applications. Thus, polymeric carrying systems such as nanoparticles have had a remarkable progress development in the last decades. Such polymeric nanoparticles are suitable for hosting, protection, transporting and even targeting of desired sites of the body [1-3]. In case of various diseases and affections the administration of required drugs is limited by their poor solubility, low bioavailability, degradation, reduced localization or non-specific toxicity. The clinical therapy of cancer is no exception of these limitations [4-8]. There were developed various polymeric nanoparticles systems in the range size of 50-500 nm for preferentially accumulation in the tumor. The success of the polymeric nanoparticle-based therapy is direct related with nature of the polymer. The polymer nature is given by chemical structure which places it into a more hydrophilic or more hydrophobic side. One of the most important characteristics

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is the ability to generate particles with the required sizes. Another important characteristic is the possibility to develop and formulate stealth nanoparticles [8-11]. The hydrophilic surface designed nanoparticles or surface coated by hydrophilic molecules are expected to provide stealth properties. The nanoparticles with stealth properties could avoid the mononuclear phagocytic system with higher plasma stability and a longer circulation into blood system [12-17]. The chemical structure of the polymer provides also the nature of interaction between polymer-drug-water. The triad interaction shapes the release mechanism of the drug. Being a highly amount component of the body, the water is essential for release of the drugs from nanoparticles. Thus, all the *in vitro* drug release studies are required to be carried out in aqueous environment. The enzymatic activity plays a key role for the release of the drug in the living bodies and *in vitro* tests by acting on specific sites from polymer structure to provide smaller molecular weight chains up to monomers [18, 19]. Polymers such as proteins or polyesters with specific amidic and ester backbone are preferentially attacked by specific enzymes such as protease an esterase. Natural polyester poly(3-hydroxubutyrate-co-3-hydroxyvalerate) (PHBHV) is an extensively used biocompatible and biodegradable polymer for development of wide types of carriers for drug release and other medical applications [20, 21]. We developed in a previous paper some PHBHV/drug systems for the release of hydrophilic drug and hydrophobic drugs [22-23]. Both systems showed release efficiencies below 50%. In the present research we show a further investigation on the drug release mechanism of drug-loaded nanoparticles for two types of binary systems based on PHBHV/ 5-Fluorouracil and PHBHV/silymarin.

2. Experimental

2.1. Materials

Poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV) with a molecular weight of 67000 g/mol and 2 wt.% 3-hydroxyvalerate was provided by Good Fellow. Polyvinyl alcohol (PVA) stabilizer with a molecular weight of 88000 g/mol, 88% hydrolyzed, and chloroform (solvent) were supplied from Sigma Aldrich. Buffer medium of sodium hydroxide and potassium phosphate together with 5-Fluorouracil and silymarin were provided by Sigma Aldrich. Rabbit liver esterase (ERL, Sigma Aldrich) having a molecular weight of approximately 60000 g/mol and an enzymatic activity of 80 units/mg of protein could hydrolyze carboxylic ester to carboxylate and alcohol in the presence of water. Porcine liver esterase (EPL, Sigma Aldrich) having a molecular weight of approximately 168000 g/mol with an enzymatic activity of 18 units/mg of protein could catalyze the conversion of esters to carboxylic acid and alcohol.

2.2. Obtaining of drug-loaded polyester nanoparticles

The two types of PHBHV loaded nanoparticles with 5-FU and silymarin were developed according to literature [22, 23]. PHBHV/silymarin nanoparticles were obtained by nanoprecipitation. Briefly, a solution of 2% PHBHV in chloroform (organic phase) was obtained. The hydrophobic silymarin drug was added into organic phase for dissolution. A stabilizer solution of 2% PVA in water was prepared. The organic phase with loaded silymarin drug was dropwise added into aqueous phase to obtain PHBHV nanoparticles loaded with silymarin [23]. PHBHV/5-fluorouracil nanoparticles were obtained by emulsion-diffusion method. Briefly, a solution of 2% PHBHV in chloroform (organic phase) was obtained and a stabilizer solution of 2% PVA in water (aqueous) was prepared. The more hydrophilic 5-FU drug was added into aqueous phase for dissolution. The organic phase was dropwise added into aqueous phase followed by sonication, water washing and centrifugation to obtain PHBHV/5FU nanoparticles [22].

2.3. Drug release behaviour in the presence of enzymes

Drug release tests for PHBHV/silymarin and PHBHV/5-FU were carried out in enzymatic conditions. PHBHV nanoparticles loaded with silymarin were subjected to enzymatic degradation with ERL and EPL enzymes at 7.45 pH and 37 °C. Silymarin loaded nanoparticles were dispersed in a volume of 5 ml of buffer solution with esterase with a fixed enzymatic activity of 3.6U/ml of solution for EPL and 16U/ml of solution for ERL. 1 mg of EPL and 1 mg of ERL was used according with the enzymatic activity, 18 units/mg protein for EPL and 80 units/mg protein for ERL. PHBHV nanoparticles loaded with 5-FU were also tested for enzymatic degradation with EPL and EPR. Therefore, nanoparticles loaded with each 3.5 mg of 5-FU were used for in vitro release at pH 7.45, 37 °C in EPL conditions and ERL conditions. 5-FU loaded nanoparticles were dispersed in a volume of 5 ml of buffer solution with esterase with a fixed enzymatic activity of 3.6U/ml of solution for EPL and 16U/ml of solution for ERL. There were used 1 mg of EPL and 1 mg of ERL according with the enzymatic activity. Both systems of nanoparticles were dispersed in buffer solution for 2 hours and then subjected to drug release tests with renewal of enzyme activity at every 12 hours. Nanoparticles/drug/enzyme systems were put in cellulose membrane bags (molecular weight cut off, MWCO of about 14 kDa) that were immersed in 35 ml phosphate buffer solution (PBS). Volumes of PBS were collected to evaluate the release of products through cellulose membrane.

2.4. FTIR structural analysis for degradation products

FTIR-ATR spectra of polyvinyl alcohol, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and degradation products were recorded with a Bruker Vertex

70 FT-IR spectrophotometer using Attenuated total reflectance (ATR) accessory. FT-IR spectrophotometer used 32 scans and a resolution of 4 cm^{-1} in mid-IR region 4000-600 cm^{-1}. FTIR spectra were recorded for degradation products generated under enzymatic esterase activity at 1 and 3 days. Separate PHBHV and PVA were immersed into volume of PBS under enzymatic activity. The mixtures were placed into cellulose membrane bags which were added in flasks with more PBS. After 1 day of test there were collected PBS volumes which contained degradation products passed through membrane wall. The collected PBS was dried in order to recuperate the degradation products. The degradation products at 3 days were recuperated by the same method.

3. Results and discussions

3.1. Drug release profile via degradation with esterase

The drug loading mechanism and efficiency are different for each binary system. The drug loading efficiency of the silymarin loaded nanoparticles was 61.1% and the release efficiency was about 38% [23]. For the second system, the 5-FU loading efficiency was 35% and the release efficiency was about 20.1% [22]. These results were obtained at pH 7.45 and temperature of 37 °C. Drug release profile by enzymatic degradation showed a different release mechanism with higher release efficiency.

PHBHV/5-Fluorouracil system

5-FU release profile by enzymatic degradation (Fig. 1) assumes a release efficiency of 81% for EPL and 67% for ERL. Both enzymes induce a release time of about 38 hours with close release rate profile. The normal *in vitro* study revealed a release profile with a 20% release efficiency from the total amount of encapsulated 5-FU during the first 42 hours. Therefore, the drug release profile showed a faster release for the first 6 h and a slower release for the next 36 hours [23]. In case of enzymatic degradation, the 5-FU profile revealed a fast release during the first 4 hours and a slower release for the next 34 hours. The different time-dependent release profile can be explained by the drug-loaded into different areas of the nanoparticles such as PVA shell and PHBHV core. The hydrophilic 5-FU allows higher interactions and loading within stabilizer shell. Thus, the 5-FU entrapped into hydrophilic shell is expected to exhibit a faster release with respect to the core loaded 5-FU. The 5-FU release profile by enzymatic degradation is also similar to the release profile by normal *in vitro* conditions. The results reveal the same fast release for the first hours for shell drug loaded and slow longer release for core drug loaded but with higher efficiency for each step. These results could sustain the fact that the esterase enzymes act for specific ester sites from PHBHV backbone but also on PVA shell.

PHBHV/Silymarin system

Silymarin release profile (Fig. 1) shows a release efficiency of 77% for EPL and 81% for ERL.

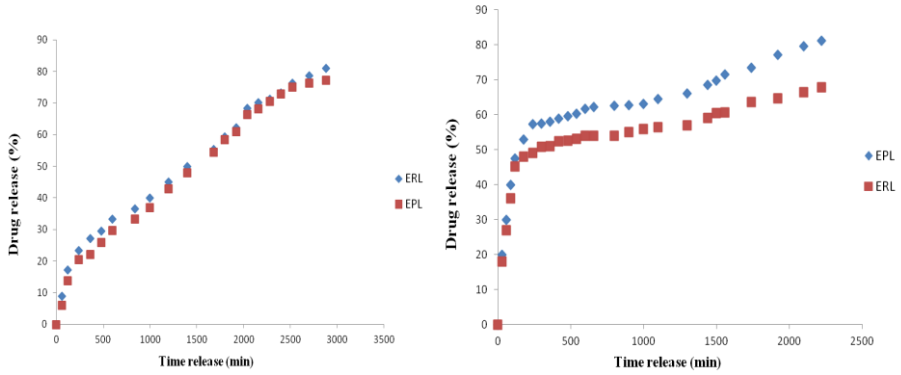

Fig. 1. Drug release profile for PHBHV/silymarin (left) and PHBHV/5-FU (right) in enzymatic degradation

Enzymes induce a release time of about 48h with a close release rate profile but longer as compared to PHBHV/5-FU system. The release profile for both enzymes shows a fast release step for about 2 hours followed by a slower sustained release for the next 46 hours. The normal *in vitro* study revealed a total release investigation lasted 48 hours and supposes a faster release for the first 2.5 hours reaching about 32% efficiency and slower release for the next 20 hours reaching a maximum 38%. The enzymatic degradation study results reveal a different release profile as compared to normal *in vitro* conditions that exhibited a fast release for a longer period. Furthermore, the release time by enzymatic degradation is almost 2.5 times longer than normal *in vitro* conditions. The hydrophobic silymarin allows a higher interaction and compatibility with highly hydrophobic PHBHV core within a higher entrapping as compared to PVA shell. Therefore, the results show an enzymatic activity on PVA shell similar to PHBHV/5-FU system. Reaching highly hydrophobic PHBHV core by the enzyme takes places only after the degradation of hydrophilic PVA shell. The higher release efficiency can be attributed to PHBHV core degradation by leakage of ester backbone bonds and releasing of shorter fragment chains together with silymarin. The slower rate release with respect to *in vitro* normal conditions could be attributed to silymarin-enzyme interaction that can delay the release of the drug.

3.2. FTIR structural analysis for degradation products

FTIR analysis for PVA degradation

The physico-chemical analysis of synthetic PVA polymer and degradation products was carried out to show the enzyme activity mechanism within polymeric backbone. FTIR spectrum of crude PVA clearly shows the major peaks associated to its chemical structure. Fig. 3 depicts wide intense peak at 3330 cm^{-1} specific to O-H stretching bonds from PVA side chains. The peak at 2939 cm^{-1} is associated to asymmetric and symmetric stretching of C-H bonds from the chemical backbone and side chains. The intense peak at 1733 cm^{-1} is specific to C=O from un-hydrolyzed acetate side chains. The peak at 1451 cm^{-1} is specific to C-H bending from polymer backbone. Peak at 1393 cm^{-1} is related with bending of CH_3 within the side chains or ends of macromolecular chains and peak at 1250 cm^{-1} is atributed to the structural backbone. The intense peak at 1089 cm^{-1} covers the specific C-O and C-O-C stretching bonds. The peaks lower than 1000 cm^{-1} are associated with motion of the carbon skeleton [24-26]. The FTIR spectra of degradation products generated after 1 day reveals (Fig.1) the specific peaks of pure PVA and an extra peak at 1568 cm^{-1} which can be associated with C=C bond from chain ends. The shifting of C=C bond lower than normal 1600 cm^{-1} can be due to direct bond with acetate group. Also, the peak at 1250 cm^{-1} is shifted at 1239 cm^{-1} with a reduced intensity reported at 1089 cm^{-1} peak. This fact can be explained by fragmentation of macromolecular chains with reduction of molecular weight. The breaking points of macromolecular can be the carbon bound with side acetate with generation of ends carbon double bonds on new fragmentation chains. Furthermore, the peak at 1393 cm^{-1} reduced intensity and was covered by the one at 1451 cm^{-1} suggesting the reduction of CH_3 methyl groups. The FTIR spectra of degradation products generated after 3 days reveals a wide reduced peak around 3300 cm^{-1} suggesting the high reduction of O-H bonds. The area related with C-H stretching highlights split intense peaks at 2956 cm^{-1} assigned to CH_3 asymmetric stretching and another intense peak at 2918 cm^{-1} assigned to CH_2 asymmetric stretching from backbone chains. The last two peaks at 2871 cm^{-1} and 2850 cm^{-1} can be assigned to CH_3 symmetric stretching, respectively CH_2 symmetric stretching from backbone chains. The peak at 1735 cm^{-1} specific for C=O with a lower intensity suggests the high reduction of this group from acetate side chains. Also, the peak at 1250 cm^{-1} reduced intensity being probably covered by the broader peak at 1089 cm^{-1} suggesting further chains fragmentation and appearing of new ether C-O-C bonds. The FTIR spectra for 3-days degradation products suggest the formation of C-O-C ether bonds with generation of acetal bridges from acetate groups. These structures also explain the high reducing of O-H and C=O bonds. The split and increased intensity of the C-H stretching area can be explained by the fragmentation of macromolecular chains with reducing of

molecular weight. The water solubility of degradation products is low even though the chains were fragmented, probably due to the formation of intermolecular acetal bridges with obtaining of partially crosslinked structures.

FTIR analysis for PHBHV degradation process

The physico-chemical analysis of PHBHV and degradation products by esterase activity were also carried out to reveal the new generated chemical structures. In this case the used esterase enzyme was specific for cleavage of polyester backbone comparing with non-specific activity on PVA. Also, the esterase enzymatic activity on PHBHV was evaluated after 1 and 3 days. The FTIR spectra of pure polyester PHBHV in Fig. 2:

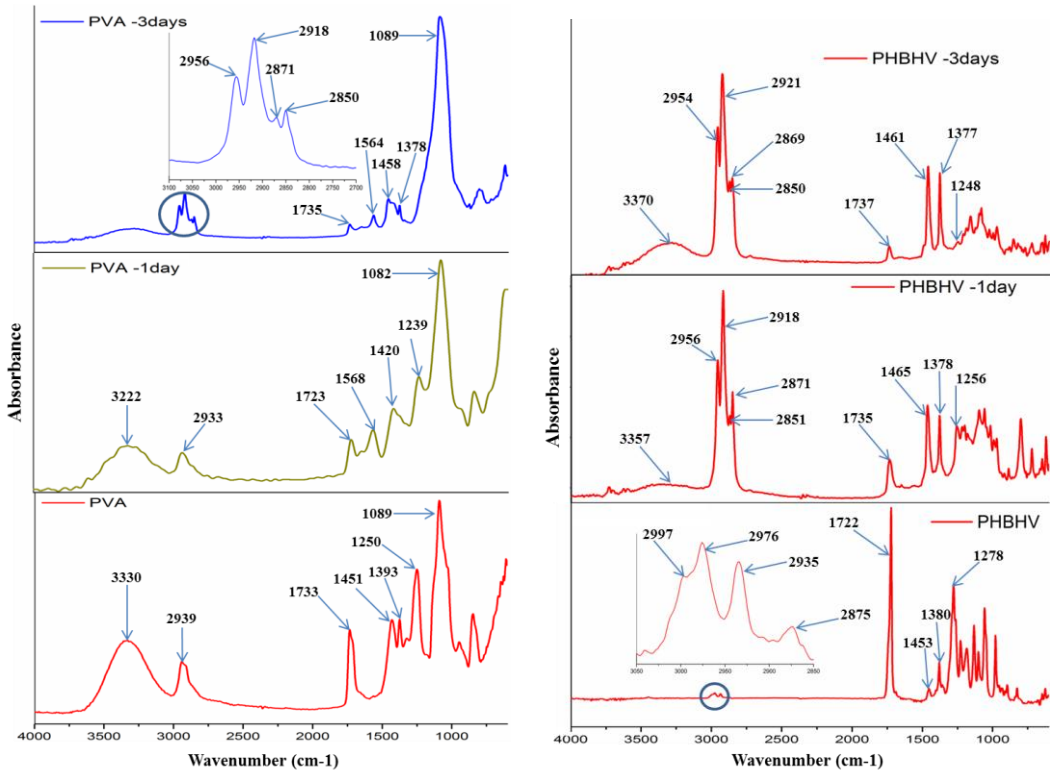

Fig.2. FTIR spectra for degradation products resulted from PVA (left) and PHBHV (right)

shows the characteristic peaks starting from range 2800 cm-1 to 3000 cm-1 specific for C-H motion. The O-H stretching vibration from hydroxyl and carboxyl groups present at the end of macromolecular chains is so weak and its contribution to the spectra can be considered negligible. In this range the spectra reveal four peaks at 2997 cm^{-1}, 2976 cm^{-1}, 2935 cm^{-1}, 2875 cm^{-1} characteristic to CH_3 asymmetric stretching, CH_2 asymmetric stretching, CH_3 symmetric stretching

and, CH_3 symmetric stretching, respectively. The next major peak at 1722 cm^{-1} corresponds to C=O from ester bond. The peaks in the range 1500 – 800 cm^{-1} are specific for CH_3, and CH_2 bending vibration and C-O-C, C-O and C-C stretching vibration with a high overlapping. The peaks at 1453 cm^{-1} and 1380 cm^{-1} are assigned to CH_3 and CH_2 asymmetric bending vibration, and CH_3 and CH_2 symmetric bending vibration, respectively. The next peaks at 1278 cm^{-1} and 1228 cm^{-1} are related to C-O-C stretching vibration in ester. The peaks at 1132 cm^{-1}, 1101 cm^{-1} and 1057 cm^{-1} are related to C-O vibration in ester and C-C stretching [27-30]. The spectra for PHBHV products generated after 1 day reveals (Fig. 2) the specific peaks of pure PHBHV in the range 2800 - 3000 cm^{-1} specific for C-H motion with a higher intensity and shifted to lower wavenumber.

Furthermore, a new broad peak appears at 3357 cm^{-1} due to hydroxyl and carboxyl groups present at the end of chains. In the case of chain cleavage will be generated more chains with lower molecular weight so there are more available ends hydroxyl and carboxyl groups. The peak at 1722 cm^{-1} corresponded to C=O in ester bond decreased intensity and shifted to higher wavenumber. The peak decreasing intensity suggests that the contribution of ester bond in backbone in spectra is lower probably due to chain cleavage. The peak shifting to higher wavenumber also suggests the chain cleavage by decreasing of crystalline degree and generation of new unorganized amorphous phase and also the contribution of C=O in new carboxyl groups formed [27]. The spectrum for degradation products at 3 days is similar to the spectrum at 1 day on the 3000-2800 cm^{-1} range. The peak corresponded to O-H stretching shifted at 3367 cm^{-1} and increased intensity due to contribution of more hydroxyl and carboxyl groups available by chains cleavage. The peaks attributed to C=O at 1737 cm^{-1} and C-O-C in ester bond also decreased intensity due to chain cleavage.

The possible generated structures of the degradation products for both polymers are shown in Figs. 3 and 4.

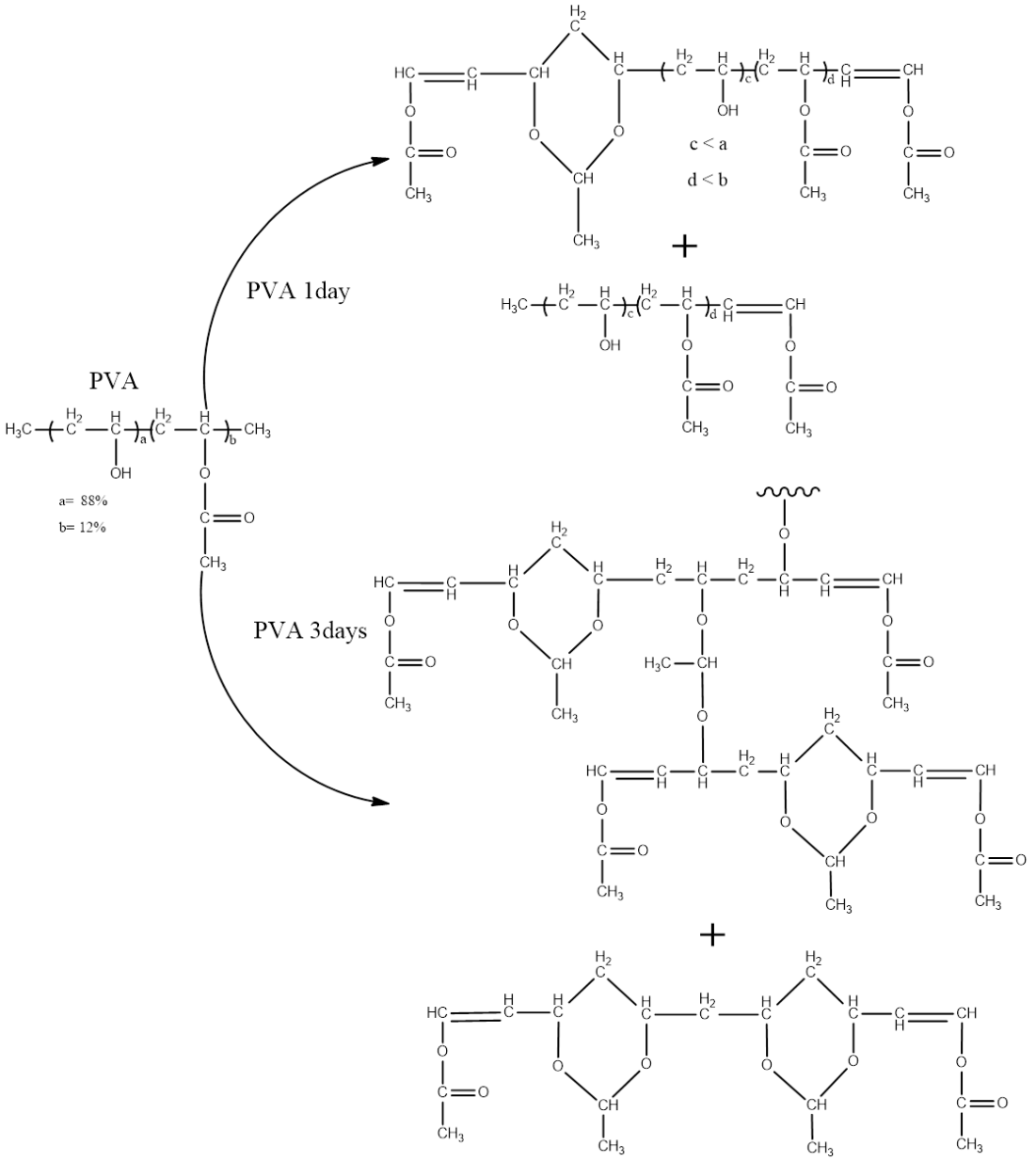

Fig.3. Possible chemical structures for degradation products resulted from PVA at 1day and 3 days (enzymatic degradation)

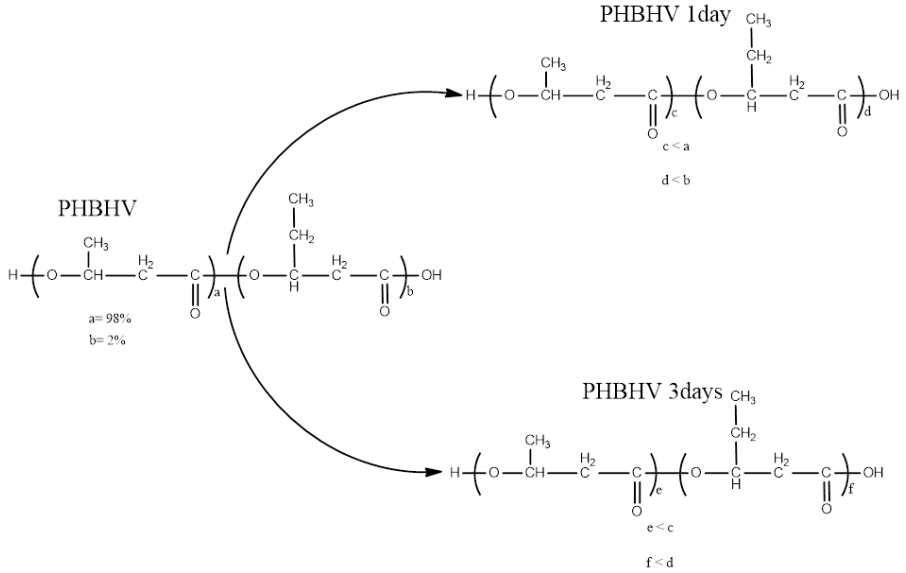

Fig.4. Possible chemical structures for degradation products resulted from PHBHV at 1day and 3 days (enzymatic degradation)

4. Conclusions

The aim of the paper was to demonstrate the enzymatic activity on PHBHV polymeric chains. For this purpose, we used specific esterase enzyme capable to act on the ester bonds. Esterases were from animal origin for higher similarity with the living body conditions. FTIR analysis revealed the cleavage of the molecular chain backbone and side chains with generation of new structures with lower molecular weight. The decrease of molecular weight was sustained also by the fact that starting from 88 kDa (PVA) and 67 kDa (PHBHV) the degradation products were released through cellulose membrane wall with MWCO of 14kDa. The membrane allowed only the passage of structures with this molecular weight or lower. The enzymatic degradation of PVA generated shorter chains with ends double bonds and side acetal bridges by a new binding of acetate with side oxygen. The enzymatic degradation of PHBHV generated shorter hydroxyl-acids chains with more terminal hydroxyl and acidic groups available. The possible chemical structures of degradation products for both polymers were evidenced in the manuscript. The chains cleavage led to smaller molecular weight fragments and higher release efficiency of the drugs with respect to *in vitro* normal condition release.

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