

Polyurethane Structures Used as a Drug Carrier for Epigallocatechin Gallate

AUREL MARIN¹, MARIA ALINA MARIN^{2*}, IOAN ENE³, MARIOARA POENARU⁴

¹"Victor Babes" University of Medicine and Pharmacy, General Medicine, ENT Department, 2 Eftimie Murgu Sq, 300041, Timisoara, Romania

²"Victor Babes" University of Medicine and Pharmacy, General Medicine, ENT Department, 2 Eftimie Murgu Sq, 300041, Timisoara, Romania

³Emergency Pediatric Hospital, ENT Department, 68 Motilor Str, 400001, Cluj Napoca, Romania

⁴"Victor Babes" University of Medicine and Pharmacy, General Medicine, ENT Department, 2 Eftimie Murgu Sq, 300041, Timisoara, Romania

Abstract: *The current study was proposed in order to obtain, to characterize by usually physico-chemical methods and to assess in vitro, the potential cytotoxic effect of polyurethane microstructures loaded with epigallocatechin gallate (PU_MS) on human pharyngeal carcinoma cells (Detroit 562) and squamous cell carcinoma (SCC-4). The results showed that polyurethane microstructures obtained are stable and have dimensions that make them biocompatible with the biological environment. The cytotoxicity of test samples is dependent on concentration and 72 h after stimulation at the highest concentration tested the viability of tumor cells was below 50%, the decrease being much more pronounced in the case of squamous cell carcinoma. The novel nanostructures loaded with epigallocatechin gallate induced an augmented cytotoxic effect, suggesting that this drug carriers are suitable to enhance the antitumor effect of epigallocatechin gallate.*

Keywords: *drug transporter, polyurethane structures, thermal degradation, cell viability, carcinoma cells*

1. Introduction

The discovery of active molecules against cancer has grown in the last decade. Despite all the efforts made, the affected population is increasing and the survival rate in the case of patients varies greatly depending on the type of cancer, stage, age, country, etc. In general, the survival rate in developed countries is significantly higher compared to poorly developed or developing countries. Compounds from natural sources are an alternative used effectively in the study of anticancer drugs. The most relevant examples of anticancer drugs obtained from natural sources are given by Paclitaxel, vincristine and doxorubicin [1-3]. Despite the efforts, obtaining medicines from natural sources is not an easy one and depends on a series of factors such as: correct selection of isolation and separation methods, the influence of yield, evaluation method, legislation in force, etc.

The major chemical compounds of tea are represented by the class of catechins composed of a benzopyran backbone substituted with a phenyl (position 2) and hydroxyl or ester groups (position 3), respectively [4,5]. Polyphenols identified in green tea have been studied for years due to their influence on several important biological pathways and also the anticarcinogenic effects they show. Most of these effects are mainly attributed to the most abundant chemical compound (-) – EGCG (Figure 1).

Polyurethane materials have a long history since Prof. Bayer and his research team have developed the first foams in their laboratory from Leverkusen, Germany, more than 80 years ago; the first medical applications have been developed by Pangman in 1958 (a composite breast prostheses covered with a polyester-urethane foam) and in the same year, Mandarino and Salvatore have obtained a rigid polyurethane foam used for in situ bone fixation (Ostamer™) [6,7]. The possibility to use polyurethanes as drug delivery systems was first described by Levy in 1988 when he has presented the obtaining of a poly(ether urethane) carrier used for the controlled release of lidocaine to normalize the cardiac rhythm; later, Hafeman has discovered another carrier used for a sustained release of tobramycin, Moura has

*email:alinamaria.muntean@yahoo.com

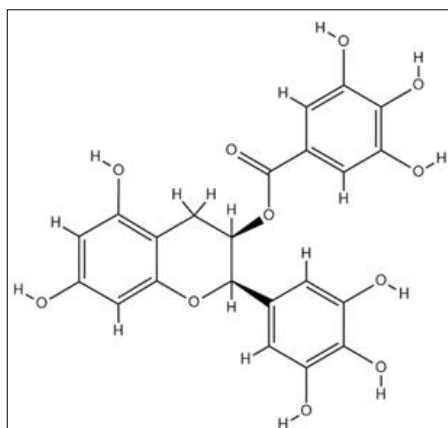


Figure 1. Chemical structure of Epigallocatechin gallate

reported the synthesis of dexamethasone-loaded polyurethane matrices etc. [8,9]. The advantages of these carriers are the following: a reduced cost of the raw materials, the possibility to modify the degradation period of macromolecular chains by balancing the ratio between the ether- and ester-polyols used in the aqueous phase, and the possibility to modify the size of particles by the use of different amounts of chain extenders (diols and diamines with low molecular weight) [10,11].

The aim of the current study was to obtain polyurethane structures loaded with epigallocatechin gallate and to realize a preliminary evaluation of the compounds obtained on pharyngeal carcinoma cells and squamous cell carcinoma.

2. Materials and methods

2.1. The reagents

The raw materials used in the synthesis of the polyurethane microstructures (PU_MS) are: monoethylene-glycol (MEG) from Lach-Ner s.r.o. (Czech Rep.), 1,4-butanediol (BD) from Carl Roth GmbH (Germany), the solvent of the organic phase (acetone) from Chimopar S.A. (Romania), while the others: polyethylene glycol (PEG, average mol wt 200), the surfactant (Tween[®]20), isophorone-diisocyanate (IPDI), and hexamethylene-diisocyanate (HMDI) were obtained from Merck (Germany) and epigallocatechin gallate (EGCG) was obtained from Sigma-Aldrich (Germany). All substances, high-purity reagents, were maintained under the conditions specified by the manufacturer and they were used as received, without any previous purification.

Inorganic salts of Na and K, such as chlorides, mono- and diphosphates, NaHCO₃, and HCl from Chimopar S.A. (Romania) were used to obtain a simulated body fluid (SBF) which is described in the literature [12,13] as a proper medium to study the degradation of an organic drug delivery system and its release profile. They were previously heated at 110°C for 90 min in order to remove the crystallization water.

2.2. The chemical synthesis

A protocol based on five steps was used to synthesize PU_MS according to previous papers [11,14–16]: (i) A non-aqueous phase based on a mixture of IPDI and HMDI in acetone (Table 1) was prepared under magnetic stirring (450 rpm) at 35°C for 15 min; (ii) A hydroxylic phase based on a mixture of MEG, BD, PEG, and Tween[®]20 in deionized water was homogenized (450 rpm) at 35°C for 10 min; (iii) The organic phase was rapidly injected in the aqueous phase (1: 1.4, v/v) under magnetic stirring (550 rpm) at 40°C and the stirring continued for 5h to ensure the maturation of the PU_MS macromolecular chains; (iv) The obtained suspensions were purified by repeated washing - slight centrifugations using a water-acetone mixture (1: 1.2, v/v); (v) The products were dried as thin layers in Petri dishes at 65°C for more than 24h.

The experiment was repeated three times to synthesize different samples for further comparative characterizations: PU_MS_0 (structures without EGCG), PU_MS_1 (structures with low amount of EGCG), and PU_MS_2 (structures with high amount of EGCG).

Table 1. The ratios between the raw materials.

Sample	Aqueous phase (mL / 50 mL)				EGCG (mg / 50 mL)	Organic phase (mL / 50 mL)	
	MEG	BD	PEG	Tween		IPDI	HMDI
PU_MS_0	0.40	0.60	2.15	1.50	0.00	1.85	3.15
PU_MS_1	0.40	0.60	2.15	1.50	5.50	1.85	3.15
PU_MS_2	0.40	0.60	2.15	1.50	11.00	1.85	3.15

2.3. Characterization of the samples

The efficacy of the drugs encapsulation can be calculated by reporting the untrapped active substances to the total amount that was added in that synthesis according to an old study on a polyurethane drug delivery system [17]. UV spectrometry using an UVi Line 9400 Spectrophotometer (SI Analytics, Germany) based on the difference between the maximum absorption (276 nm for EGCG and 353 nm for PU_MS_0), a calibration curve and the Beer-Lambert law were involved to establish the encapsulation efficacy. The release kinetics was determined by maintaining the samples PU_MS_1 and PU_MS_2 in a degradation medium, SBF from the recipe of T. Kokubo, much more proper to be used in different assessment based on the degradation of biomaterials [18,19] for ten days; 3 aliquots of each 0.2 mL medium were replaced every second day with fresh medium and their UV absorption was read at 276 nm; average values +/- standard errors were used to present the release profile [20].

The electric charge of PU_MS surface and their average size were evaluated using a Vasco Particle Size Analyzer and a Wallis Zeta potential Analyzer (both from Cordouan Technology, France); the following parameters were set: assessment temperature (25±1°C), interval of time (15±3 µs), number of channels (around 435), power of laser (75-90 %), acquisition mode (continuous), analysis mode (Pade-Laplace), Wallis resolution (medium), and Smoluchowski model as Henry function.

The thermal behavior of PU_MS was studied using a Mettler-Toledo DSC1 instrument (Mettler-Toledo, Switzerland) and aluminum crucibles with perforated lids, between 40-280°C in an inert atmosphere (10°C/min).

2.4. In vitro evaluation of the samples

The cells employed in the current experimental study were: pharyngeal carcinoma cells (Detroit 562: ATCC® CCL-138™) and squamous cell carcinoma (SCC-4: ATCC® CRL-1624™) acquired as frozen items from ATTC and stored in liquid N₂ until the experimental use. The specific reagents required for cell culture were: EMEM and FBS 10% for pharyngeal carcinoma cells and DMEM:F12 (contains L-glutamine, HEPES, sodium pyruvate, and sodium bicarbonate.), hydrocortisone and FBS 10% for squamous cell carcinoma were provided from ATTC. The cell culture with a confluence of minimum 80% was obtained according to manufacturer's specifications [21,22]

The analysis of cell viability was assessed by the means of MTT assay after 72h post-treatment with different concentrations of test samples [23,24]. In short, 10,000 cells/well were seeded in a 96/well plate, after 24h cell media was remote and the cells were stimulated with test samples; blank or control cells were stimulated only with specific media; cell viability was obtained after the plates were spectrophotometrically analyzed at 570nm wavelength, with a microplate reader.

3. Results and discussions

3.1. The encapsulation efficacy and the release kinetics

A calibration curve between 0.05 and 2.50 µg / mL EGCG ($y = 0.42x - 0.01$; $r=0.9981$) was used to calculate the encapsulation efficacy and the drug release. The relation between the level of the maximum adsorption and EGCG concentration (the Beer-Lambert law) indicates high encapsulation efficacy (62.93

for PU_MS_1 and 77.18 for PU_MS_2). Polyurethane carriers, based on hollow nano- and micro-particles, have a very good capacity to encapsulate large amounts of active substances: 67.9 % was found in the case of a chili pepper extract [11] and 82.9% for a ginger extract [16].

Figure 2 presents the EGCG release inside the degradation media for the two samples of polyurethane microstructures containing different amounts of EGCG.

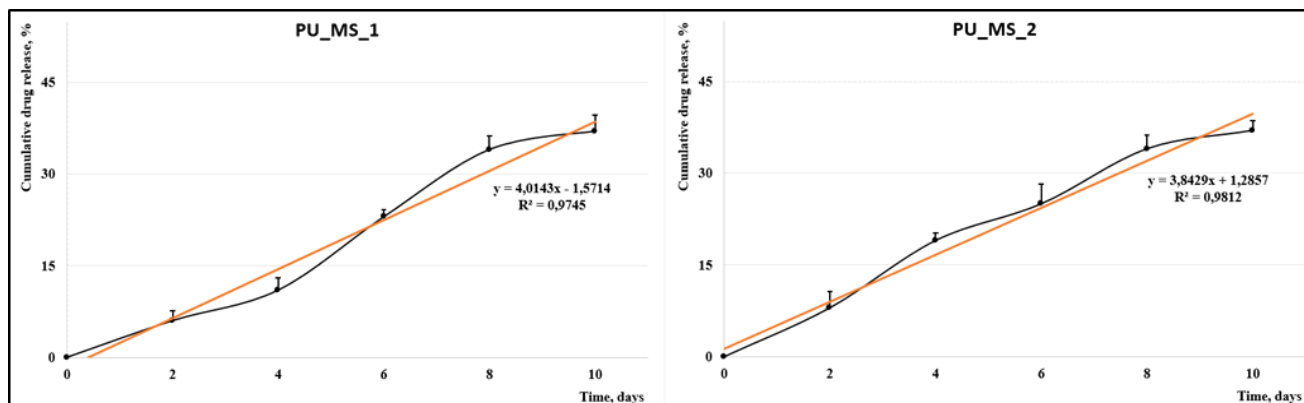


Figure 2. The release profile for EGCG from samples of PU_MS with active substance

The drug release depends on the degradation of the carrier macromolecules. Computational models play a key-role in the description of the release profiles; the trend of these PU_MS release can be described by the Equation 1 and 2. No important difference is described by these two equations; the cumulative drug release is around 37 % for both samples after 10 days, but a slight increased value was determined in the fourth day in the case of the sample with an increased amount of EGCG used in the synthesis.

$$y = 4.0143 x - 1.5714 (R^2 = 0.9745) \quad (1)$$

$$y = 3.8426 x - 1.2857 (R^2 = 0.9812) \quad (2)$$

The causes of transport phenomena are imbalances inside many systems or between the parts of different systems. The natural evolution is towards the uniformity of the properties, characterized by physical parameters that are constant in time and space. The drug release at a specific target is based on a transport process and taking into account that molecules have a mass, this process can be described by the Fick law about the mass diffusion - the mass flux (J_m) is proportional to the mass density gradient (ρ_m) [25] according to Equation 3.

$$J_m = - D_m \cdot \quad (3)$$

where D_m is the coefficient of molar diffusion.

Kakar and collaborators have described a more useful model for the drug release from polymeric systems [26]; they present the Korsmeyer peppas model based on the fact that the fraction of drug released has a constant rate if it is expressed as logarithm of percent of cumulative drug release in function of the logarithm of time ($\text{Log}\% \text{CDR} = f(\text{LogTime})$). In biomedical applications, taking into account drug release, these transport nanotypes are safe and can be used as intelligent drug delivery matrices for targeted delivery [27,28].

3.2. Zetasizer characterization

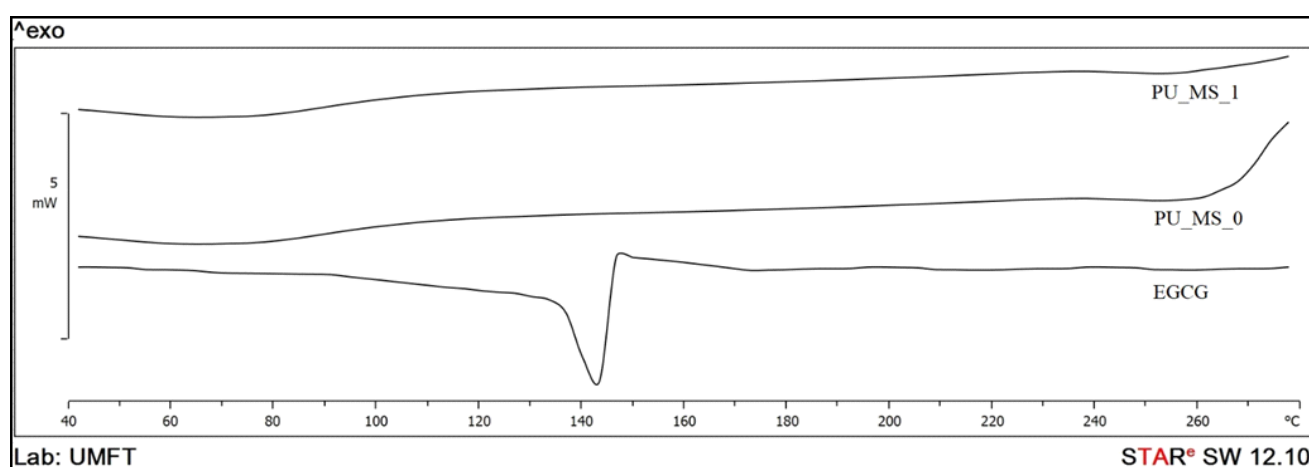
Table 2 presents the PU_MS characterization that was obtained during the Zetasizer assessment. The decreased values of the polydispersity index (PDI) indicate the obtaining of almost homogeneous samples, while the Zeta potential values show a medium tendency of these structures to form agglomerations according to Gallardo *et al.* [29].

Table 2. Experimental values of the Zetasizer characterization

Sample	Size of structures (nm)		Zeta potential (mV)
	Mean \pm SD	PDI	
PU_MS_0	174 \pm 15	0.2	+29.12
PU_MS_1	229 \pm 17	0.4	+25.79
PU_MS_2	232 \pm 21	0.4	+24.91

3.3. The thermal stability

The investigation on the thermal stability of samples were done comparatively between the samples of the polyurethane carrier with and without the active agent against its EGCG sample (Figure 3). The glass transitions of the polymer structures were not observed inside the studied range of temperature, but it can be seen that a slight degradation starts around 270°C. No significant peaks can be noticed in the thermograms of PU samples; the absence of the drug's characteristic peak indicates the complete encapsulation inside the PU carrier [30].

**Figure 3.** DSC thermograms of samples with and without drug vs. EGCG

The melting point of EGCG is between 140-142 °C and it was found during these analyses; on the other hand, the absence of this peak from the thermogram of sample PU_MS_1 confirmed the good encapsulation of the active compound.

3.3. *In vitro* tests on tumoral cells

A number of *in vitro* studies have shown that polyphenols in green tea or (-) - pure EGCG have influenced oral squamous cell carcinoma cells and other oral epithelial cells.

The cytotoxicity of test samples, was assessed on pharyngeal carcinoma cells (Detroit 562) and squamous cell carcinoma (SCC-4) by MTT colorimetric method. The evaluation was realized with various concentrations (5, 10, 25, and 50 μ M) of samples after a stimulation time of 72h.

The data (Figure 4 and 5) indicated that test samples exerted different influences on cell viability. At low concentration (5 μ M) samples did not induced significant cell viability reduction. The lowest cell viability percentages were recorded after exposure of SCC-4 cells to 50 μ M of PU_MS_2 (~21%) and PU_MS_1 (~21%), followed by treatment of Detroit 562 cells with PU_MS_2 at concentration of 50 μ M (~50%), EGCG at concentration of 50 μ M (~52%) and PU_MS_1 at concentration of 50 μ M (~58%) as can be seen in Figures 4 and 5.

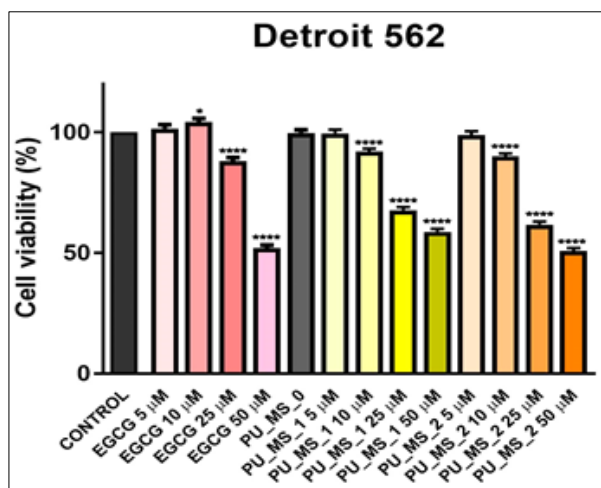


Figure 4. Viability percentage of pharyngeal carcinoma cells after stimulation with test samples (5, 10, 25 and 50 μM), at 72h post-stimulation

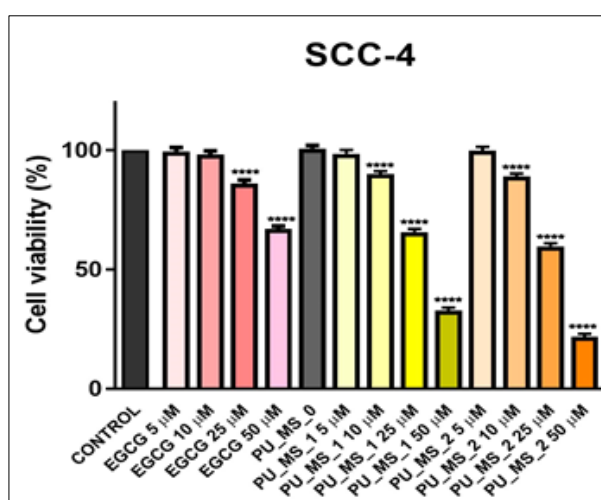


Figure 5. Viability percentage of squamous cell carcinoma after stimulation with test samples (5, 10, 25 and 50 μM), at 72h post-stimulation

Masuda *et al.* have highlighted that among the many bioactive molecules EGCG seems to be one of the most promising, especially due to its clinical efficacy but also the biological effects on certain lipid layers [31]. EGCG also has been shown to prevent the invasion and metastasis of the OC2 and SCC-9 cells by inhibiting MMP2 and MMP9 matrix metalloproteinase and suppressing urokinase plasminogen activator activity [32,33]. Another study conducted by Irimie *et al.* showed that EGCG can induce the activation of cell death receptors in the case of SCC-4 cells, suggesting the importance potential of the compounds as natural drug candidate [34].

4. Conclusions

The purpose of this study was achieved by the obtaining of a polyurethane drug delivery system used for the transmembrane transfer of Epigallocatechin gallate; the carrier consists of almost homogeneous particles with a high encapsulation efficacy, diameters around 200 nm, with a medium tendency to form agglomerations, very stable during heating processes. Furthermore, the data obtained from cell viability evaluations indicated that polyurethane structures loaded with epigallocatechin gallate exerts a cytotoxic which deserves to be studied in more detail and *in vivo* studies.

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