EFFECT OF POLYLACTIDE MOLECULAR WEIGHT ON DOXORUBICIN AND TEMOZOLOMIDE RELEASE FROM CHITOSAN-GRAFTED POLYLACTIDE NANOPARTICLES

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Abstract

The purpose of this study was to examine low molecular weight chitosan-grafted polylactide nanoparticles as a drug delivery system for doxorubicin and temozolomide. We investigated the effect of polylactide molecular weight on the release kinetic and encapsulation efficiency of both drugs. Polylactide (10 kDa and 60 kDa) has been linked to chitosan via coupling reaction. Chitosan-grafted-polylactide nanoparticles were prepared via ionotropic gelation method with tripolyphosphate as a linking agent. Results such as size (150-350 nm), ζ-potential (+25-35 mV), efficient encapsulation and prolonged release kinetic suggested that chitosan-grafted-poly lactic acid could be a great system for co-delivery of doxorubicin and temozolomide in Glioblastoma Multiforme treatment.

Keywords: Chitosan, Polylactide, Doxorubicin, Temozolomide, *in vitro* release

1. INTRODUCTION

Nanoparticles have become an important area of research in the field of drug delivery, a wide number of polymeric nanoparticles have been synthesized and studied as promising delivery systems for improvement of drug delivery efficiency and reduction of side-effects of drugs in the past few years [1]. CS is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine. CS-based materials have drawn considerable attention in view of its excellent biocompatibility, biodegradability, and reactive surface functional groups [2]. PLA is a linear synthetic and biodegradable polymer with good mechanical properties and it has been used widely in drug delivery [3]. In the present study, CS based nanoparticles were prepared by grafting PLA on CS backbone to obtain an amphiphilic system, CS-g-PLA, to serve as a drug carrier for prolonged drug release. CS-g-PLA nanoparticles were prepared via ionotropic gelation for encapsulation and co-encapsulation of two anticancer drugs, Doxorubicin (DOX) and Temozolomide (TMZ). Doxorubicin is commonly used to treat different forms of cancer such as breast, stomach, lung, brain and others, while temozolomide is particularly indicated for treatment of Glioblastoma Multiforme, one of the most aggressive malignant primary brain tumor involving glial cells. A co-therapy of DOX and TMZ has shown great results for Glioblastoma Multiforme therapy in various studies in the last years [4]. CS-g-PLA was obtained by coupling reaction between PLA carboxylic group and CS amino group using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as a condensing agent and N-Hydroxy Succinimide (NHS). For this purpose PLA with various molecular weights (10 kDa, synthesized from L-lactic acid via polycondensation reaction [5] and 60 kDa) was used. The chemical structure and bonding were studied by nuclear magnetic resonance (^{1}H-NMR) and FTIR-ATR spectroscopy.

2. MATERIALS AND METHODS

2.1 Materials

Low molecular weight chitosan (D.D 75-85 %), Polylactide (60 kDa), Sodium tripolyphospate-Technical grade 85 %; N-HydroxySuccinimide; Temozolomide-Technical grade >98 %; Doxorubicin hydrochloride

98.0-102.0%, N-(3-Dimethylaminopropyl)-N'-Ethylcarbodiimide hydrochloride, commercial grade, powder, Stannous 2-ethylhexanoate ($Sn(Oct)_2$) (95%) and N,N-Diethylformamide 99% were supplied by Sigma Aldrich. L-Lactic Acid $C_3H_6O_3$, 80% water solution, optical rotation 10.6` was purchased from Lachner Neratovice, Czech Republic. The solvents acetone C_3H_6O and ethanol C_2H_6O were bought from IPL Lukes, Uhersky Brod, Czech Republic. Chloroform $CHCl_3$ (HPLC grade) was purchased from Chromspec, Brno, Czech Republic.

2.2 Synthesis of PLA and chitosan-grafted-polylactide

Low molecular weight polylactide (10 kDa) was synthetized according to the procedure described by P.Kucharczyk et al.[6] and the amphiphilic polymer chitosan grafted polylactide was prepared as described by J. Li et al.[7]

2.3 CS-g-PLA and CS-g-PLA-drugs preparation

CS-g-PLA nanoparticles with and without drugs were prepared based on the ionic-linking with TPP according to the procedure described by Wu et al [8]. CS-g-PLA was dissolved in an aqueous solution of acetic acid (pH 3.5) to form a 1mg/ml solution. TPP was dissolved in deionized water at concentration of 1mg/ml and 5 ml of the solution was added drop-wise to 5 ml CS-g-PLA solution under stirring and slight heating. An aliquot (2ml) was withdrawn for subsequent DLS analysis, and the remaining was freeze dried. The same procedure was followed for synthetized CS-g-PLA loaded with DOX and TMZ separately and together. DOX (1mg/ml) was dissolved in CH_3COOH solution (pH 3.5) instead of TMZ (1mg/ml) in ethanol. Solution containing drugs and TPP was added to CS-g-PLA solution.

2.4 TEM and Dynamic Light Scattering analysis

Transmission electron micrographs were taken by Tecnai G2 Spirit (FEI). Particle size and ζ-potential of all formulation were analyzed through DLS with Nano ZS Malvern. The analysis were performed in triplicate at various temperatures (4, 25 and 37 ˚C) and pH 3.5

2.5 Swelling Index (SI):

6.5 mg of lyophilized powder sample was immersed in 1.5 ml of human serum (HS) for 24h at 37˚ C until a swollen equilibrium was achieved. The swollen samples were collected by filtration and blotted with filter paper. The swelling index was calculated as follows:

$$SI = \left(\frac{W_s - W_d}{W_d} \right) \times 100 \quad (1)$$

W_s = average weight of swollen samples (g), W_d = average weight of dry samples (g)

2.6 Drug encapsulation efficiency

Encapsulation and co-encapsulation efficiency of DOX and TMZ in all systems at different pH (3.5, 7.4 and 9) was determined via UV spectrophotometer HEλIOS Thermo Scientific. The fluorescence intensity of DOX and TMZ was measured at 480 nm and 325 nm respectively. The amount of drug was calculated from a calibration curve, prepared by measuring the fluorescence intensity of the known drug concentration. The drug entrapment efficiency (EE) was calculated as follows:

$$EE = \left(\frac{D_t - D_f}{D_t} \right) \times 100 \quad (2)$$

D_t= Total amount of drug (µg/ml); D_f= Amount of drug untrapped (µg/ml)

2.7 *In-vitro* drug release studies

The *in vitro* drug release test was carried. 10 mg of each samples was suspended in 10 ml of buffer at pH 3.5, 7.4 and 9 at 37˚C. At predetermined time intervals, an aliquot (1ml) was withdrawn and the concentration of drug released was monitored by UV spectrophotometer at 325nm for TMZ and 480 nm for DOX. The dissolution medium was replaced with fresh buffer to maintain the total volume. The percentage of drug released (DR) was determined by the following equation

$$DR = \left(\frac{D_t}{D_0} \right) \times 100 \quad (3)$$

Where $D_{(t)}$ represents the amount of drug released at a time t (µg) and $D_{(0)}$ the amount of drug loaded (µg). All studies were done in triplicate.

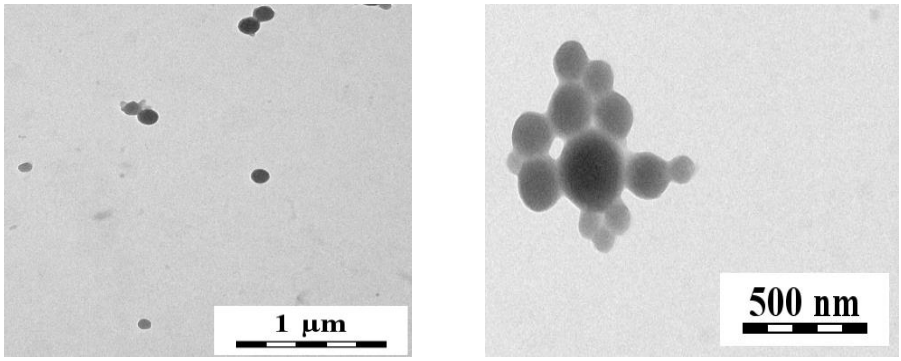
3. RESULTS AND DISCUSSIONS

3.1 Physicochemical characterization of nanoparticles

Nanoparticles were formed spontaneously upon the incorporation of TPP solution to the CS-g-PLA solution under magnetic stirring. CS-g-PLA nanoparticles obtained by ionic gelation, a simple and solvent free process, are formed by electrostatic interaction between the positively charged chitosan chains and TPP employed as a cross linker. The nanoparticles size was found in the range 150-350 nm (Tab.1). CS grafted with PLA 10 kDa showed best results in terms of size compared with nanoparticles obtained with CS-g-PLA 60 kDa at all tested temperatures. In both systems a slight increase in size occurred when drugs were encapsulated, in particular together. ζ- potential of all formulated nanoparticles was in the range 25-48 mV (Tab.1). The presence of drugs has decreased the ζ-potential values, probably through a shielding effect as both drugs carry a positive charge. No significant differences were obtained between CS-g-PLA made with PLA 10 kDa and PLA 60 kDa in terms of ζ- potential in case of all formulations.

Tab.1 Size and ζ-pot. of all formulations at different temperature and pH 3.5

	Temp. 4˚C		Temp. 25˚C		Temp. 37˚C	
Sample	**size (nm)**	**ζ(mV)**	**size (nm)**	**ζ(mV)**	**size (nm)**	**ζ(mV)**
CS-g-PLA$_{10kDa}$	155±10	42±1.2	193±14	45±0.4	180±8	41±0.2
CS-g-PLA$_{10kDa}$ DOX	256±8	41±0.9	262±9	39±0.6	280±12	43±0.5
CS-g-PLA$_{10kDa}$ TMZ	249±11	34±0.9	229±6	37±0.2	283±4	33±0.9
CS-g-PLA$_{10kDa}$ DOX TMZ	333±15	26±1.3	358±10	24±1.1	305±6	29±0.3
CS-g-PLA$_{60kDa}$	235±28	47±1.5	310±23	44±0.7	237±11	48±1.2
CS-g-PLA$_{60kDa}$ DOX	309±19	36±0.5	312±25	33±0.9	299±24	35±0.8
CS-g-PLA$_{60kDa}$ TMZ	285±23	35±0.8	302±27	39±0.3	320±25	30±0.8
CS-g-PLA$_{60kDa}$ DOX TMZ	311±21	29±1.1	320±16	28±0.2	317±25	32±1



a **b**

Fig.1 TEM image of CS-g-PLA-DOX-TMZ nanoparticles. a) CS-g-PLA 10 kDa, b) CS-g-PLA 60 kDa

TEM analysis confirmed the spherical shape and the nanometric size of the nanoparticles (Fig.1). However, particles obtained from CS-g-PLA 60 kDa showed higher polydispersity than those obtained from CS-g-PLA 10 kDa. To understand the cross-linking and how the chains are located in the core, density swelling studies in human male serum were carried out (Tab. 2). Results demonstrated that PLA chains, DOX and TMZ influence the cross linking formation through interaction with the negative charges of TPP causing a reduction of the swelling index and changing the core structure of the nanoparticles. The length of the PLA chains can also influence the swelling index when drugs are loaded, but not significantly when they are absent.

Tab.2 Swelling Index in CS and CS-g-PLA and derivatives in Human Serum

Sample	Weight dry(mg)	Weight wet(mg)	Swelling Index %
CS	6.5±0.1	53.9±1.07	729±13
CS-g-PLA$_{10kDa}$	6.5±0.1	49.6±1.48	663±19
CS-g-PLA $_{10kDa}$ DOX	6.5±0.1	42.7±1.28	556±17
CS-g-PLA $_{10kDa}$ TMZ	6.5±0.1	44.9±1.79	590±23
CS-g-PLA $_{10kDa}$ DOX TMZ	6.5±0.1	41.2±1.23	533±16
CS-g-PLA $_{60kDa}$	6.5±0.1	48.3±1.44	643±19
CS-g-PLA $_{60kDa}$ DOX	6.5±0.1	37.4±1.49	475±19
CS-g-PLA $_{60kDa}$ TMZ	6.5±0.1	35.8±1.79	450±22
CS-g-PLA $_{60kDa}$ DOX TMZ	6.5±0.1	39.2±1.17	503±15

3.4 Encapsulation Efficiency

Drugs were loaded into nanoparticles during the preparation. It is also possible to load them following the nanoparticles formation but the efficiency is low. In most nanoparticle delivery systems, the drug carrying capacity is defined in terms of encapsulation efficiency. Tab. 3 shows the encapsulation and co-encapsulation efficiency (EE) in both systems at different pH.

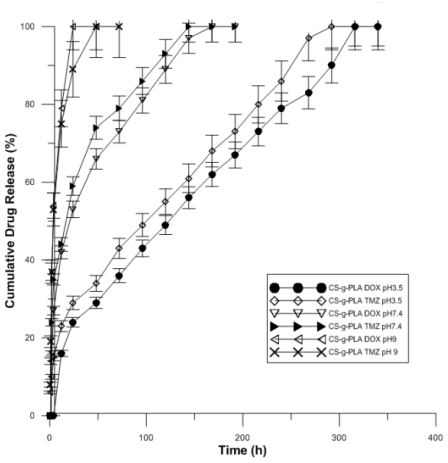
Tab.3 Encapsulation and co-encapsulation efficiency of DOX and TMZ in terms of µg drug/ mg polymer

	Encapsulation		Co-encapsulation				
Sample	**DOX**	**TMZ**	**DOX**	**TMZ**	**DOX+TMZ**	**pH**	**T. (˚C)**
CS-g-PLa10KDa	520±0.32	470±0.25	450±0.19	390±0.38	840	3.5	25
	90±0.54	120±0.49	60±0.64	80±0.47	140	7.4	25
	12±0.63	16±0.72	24±0.92	13±0.75	37	9	25
CS-g-PLA60kDa	480±0.18	390±0.41	320±0.35	270±0.31	590	3.5	25
	120±0.23	140±0.37	70±0.29	100±0.33	170	7.4	25
	11 ±0.44	13±0.25	19±0.81	15±0.68	34	9	25

The encapsulation efficiency is strictly correlated with the pH of the medium. In fact, maximum encapsulation and co–encapsulation values were obtained at pH 3.5, while lower values were obtained at pH 9 in both systems, because the amino group and TPP, responsible for the electrostatic interaction that allows to keep the system up, are a charge making mesh-like structure that allows to hold the drugs back inside.

3.5 Release Kinetic

In vitro release analysis of DOX and TMZ in buffer solutions at pH 3.5, 7.4 and 9 from CS-g-PLA 10 kDa and CS-g-PLA 60 kDa nanoparticles are presented in Fig.2. Results indicated that they exhibited sustained release behaviour related with the pH of the environment. We detected that the drug release rate increases proportionally with the pH of the environment. However, the molecular weight of grafted PLA seems to influence the release kinetics in particular at pH 3.5. In fact, the release rate of DOX and TMZ from CS-g-PLA10 kDa showed greater homogeneity compared with CS-g-PLA 60 kDa. Indeed 50% of drugs were released in about 200 hours. In case of CS-g-PLA 60 kDa, 50% of DOX was released in about 150 hours instead of the same amount of TMZ in less than 100 hours. At low pH amino group from chitosan, DOX and TMZ are charged and can interact with TPP, increasing the pH of the environment, elecrostatic interactions become less strong and this allows a faster release of the drugs. We found out that TMZ is released faster than doxorubicin at pH >3.5 in both systems. It is probably the molecular structure of TMZ that enables easy diffusion through the mesh-like system. As showed in Fig.2 at pH 9 the whole amount of drug is released in less than 2 days, on the contrary at pH 3.5, 90% is released in almost 13 days (300 hours) at pH 3.5. This allows a modulation of the release rate just by changing the pH.



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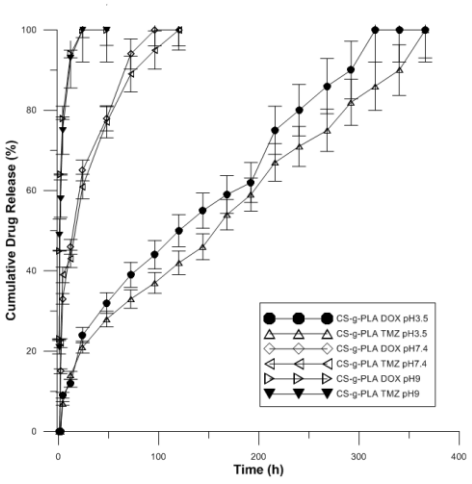

b

Fig.2 Release profile of DOX and TMZ at different pH from a) CS-g-PLA 10 kDa and b) CS-g-PLA 60 kDa

CONCLUSION

In this study, amphiphilic nanoparticles made by grafting PLA with different molecular weight (10 kDa and 60 kDa) to chitosan backbone were obtained. Polylactide synthetized by polycondensation technique was linked to chitosan through coupling reaction between amino group of CS and carboxylic group of PLA. The amide bond formation was confirmed by FTIR-ATR and ^{1}H-NMR analysis. Nanoparticles were obtained via ionic gelation technique with the average size of 150-350 nm and a z-potential in the range 25-48 mV which suggested that nanoparticles are substantially stable at different pH and temperature and PLA chains are located in the core of the NPs, which interact with the drugs helping to keep them inside. CS-g-PLA 10 kDa has showed better encapsulation and co-encapsulation efficiency for doxorubicin and temozolomide in particular at pH 3.5 around 50%. *In vitro* release studies showed a sustained and controlled release of both drugs, particularly at pH 3.5 where 90% of the drugs were released in almost 13 days. Additionally, release profile demonstrated significant dependence on pH, leading CS-g-PLA nanoparticles as good candidates for use in localized drug delivery, in fact decreasing the pH, both drugs are released slowly and this is important for controlling the drug concentration in the targeted site. DOX and TMZ encapsulated in CS-g-PLA nanoparticles would not only offer several advantages over conventional drug therapies but it is also expected to overcome side effects related with DOX and TMZ and there is a chance to carry out a co-therapy, which seems to be effective in Glioblastoma Multiforme treatment.

ACKNOWLEDGEMETS

This work was supported by the Research and Development for Innovations Operational Programme co-funded by the European Regional Development Fund (project CZ.1.05/2.1.00/03.0111). The authors are also grateful to the Internal Grant Agency of Tomas Bata University in Zlin (grant IGA/FT/2013/004) for co-funding.

REFERENCES

[1] K.E.UHRICH, S.M.CANNIZZARO, R.S.LANGER, *Polymeric systems for controlled drug release*. ChemRev.Vol.99.1999;pp.3181-98

[2] DODANE and VILIVADAM, *Pharmaceutical applications of chitosan* Pharm Sci Technol Today. Vol. 1. 1998

[3] M.BISHAL, D.SWARNALOK, A.BASUDAN, R.BANERJEE, *Copolymerization of Lactic Acid for cost-effective PLA synthesis and studies on its improved characteristics*.Food Sci.Biotechnol. 22(5):73-77(2013)

[4] M.CARAGLIA, R.ADDEO, R.COSTANZO, L. MONTELLA , V. FAIOLA , M. MARRA , A. ABBRUZZESE , G. PALMIERI , A.BUDILLON, F. GRILLONE , S. VENUTA , P. TAGLIAFERRI , S. DEL PRETE .*Phase II study of temozolomide plus pegylated liposomal doxorubicin in the treatment of brain metastases from solid tumours*.; Cancer Chem. Pharmacol. 2006 jan;57(1):34-9 EPUB 2005 Nov

[5] P.KUCHARCZYK,V.SEDLARIK,I.POLIANSEK,V.KASPARKOVA,A.SALAKOVA,J.DRBOHLAV,U.CVELBAR,P.SAHA *Functionalization of Polylactic acid through direct melt polycondensation in the presence of tricarboxylic acid*. J.of App. Polymer Science, Vol.122,1275-1285

[6] J.LI,M.KONG, X.J.CHENG, Q.F.DANG, X.ZHOU, Y.N.WEI,X.G.CHEN; *Preparation of biocompatible chitosan grafted poly(lactic acid)nanoparticles*, Int.J.of Biological Macromolecules 51(2012)221-227

[7] C.ARAL, J.AKBUGA, *Alternative approach to the preparation of chitosan beads*. Int.J.Pharm. 168(1998) 9-15

[8] J.R. EISENBREY, O.MUALEM BURSTEIN,R.KAMBHAMPATI, F.FORSBERG, J.B.LIU, M.A.WHEATLEY, *Development and optimization of a Doxorubicin loaded poly(lactic acid) contrast agent for ultrasound direct drug delivery*, Journal of Controlled Release 143(2010) 38-44