VEMURAFENIB BASED HYDROGELS AS POTENTIAL TOPICAL BIOFORMULATIONS FOR THE TREATMENT OF MELANOMA

Larisa Păduraru[1], A. Sava[1], H. Orhan[2], Cătălina Natalia Yilmaz[3], M. Apostu[1], Alina Diana Panainte[1*], Mădălina Vieriu[1], K. Atmaca[2], Nela Bibire[1]
"Grigore T. Popa" University of Medicine and Pharmacy Iasi, Romania
Faculty of Pharmacy
1. Department of Pharmaceuticals Sciences (I)
Ege University, Bornova-Izmir, Turkey
Faculty of Pharmacy
2. Department of Pharmaceutical Toxicology
Dokuz Eylül University, Izmir, Turkey
Faculty of Science
3. Department of Chemistry, Biochemistry Division
*Corresponding author. E-mail: alina-diana.panainte@umfiasi.ro

VEMURAFENIB BASED HYDROGELS AS POTENTIAL TOPICAL BIOFORMULATIONS FOR THE TREATMENT OF MELANOMA (Abstract): One of the most aggressive forms of cancer, cutaneous melanoma, occurs as a result of uncontrolled division of melanocytes. The efficacy of the used treatments can be diminished in terms of targeted action, increased toxicity and resistance to treatment. In the present study we have investigated an antitumor agent from the BRAFV600 inhibitor class, namely vemurafenib, currently approved by the FDA and EMA for oral administration only. Due to its high toxicity, we evaluated the possibility of cutaneous administration for the treatment of early-stage melanoma or post-surgical resection. **Materials and methods**: In that regard, we considered hydrogels based on chitosan, β-cyclodextrin and polyvinyl alcohol in various concentration ratios from which, after a careful analysis of the previously published data, we chose two matrices to be loaded with vemurafenib. **Results:** The inclusion complexes were obtained by PVA gelation through repeated freeze-thaw cycles and subsequently characterized by FTIR, scanning electron microscopy and swelling capacity at various pH and temperature values. **Conclusions**: The obtained results were promising, as the synthesized formulations proved their capability of controlled release for vemurafenib. **Keywords:** CHITOSAN, β-CYCLODEXTRIN, POLYVINYL ALCOHOL, HYDROGEL, VEMURAFENIB, SMART DRUG DELIVERY.

INTRODUCTION

In view of the increasing number of malignant pathologies being diagnosed, the medical world is showing a keen interest in the study of new systems for the transportation and targeted delivery of active substances with selectivity towards cancer cells, minimal toxicity to healthy tissue and fewer side effects. In that regard, numerous transport systems have been synthesized, including smart hydrogels that have gained notoriety in recent years in medical research. Invasive melanoma accounts for 1% of all skin cancer cases with very poor

prognosis, being the least common but the deadliest type of skin cancer in the incidence of which, the main involved factors are UV irradiation, prolonged sun exposure, light skin tone, family history of melanoma, higher numbers of melanocytic nevi and chronic immunosuppression. Death occurs as a result of metastasis, a stage characterized by increased tumor cell invasion and migration of cancer to other organs. The main method of treatment is surgical resection with excellent long-term prognosis, but in advanced melanoma (stage III-IVB), that approach is not sufficient and must be complemented by other therapeutic methods considering the spread of cancer cells to several organs (1, 2). Although surgical resection remains a gold standard for the treatment of melanoma, certain cases in which that approach is not possible, such as those in which patients are not suitable surgery-candidates depending on tumor/patient's characteristics, must also be considered as a second-line therapy. In that context, two possible options are the topical application of a specific drug or the radiation therapy, being the only ones that have proven a certain therapeutic efficacy. Other therapeutic approaches such as liquid nitrogen cryotherapy, electrodesiccation, curettage, laser surgery, and fluorouracil 5% cream have been linked with a high risk of recurrence and are not recommended. Currently, the only cream available on the pharmaceutical market but used off-label for the treatment of melanoma is imiquimod 5%, as adjuvant therapy (3).

Targeted therapy of cutaneous melanoma has developed based on the observation that more than 66% of malignant cases presented somatic missense mutations of protein kinase b-raf (BRAF). 69 BRAF mutations result in high serine/threonine kinase activity and mediate cell proliferation through the regulation of the mitogen-activated protein kinase (MAPK) signaling pathway. The most widespread variant is a val-to-glu point mutation at codon 600 (V600 E), which benefits from the treatment with VEM, which has been designated an orphan drug by the FDA for the treatment of BRAF V600E mutation-positive stage IIB to IV melanoma and it is not indicated for use in patients with wild-type BRAF. When evaluating physico-chemical properties of numerous antitumor drugs, specifically their solubility, we observed that most of them had low solubility in water, raising bioavailability issues (6). Attempts on the topical application of VEM have also been made in other studies, e.g., in the form of a solid-in-oil nanodispersion as an efficient and safe way to deliver VEM in early-stage melanoma (7). Peuvrel *et al.* (8) reported the appearance of skin rashes as a toxic response to a prolonged dermal treatment with VEM. That problem could be solved through formulation related strategies. One of them could be the inclusion of the drug within a biopolymeric matrix which can improve targeted drug delivery and diminish its cutaneous toxicity. This could not only target better VEM to the site but also to buffer its skin toxicity.

Analyzing the option of topical application, we have considered the synthesis of a chitosan (CS)-based hydrogel combined with β-cyclodextrin (β-CD) to allow controlled and targeted release of VEM and to counteract its limitations imposed by low water solubility and by its toxicity.

Due to its structural properties, in particular the presence of amino groups on the polysaccharide chain, CS is a biopolymer that exhibits certain properties that make it

suitable for biomedical applications such as high biocompatibility, low immunogenicity and allergenicity. CS is obtained by the deacetylation of chitin and it is a polysaccharide biomaterial composed of β-(1-4)-2-acetamido2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose. The combination of CS with β-CD can provide unique benefits, particularly when administering hydrophobic medications. Cyclodextrins are produced by *Bacillus macerans* and from a chemical point of view they are a particular category of oligosaccharides obtained from the enzymatic degradation of starch. β-CD is a derived oligosaccharide with electron-rich glycosidic oxygen atoms, that has a hydrophobic central cavity that can integrate hydrophobic molecules such as VEM in the presence of water and thus forming inclusion complexes with various drugs and bioactive molecules. Moreover, its ability to improve the solubility of certain drugs and the permeability of some macromolecular substances have also been demonstrated (9).

As Wang *et al.* (10) demonstrated in their study, the copolymer chitosan-grafted β-cyclodextrin (CS-g-β-CD) showed an improved hydrophilicity as its porous structure is advantageous for the complete exposure of polar functional groups (amino and hydroxyl groups) on its surface, being a smart drug carrier for etoposide, another anticancer drug.

The present study aimed to develop a method that combined CS and β-CD as hydrogels within an optimal composition to include VEM as the active substance. The obtained formulation was intended for topical delivery of VEM, the matrix being analyzed as a potential alternative to the topical preparations that are currently used. The preparation of the CS/β-CD complex is based on CS's ability to act as a cationic polymer to generate hydrogels (11,12) and to modulate the complex's properties, such as controlled release of drugs (13) by varying the external stimuli (i.e., pH), leading to smart delivery systems. An additional crosslinking was necessary because of the requirement that the biopolymeric matrix must be resistant to enzyme activity and other physicochemical factors. A non-invasive crosslinking strategy is physical crosslinking with the help of polyvinyl alcohol (PVA) involving the hydrogen bonding process, leading to a strong gel without external crosslinking agents. That type of gelation may provide biocompatible hydrogels of very high purity, with enhanced mechanical strength, with recently discovered inherent self-healability, that are extremely useful for post-surgery applications (14-16). The present study had its premises in a previous work done by Cheaburu *et al.*, where the behavior of an antimycotic drug with unfavorable pharmaco-toxicological profile for oral and/or parenteral administration was studied by loading it into a similar CS-based hydrogel, namely a CS-g-PNIPAAm/PVA copolymer, intended for topical mucosal application, with promising results (17).

MATERIALS AND METHODS

Materials. In our experiment, we have used the following materials: vemurafenib (VEM), chitosan (CS, 75-80% degree of deacetylation), β-cyclodextrin (β-CD), polyvinyl alcohol (PVA, 99% hydrolyzed with Mw of 89,000-98,000 Da), and phosphate buffered saline tablets pH = 7.4 (PBS), all purchased from Sigma-Aldrich, St. Louis, MO, USA. Other materials not mentioned were of analytical grade and used without further purification. All aque-

ous solutions were prepared using ultrapure water (Milli-Q, Millipore).

Preparation of Freeze-Thaw Hydrogels CS/β-CD/PVA. For the preparation of the hydrogels, we synthesized chitosan and β-cyclodextrin complexes (CS/β-CD) by solution mixing of 2 wt.% solution of CS in 0.5% acetic acid solution and 2 wt.% solution of β-CD in various ratios to obtain complexes with various compositions. Freeze-thaw hydrogels were prepared by mixing the prepared solutions of CS/β-CD with 5 wt.% PVA solution. Gelation strategy involved the freeze-thaw technique conducted by exposing the product to freezing temperatures (approximately -20°C) for 20 hours, and then allowing it to thaw at room temperature (RT) for 4 hours. The process of freezing and thawing (F-T cycles) was repeated three times. The number of F-T cycles was determined considering the previously obtained results of texture profile and rheological analyses of the grafted chitosan crosslinked with PVA being suitable for topical application and requiring low hardness and good adhesion properties. Additionally, freeze-thaw hydrogels chitosan/PVA and β-CD/PVA were prepared as reference matrices with the same compositions. Table I details the list and the precise composition of each prepared hydrogel.

TABLE I.
List of synthetized hydrogels

CODE	FORMULATION
CS/β-CD/PVA 25/70/5 + VEM	Hydrogel CS/β-CD physically crosslinked with PVA (25/70/5 combination ratio); loading with VEM was done by solution mixing after hydrogel preparation
CS/β-CD/PVA 25/70/5	Hydrogel CS/β-CD physically crosslinked with PVA (25/70/5 combination ratio), without VEM
CS/β-CD/PVA 20/75/5 + VEM	Hydrogel CS/β-CD physically crosslinked with PVA (20/75/5 combination ratio); loading with VEM was done by solution mixing after the hydrogel preparation
CS/β-CD/PVA 20/75/5	Hydrogel CS/β-CD physically crosslinked with PVA (20/75/5 combination ratio), without VEM
CS/β-CD/PVA 45/50/5	Hydrogel CS/β-CD physically crosslinked with PVA (45/50/5 combination ratio), without VEM
CS/β-CD/PVA 5/90/5	Hydrogel CS/β-CD physically crosslinked with PVA (5/90/5 combination ratio), without VEM
β-CD/PVA 95/5	Hydrogel containing only β-CD with PVA (95/5 combination ratio)by three cycles of −20/25°C freeze-thaw, without VEM
where: CS/β-CD - chitosan and β-cyclodextrin complexes; PVA - polyvinyl alcohol; VEM - Vemurafenib.	

Preparation of Vemurafenib loaded hydrogels. The biopolymeric matrices' characteristics were analyzed by means of FT-IR spectroscopy and swelling tests. The most suitable hydrogels for VEM loading designated for topical application were determined to be CS/β-CD/PVA 25/70/5 and CS/β-CD/PVA 20/75/5.

The drug loading was done by dissolving 1.5 mg VEM in 100 μL of DMSO (due to low water solubility) and mixed with β-CD solution and then complexed with CS and crosslinked with PVA. The proposed reaction mechanism is illustrated in figure 1.

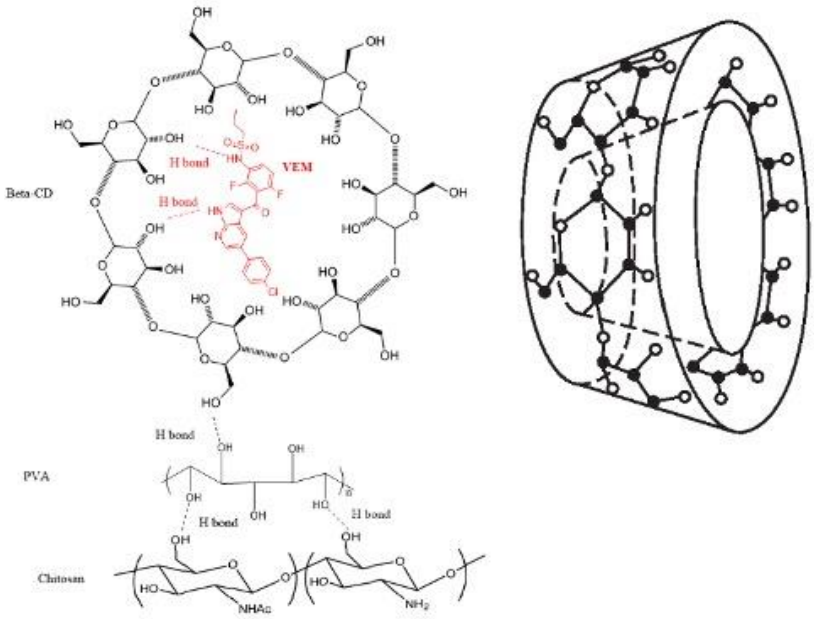


Fig. 1. Proposed preparation mechanism of biopolymeric based hydrogels

Characterization methods

The prepared complexes with various compositions in CS and β-CD with and without VEM and finally gelified by cross-linking with PVA were analyzed by means of structural identity, ability to swell on a substrate, morphological observations and drug loading capacity.

1. FTIR Spectroscopy

The chemical characterization of the obtained final hydrogels and individual components was accomplished by employing a Perkin Elmer Spectrum-100 instrument (Shelton, CT, USA), through reflection on a diamond crystal with a 45° angle, 4 cm^{-1} resolution of and over the 4000-400 cm^{-1} range

2. Hydrogel swelling behavior

The swelling behavior of the freeze-dried hydrogels was evaluated by direct immersion of formulations in acidic medium, water, and phosphate buffer saline (PBS, pH = 5.5) at various temperature values to simulate medium conditions for further *in vitro* experiments. The samples were periodically removed from the solution, wiped with a soft tissue to remove surface solution, weighed, and then carefully placed back into the beaker as quickly as possible.

The swelling degree (Q%) was calculated according to Equation (1):

$$Q\% = \frac{w_t - w_d}{w_d} \times 100 \quad (\mathbf{1})$$

where w_t is the weight of the swollen samples at time t and w_d is the weight of the dry sample.

3. Morphological observations characterization of the hydrogels

The scanning electron microscopic (SEM) images of cross-sections of the lyophilized hydrogels were captured using a Hitachi SU 1510 Scanning Electron Mi-

croscope (Hitachi Company, Japan), while the samples were set on an Aluminum stub and coated with a 7 nm thick gold layer using a Cressington 108 device before observation, at various magnification levels.

4. Drug loading capacity (DL) and drug entrapment efficiency (DEE%) of the formulations

The amount of VEM loaded in the hydrogels was determined by using a high-pressure liquid chromatography (HPLC) system Shimadzu Nexera LC-40-XR (Shimadzu, Kyoto, Japan) equipped with a serial dual-plunger pump, an autosampler (SIL 40 XR), a SPD-40V series UV-Vis, and a RF-20Axs fluorescence detector. The chromatographic separation of VEM was performed on a Waters CORTECS C18 column (2.1 × 100 mm, 2.7 μm) using as mobile phase a mixture of: A (water/formic acid - 99.9/0.1, *v/v*), B (acetonitrile) and C (methanol). Before use, the solvents were filtered through a 0.22 μm filter and degassed by ultrasonication. The injection sample volume was 10 μL, the run time was 9 min in isocratic mode (1 mL/min), and the optimal mobile phase ratio was A (40%), B (55%) and C (5%). The column temperature was maintained constant at 30°C during the chromatographic operation with UV-Vis detection for VEM (λ = 252 nm). The quantitative and qualitative analysis of VEM was based on its retention time and peak areas, respectively. The LabSolutionDB software 6.106SP1 was used for peak integration.

The calibration curve for VEM was obtained using the following procedure: stock solutions of VEM were prepared by dissolving 0.002 g of pure drug using acetonitrile in a 10 mL volumetric flask, from which we made serial dilutions, in the 100-0.78125 mg/L concentration range (r^2 = 0.9999). We found that VEM has a retention time of 6 min. To quantify the drug from the loaded matrices, we weighed the hydrogel samples, and after dissolution in 50/50 (*v/v %)* methanol/acetonitrile mixture using 2 mL vials, we centrifuged them, filtered the supernatants through 0.22 μm filter, and then 10 μL of each sample was injected and analyzed using the HPLC method described above. We also determined the limit of detection, LOD = 10.7982 μg/mL and the limit of quantification, LOQ = 32.7216 μg/mL. The amount of loaded VEM was calculated using Equation (2) derived from the calibration curve:

$$y = 45988x + 11405;\ r^2 = 0.9999 \quad \textbf{(2)}$$

The drug entrapment efficiency (DEE) represents the quantity of loaded drug (%) that is encapsulated in the formulation. The DEE of VEM from the hydrogel was calculated using Equation (3).

$$\textbf{DEE} = \frac{\text{Actual drug (VEM) content loaded in the matrix}}{\text{Theoretical drug content (VEM)}} \times 100 \quad \textbf{(3)}$$

RESULTS AND DISCUSSION

Given that the aim of the study was to optimize a treatment option for cutaneous melanoma for topical application and that semisolid forms are the most commonly used formulations for the transport of active substances through skin (18), we chose the option of incorporating an antitumor drug (VEM) in a hydrogel. The advantages are multiple, first of all the reduced risk of side effects, an enhanced patient compliance to treatment given the non-irritant properties of the hydrogels and their ability not to disrupt skin functions.

We selected the hydrogels' preparation method using PVA, a physical cross-linking agent with high biocompatibility and high mechanical strength, due to the simplicity of their synthesis process. We chose the typical preparation method by three freeze-thaw (F-T) cycles. As it is known, the number of cycles is correlated with their stability, so including a higher amount of PVA should be advantageous in the gelation process.

Structure Identification of Hydrogels

FTIR Spectra. The FTIR spectra of CS, β-CD, CS/β-CD/PVA and of the loaded hydrogels are presented in figure 2. We can observe in the graph the following representative aspects for β-CD: broad transmittance peak at 3303 cm^{-1} due to -OH stretching vibrations, asymmetric stretching vibrations of -CH were represented at 2928 cm^{-1} whereas asymmetric stretching vibrations of C-O were represented at 1633 cm^{-1}. A band at 1021 cm^{-1} was due to coupling vibrations of C-O and C-C. A characteristic band observed at 897 cm^{-1} was due to the vibration of α-(1-4) glucopyranose ring of β-CD, indicating the success of complexing β-CD to the polymeric backbone of CS. As far as the characteristic peaks of CS are concerned, from the graph we can deduce the following aspects: characteristic peaks at 2921-2887 cm^{-1} ($-CH_3$, $-CH_2$), 1636 cm^{-1} (C=O stretch vibration), 1548 cm^{-1} (secondary amide), 1655 cm^{-1} (amide band I) and 1068-1020 cm^{-1} (C-O stretching of saccharide moiety). The IR spectra of VEM should exhibit distinct absorption bands at 1734 cm^{-1} (C=O), 1325 and 1147 cm^{-1} (SO_2), and 1635 cm^{-1} (C=N), 817 cm^{-1} (C-Cl), not all very clearly observed because of the small amount that we have used for drug loading, but consistent with other studies (19). The intermolecular hydrogen bonding formed in between the drug and β-CD were evidenced by the widening of OH groups. Moreover, the hydrogel's formation is based also on hydrogen bonding, evidenced within the spectra as well.

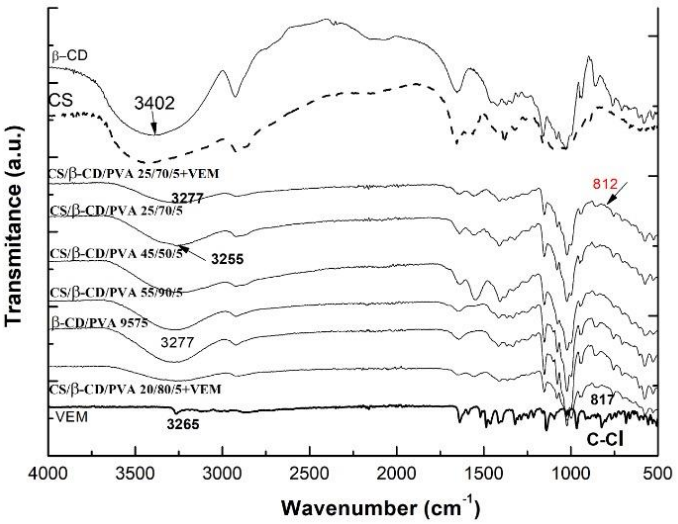
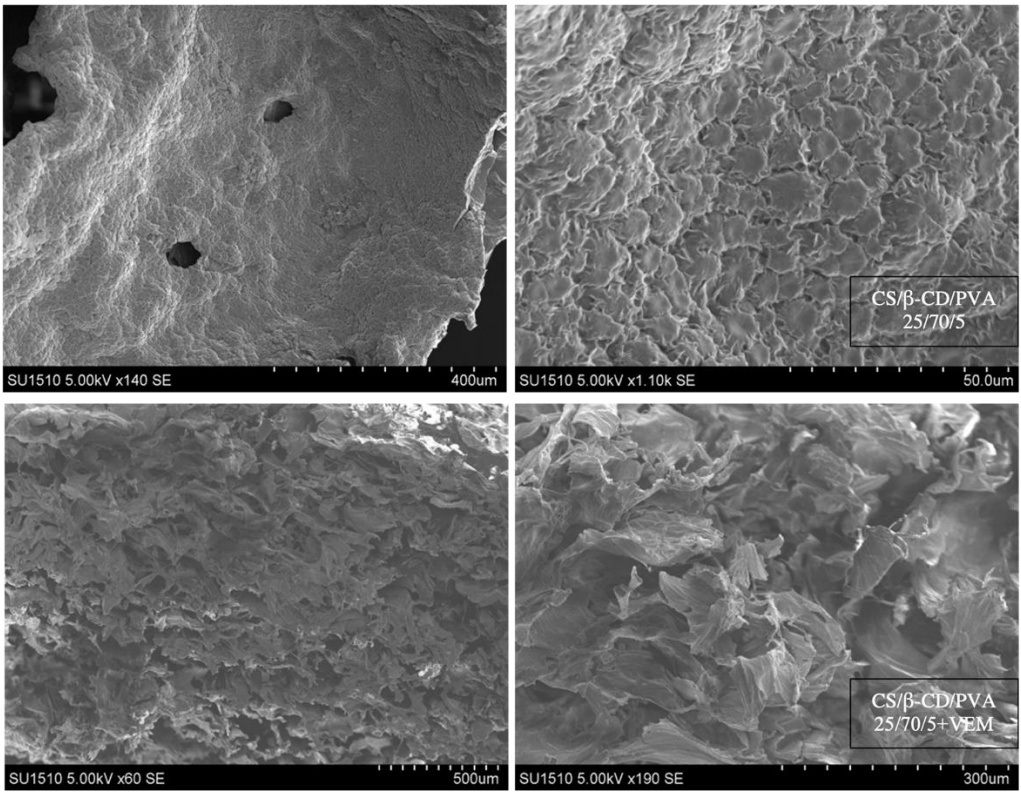


Fig. 2. ATR-FTIR spectra of the freeze-thawed hydrogels based on pure CS, β-CD, PVA, VEM and copolymers CS/β-CD/PVA loaded and unloaded with VEM

As shown within Figure 2, some characteristic vibration bands of β-CD were shifted in the inclusion complex spectra. The O-H stretching vibration from 3400 cm^{-1} of β-CD was shifted to 3250-3270 cm^{-1} within the spectra of the complexes. The shift of lower vibration bands indicated the formation of H-bonds between the OH groups of the biopolymers and PVA and thus proving the physical crosslinking.

The morphology of the prepared Hydrogels. For morphological analysis of the prepared matrices, we used Scanning Electron Microscopy (SEM) at various magnifications, as shown in figure 3.

As it is shown in the third figure, the hydrogels possess a continuous and stable network structure. Furthermore, all hydrogel samples exhibited large amounts of relatively loose and small pores, with irregular shapes and sizes providing great storage space for suitable active substances. The white dots with regular shape, that appeared in the loaded hydrogels, confirmed the presence of VEM. The roughness of the porous network that appeared on the microstructure surface of hydrogels exhibited in that figure may be attributed to the preparation and crosslinking process. The data was consistent with other SEM images found for that type of copolymer (20). The hydrogel based solely on β-CD and PVA was not taken into consideration for morphological analysis and further studies, because its very powdery texture after lyophilization was not adequate.



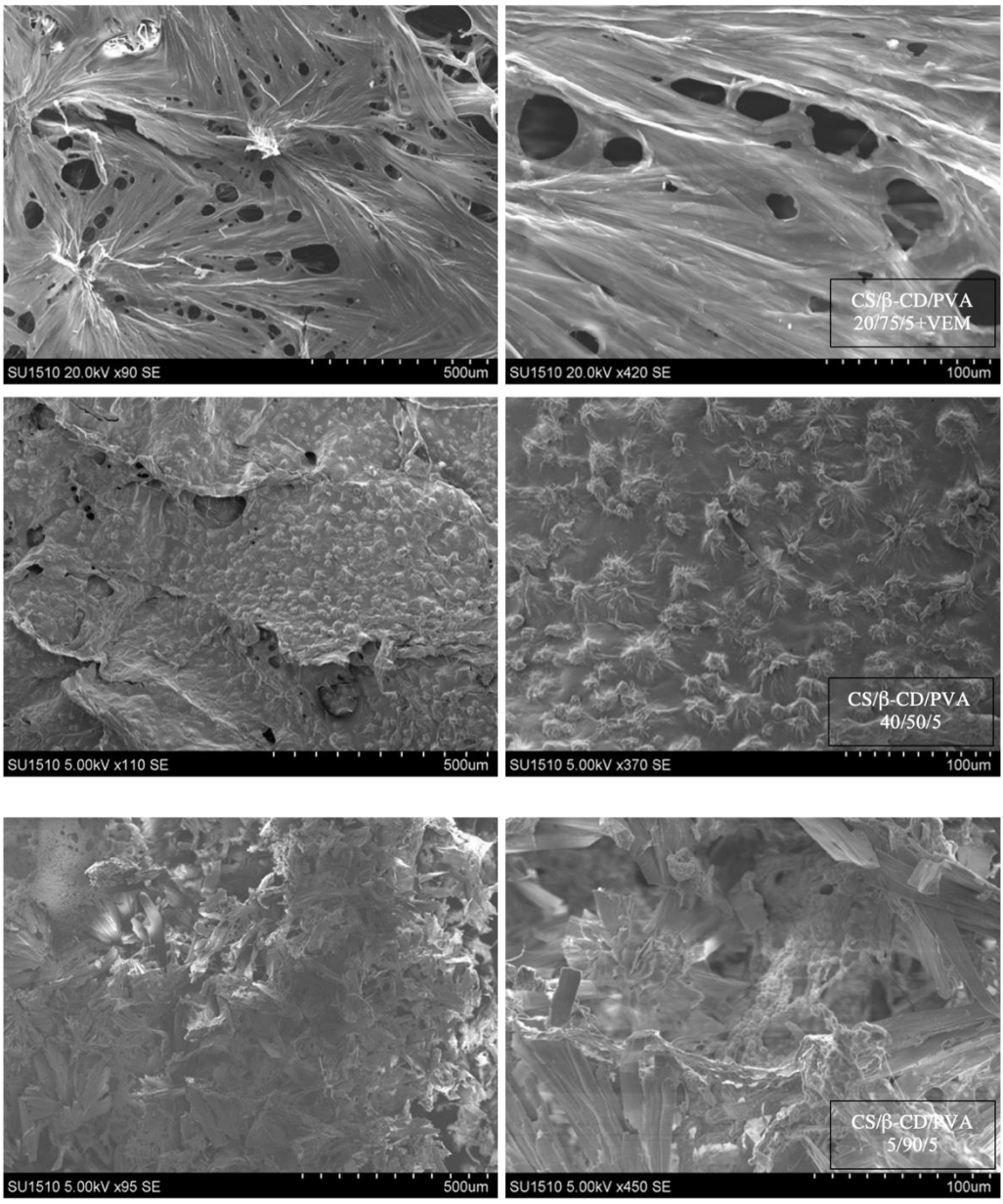


Fig. 3. SEM images for the prepared hydrogels at various magnifications

The porous aspect of the hydrogels was formed during the lyophilization process, due to the phenomenon of water sublimation from the matrix. CS/β-CD/PVA 25/70/5+VEM showed a more defined porous structure with well-interconnected pores. The CS/β-CD/PVA 20/75/5+VEM hydrogel also had a porous structure, but the diameter of the pores was larger and the walls were thicker.

The surface of the CS/β-CD/PVA 5/90/5 hydrogels was condensed with few observable pores. CS/β-CD/PVA 40/50/5 revealed spherical shapes on a smooth surface. Uncharged hydrogels exhibited a homogeneous, sponge-like morphology with well-interconnected pores.

Swelling Ability of CS/β-CD/PVA. The prepared matrices were designed to exhibit dual responsive behavior depending on various temperature and pH values at which we tested their swelling ability. The behavior of both control and drug loaded matrices were examined at various pH and temperature values as their properties may alter due to such physical changes (21).

Considering that the proposed matrices were aimed to release the active substance from a semi-solid form, the optimal pH was set at 5.5, since skin's pH is around 5.

From the graphs we observed the maximum swelling for acidic pH (being the value around which we are most interested in, considering the acidic microenvironment of tumors (22)) was around 800% due to the protonation of the free -NH_2 groups of CS, which demonstrated that optimal formulation for drug loading was CS/β-CD/PVA 25/70/5 or CS/β-CD/PVA 20/75/5 (their behavior was similar throughout the study). Similar pH-dependent swelling behavior for CS based matrices were reported in previous studies (17, 23).

Figures 4, 5 and 6 emphasize the dual responsiveness to the two stimuli, temperature and pH, for both the VEM-loaded and the control matrix. It can be observed that the loaded matrix showed a slightly lower swelling maximum, which demonstrated the incorporation of the active substance, the formation of electrostatic bonds between VEM and the polymer, and the prevention of a rapid burst release, which is not desirable, its aim being to achieve sustained drug release.

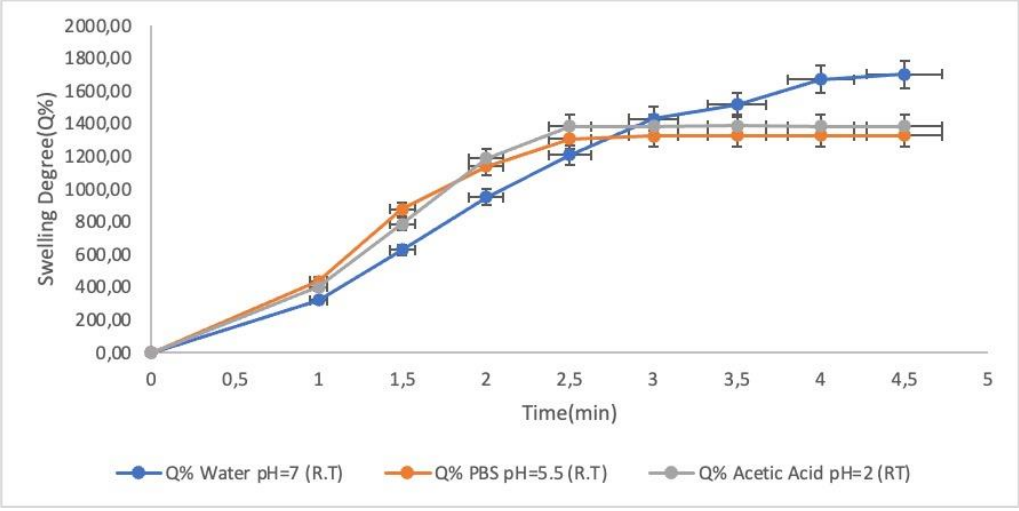


Fig. 4. Swelling behavior of the unloaded CS/β-CD/PVA 25/70/5 hydrogel at room temperature and various pH values

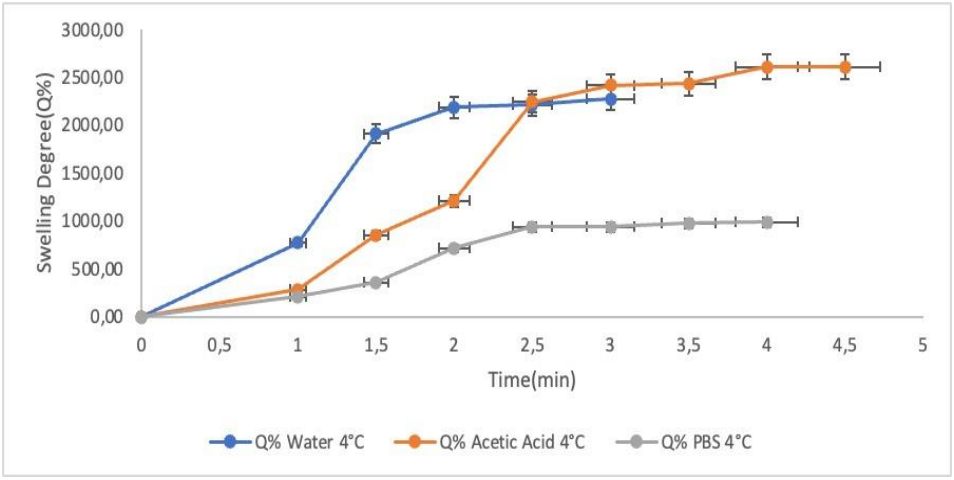


Fig. 5. Swelling behavior of the unloaded CS/β-CD/PVA 25/70/5 hydrogel at room temperature and various pH values

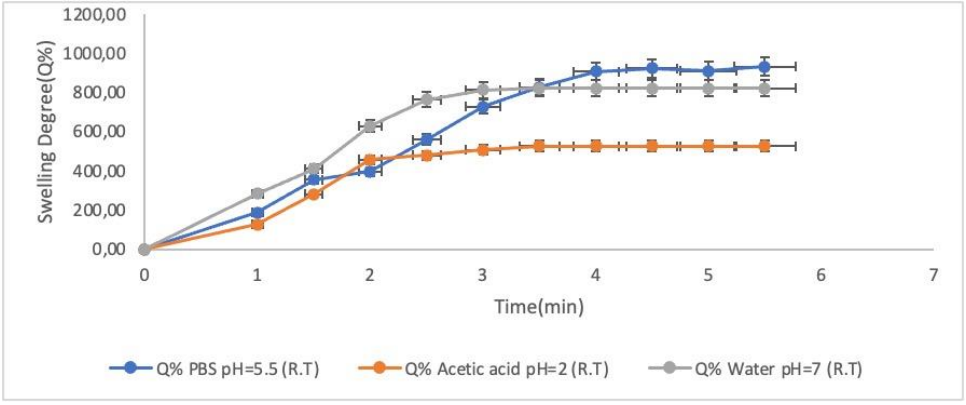


Fig. 6. Swelling behavior of the CS/β-CD/PVA 25/70/5 hydrogel loaded with VEM at room temperature and various pH values

Hydrogels are considered smart drug delivery systems precisely because of their ability to encapsulate drug substances that pose solubility problems, such as VEM. We have analyzed the drug loading capacity (DL) of our hydrogels, and we have found that for both loaded hydrogels DL was around 5.7 μg/mg, which led to 65-70% drug entrapment efficiency (DEE%), confirming a good capacity of drug encapsulation. The exact amounts and loading efficiencies are presented in table II.

TABLE II.
Hydrogels Loading VEM

Sample code	VEM loaded Hydrogel, μg/mg	VEM loading Efficiency, %
CS/β-CD/PVA 25/70/5 + VEM	5.7146	65.63%
CS/β-CD/PVA 20/75/5 + VEM	5.7672	70.93%

CONCLUSIONS

When it comes to achieving high levels of adherence to cancer treatment and drug selectivity towards cancer cells with little toxicity to healthy tissues and few unfavorable side effects, finding new smart drug delivery systems is the pillar of future therapeutic developments. The exploitation of local pH and the chemical potential of other stimuli responsive drug delivery vehicles combined with the physicochemical properties of therapeutic agents may lead to the discovery of new treatments to keep pace with the challenges brought by the increasingly aggressive forms of cancer that mankind is currently facing.

Among the various combinations of hydrogels we have experimented with, those obtained from combining chitosan, β-cyclodextrin and polyvinyl alcohol in two combination ratios (25/70/5 and 20/75/5) demonstrated excellent loading efficiency (65.63% and 70.93%, respectively).

Our proposed hydrogels have the potential to be used for that purpose, but future studies need to be performed to analyze *in vitro* and *ex vivo* release, as well as toxicity studies on melanoma cell lines.

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CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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