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In vitro release studies of poorly water-soluble compounds using the flow-through cell USP4 apparatus

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Abstract
Currently, there is a tendency for new forms of carrier-drug systems to appear with prolonged and controlled release. However, in order to design medical or pharmaceutical devices, which have to be characterized by high quality and the assumed parameters in real conditions, it is necessary to analyze this process based on *in vitro* release (IVR) testing methods. For this purpose, extracorporeal studies are carried out, which enable the determination of the release profiles of active substances using a simulated tissue-like environment. Here, we focused on the release tests of poorly water-soluble compounds (salicylic acid and fluocinolone acetonide) from the dual drug delivery system using the flow-through cell method (USP4). Additionally, bio-hybrid hydrogel matrix containing the system of thermosensitive nanocarrier with salicylic acid and fluocinolone acetonide was subjected to the following investigations: physicochemical (swelling ability, gel fraction), morphological (SEM analysis) and structural using FT-IR spectroscopy. On the basis of results, we can conclude that the USP4 method may be suitable, especially for the release tests of poorly water-soluble components introduced into modern forms of drug administration, such as polymeric matrices, hydrogels, nano- and microcarriers as well as hybrid systems.

Keywords
release study, hydrogels, poorly-water soluble compounds, USP4 method

1. INTRODUCTION

There are many methods of conducting studies on the release of the active substance from the drug form. The choice of a specific one is dictated by various factors, such as the properties of the active substance or the form of the analyzed formulation. The selection of the right method provides reliable and reproducible results, which is very important for drug quality control, as well as for determining its expected behavior in body fluids, i.e. (Kasperek, 2014). The simplest way to release the active substance from the drug form is to conduct research in a standard beaker with a stirrer. The beaker has be thermostated to keep the acceptor fluid at a constant temperature (usually 37 °C). The tested sample is also placed in the vessel. During mixing, the active substance is released, which then dissolves in the liquid. In order to determine its content, samples of acceptor fluid are taken at specified intervals and subjected to appropriate analysis (Sznitowska, 2011). Detailed and valid information on release studies is contained in the *Pharmacopoeia*. It is the official collection of standards, regulations and recommendations for pharmaceutical substances and products. It describes data on the study of the release of the active substance, which allows accurate determination of the quality of medicinal products. Several devices have been developed, the task of which is to provide conditions similar to those in which it is required for its operation in the body. According to the United States Pharmacopeia (USP), it stands out seven typically used equipment systems for releasing active substances, such as basket (USP1), paddle (USP2), cylindrical reciprocating (USP3), flow (USP4), Paddle-over-Disk (USP5), cylindrical (USP6) as well as reciprocating (USP7) apparatus (Todaro et al., 2017; Uddin et al., 2011). However, currently the flow-through cell (USP4) dissolution apparatus becomes more and more interesting, because it enables to better correlate *in vitro* data with *in vivo* situations. Moreover, USP4 method seems the proper way to carry out the research on the release of active substances from advanced modified carriers, especially hybrid hydrogels containing dual delivery systems as well as different solid dosage forms including tablets, capsules, powders, suppositories, dispersed systems, implants, and microspheres (Bhardwaj and Burgess, 2010; Browne and Kieselmann, 2010; Finnie et al., 2009; Gjellan and Graffner, 1994; Neubert et al., 2008; Nicklasson et al., 1991; Perng et al., 2003; Rawat and Burgess, 2011; Sievens-Figueroa et al., 2012; Zhang et al., 1994). The flow-through cell (USP4) device is characterized by more advanced construction than others and it includes the following elements: releasing fluid container, pump, flow cell and water bath. This type of apparatus can be operated in an open system, in which fresh acceptor media passes continuously through the test drug form. It is also possible to use a closed system, in which a constant volume of the medium flows through the analyzed sample, periodically. Additionally, release analysis can be compared using different flow rates, such as: 2, 4, 8 and 16 ml/min. A schematic diagram of USP4 apparatus is presented in Fig. 1 (Bhattachar et al., 2002; Finnie et al., 2009; Sievens-Figueroa et al., 2012).

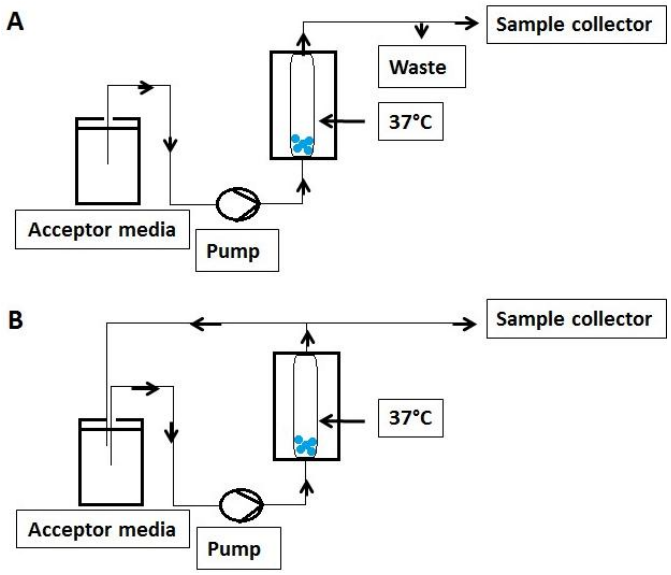

Figure 1. Schematic diagram of flow-through dissolution system: A) open mode system and B) closed mode system (adapted from Ref. (Finnie et al., 2009)).

Moreover, the flow cell may be described as the lower cone, a cylindrical portion, and the filter. Interestingly, different types of the flow chamber can be used (Fig. 2), which obviously influences the kinetics release of active substances, because of various contact between the sample and the flowing acceptor media. The following patterns can be distinguished:

a) sample is in the cone section of the cell without the 1-mm round glass beads, which causes the flow to be more nonhomogeneous;
b) sample is top of 1 mm round glass beads (6.5 g) due to more homogeneous flow;
c) sample is in the cone section, however, between 3.5 g of 1 mm round glass beads at the bottom and 3.0 g of 1 mm round beads on top, making the flow more homogeneous;
d) sample is between 6.5 g of 1 mm round glass beads and 2 g of 1 mm beads; the flow is more homogeneous;
e) sample is between the mesh and 6.5 g of 1 mm round glass beads; additionally, one millimeter round glass beads not added to the cone section; this model imparts different extent of pressure drop and flow homogenization;
f) sample is between 3.5 g of 1 mm round glass beads at the bottom and 3 g of 1 mm round glass beads on top; additionally, one millimeter round glass beads not added to the cone section by using a mesh between the cone and cylinder sections (Sievens-Figueroa et al., 2012).

In this paper, we described bio-hybrid hydrogel matrices crosslinked using different amounts of poly(ethylene glycol) diacrylate – PEGDA with M_n = 575 g/mol and after that checked its impact on the physicochemical properties of obtained materials (including the degree of cross-linking). Then, salicylic acid and both salicylic acid and fluocinolone acetonide in the thermosensitive nanocarrier system were introduced into the hydrogel matrix of the selected composition. However, the main purpose of the work was focused on the determination of the release profiles of two poorly water-soluble compounds (salicylic acid and fluocinolone acetonide) from the dual drug delivery system consisting of polymeric nanocarriers and hydrogel matrix based on sodium alginate, *Aloe vera*, and poly(vinyl alcohol) cross-linked with PEGDA 575. Moreover, *in vitro* release studies were carried out using the flow-through cell method (USP4).

2. EXPERIMENTAL PART

2.1. Materials and methods

Sodium alginate, poly(ethylene glycol) diacrylate (PEGDA) M_n = 575 g/mol (used as a cross-linking agent), N-isopropylacrylamide, N, N′-methylenebisacrylamide, salicylic

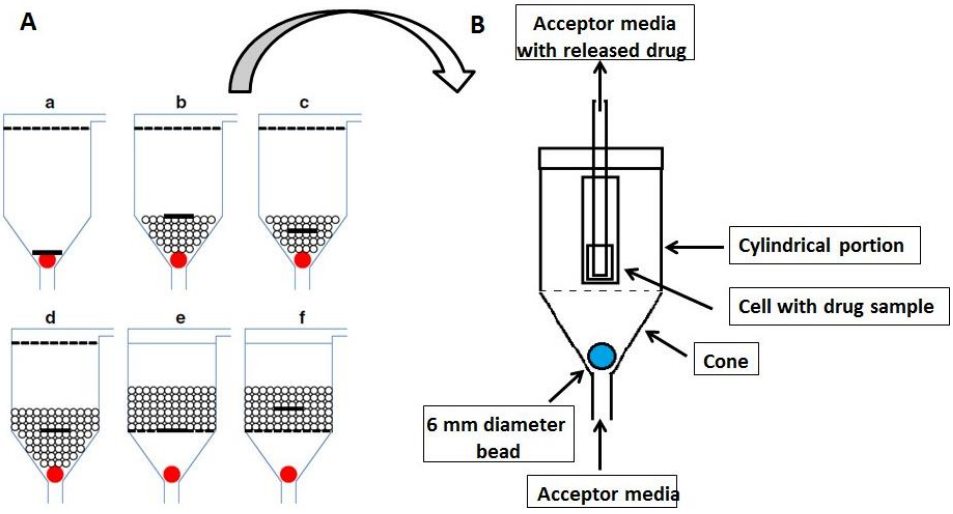

Figure 2. Schematic diagram of A – different patterns for the dissolution of films using USP4 method and B – details of a single flow chamber (adapted from Ref. (Bhattachar et al., 2002; Sievens-Figueroa et al., 2012)).

acid and fluocinolone acetonide were purchased from Sigma – Aldrich (Germany). Poly(vinyl alcohol) (Mn = 72 000 g/mol), ammonium persulphate employed as an initiator, glycerine, gum arabic were acquired from POCH SA (Poland). Phosphate-buffered saline (PBS, pH = 7.4) was purchased from Chempur (Poland), and ethyl alcohol (96%, v/v) was from Fisher Scientific. *Aloe vera* lyophilisate was purchased from a shop with cosmetics and herbal raw materials *Zrób sobie krem*, Poland.

2.2. Preparation of hydrogels

The synthesis of hydrogels was mainly based on the previously described method (Bialik-Wąs et al., 2020; Bialik-Wąs et al., 2021a, 2021b) of conventional chemical cross-linking using: 2% (w/v) of sodium alginate solution, 5% (w/v) of polyvinyl alcohol solution, *Aloe vera* lyophilizate, glycerin (1.7% (v/v), 1% (w/v) solution of ammonium persulfate as an initiator. However, in this study a drug-free reference matrix was prepared (M) using various amounts of poly(ethylene glycol) diacrylate (PEGDA, Mn = 575 g/mol) as a cross-linking agent, such as: 6.7 (M_P575_6.7), 7.5 (M_P575_7.5), 8.3 (M_P575_8.3) and 9.2% v/v (M_P575_9.2). The system of thermosensitive nanocarrier-salicylic acid (T_SA) and both salicylic acid and fluocinolone acetonide (T_SA+FA) were introduced into hydrogel matrix (M), but in the presence of 7.5% v/v of PEGDA 575. Finally, bio-hybrid hydrogels were obtained: M_P575_7.5_SA and M_P575_7.5_SA+FA. All details about synthesis of empty thermosensitive nanocarriers (118 nm) based on N-isopropylacrylamide and N, N′-methylenebisacrylamide and concerning the encapsulation process of salicylic acid and fluocinolone acetonide into thermosensitive nanocarrier (T_SA (356 nm) and T_SA+FA (369 nm)) were presented in previous research (Bialik-Wąs et al., 2020; Bialik-Wąs et al., 2021a; 2021b; Chen et al., 2014).

2.3. Characteristics of the basic matrix and bio-hybrid hydrogels incorporated with the system of salicylic acid and fluocinolone acetonide – thermosensitive nanocarriers

2.3.1. Determination of gel fraction

The obtained hydrogel materials were cut into 10 × 10 mm pieces, dried at 40 °C for 24 h and weighed (W_0). Then dried hydrogel samples were soaked in distilled water for 48 h up to an equilibrium swelling weight for removing the leachable or soluble parts from matrices. The gel materials then were dried again at 40 °C for 24 h and weighted again (W_e). The gel fraction ($\%GF$) was calculated with the following equation (1).

$$\%GF = \frac{W_e}{W_0} \cdot 100\% \quad (1)$$

2.3.2. Determination of swelling ratios

The swelling ratio ($\%SR$) was evaluated by 1010 mm of hydrogel samples immersion in the excess of distilled water or phosphate-buffered saline (PBS, pH = 7.40) at room temperature. The dried and weighed (W_d) hydrogel samples were immersed in the proper media. The swollen samples were taken out and weighed (W_s) after 3 h. The water uptake of all the tested hydrogel samples was calculated using Equation (2):

$$\%SR = \frac{W_s - W_d}{W_d} \cdot 100\% \quad (2)$$

2.3.3. FT-IR (ATR)

To identify the chemical structure of hydrogels, infrared spectroscopy was done with a Thermo Scientific Nicolet iS5 FT-IR spectrometer equipped with an iD7 ATR accessory in the range of 4000–400 cm^{-1}.

2.3.4. SEM

The surface morphology and cross-section of obtained hydrogels were analyzed using SEM (Scanning Electron Microscope) Apreo 2 S LoVac (Thermo Fisher Scientific) equipped with X-ray energy dispersion spectrometers – EDS detectors: UltraDry (Thermo Fisher Scientific) and Octane Elect (EDAX Ametek GmbH). All measurements were conducted in high vacuum. Two detectors were used to observe the sample: the ETD (Everhart-Thornley) detector for secondary electrons for operation in the high vacuum mode and the T1 (BSE) detector, which is part of the Trinity In-column Detection system for secondary and backscattered electrons. All samples were placed on the table with carbon tape and sputtered with a 5 nm layer of gold in an argon atmosphere.

2.4. The release profiles of salicylic acid from bio-hybrid hydrogels using USP4 method

The release of salicylic acid and fluocinolone acetonide from the thermosensitive nanocarrier and bio-hybrid hydrogels were conducted using the USP4 method (DZF II Flow-Through System, Erweka GmbH, Langen, Germany). The apparatus contains seven in-line flow-through diffusion cells (Fig. 3). The membrane was placed over support with an orifice of 1.5 cm diameter (diffusional area, 1.766 cm^2). The vertical cell was made of glass and was designed to have a volume into the donor compartment of 6.22 ml. The cells were placed in a cell warmer connected to the Erweka heater DH 2000i and the Erweka piston pump HKP 720. The piston pump transports the acceptor media via seven channels to the flow-through cells and automatically adapts the flow rate setting. All the determinations were made in triplicate

Figure 3. Photos of USP4 apparatus (DZF II Flow-Through System, Erweka GmbH, Langen, Germany).

for each cell. The release studies of salicylic acid and fluocinolone acetonide were carried out using a regenerated cellulose membrane Spectra/Por® Dialysis Membrane MWCO 6-8,000 Carl Roth® Company. The assays were performed in 2% ethanol in PBS (pH = 7.40) at a temperature of 37 °C. A flow rate of acceptor media was 4 ml/min. The released concentrations of salicylic acid and fluocinolone acetonide were analyzed using UV-Vis spectroscopy (Perkin Elmer Company), at the wavelength of 295 nm and 244 nm, respectively. Moreover, a closed mode system with special time intervals (0.5; 1; 1.5; 2; 24; 48; 72; 96; 120 and 144 h) was used.

3. RESULTS AND DISCUSSION

3.1. Gel fraction

Gel fraction results of obtained hydrogels are presented in Fig. 4.

First, gel fractions of hydrogels crosslinked with different amounts of PEGDA 575, such as: 6.7; 7.5; 8.3 and 9.2%, v/v, were compared. In the case of matrix with 6.7% of PEGDA 575, gel fraction is above 50% and it is quite low in relation to others. However, for samples with a higher amount of PEGDA 575, no significant changes were observed. The levels of gel fractions were very similar and they were about 70%, which also relates to our previous research involving hydrogels crosslinked using PEGDA 700 (Bialik-Wąs et al., 2021c; Bialik-Wąs et al., 2022b). That is why for further modification in order to obtain bio-hybrid hydrogels, 7.5% of PEGA 575 was selected. It turned out that introduction of T_SA system into the matrix, decreases only slightly the gel fraction to about 63%. But already the presence of a system with two drugs – T_SA+FA in the hydrogel networks caused a significant decrease of the gel fraction by up to 55%.

3.2. Swelling abilities

The results of the swelling ability of the tested hydrogels in different fluids are presented in Figure 5.

In the case of the swelling degree of the base matrices with different content of the cross-linking agent, the lowest value of the parameter is characterized by the sample containing the cross-linking agent in the amount of 6.7%. The value of

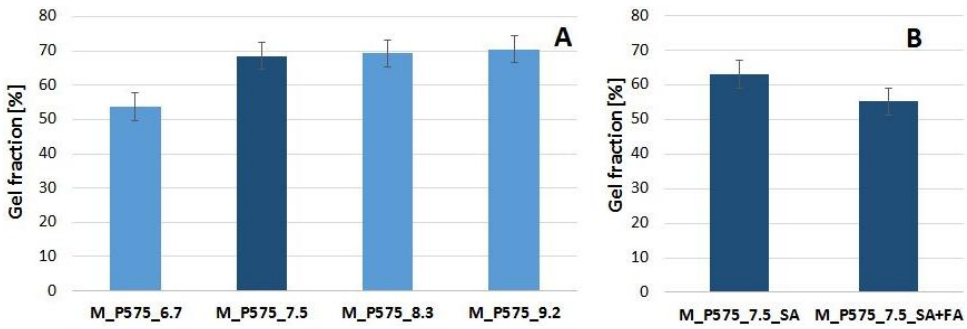

Figure 4. Gel fraction results of hydrogels crosslinked with different amounts of PEGDA 575 (A) and bio-hybrid hydrogels containing the system of thermosensitive nanocarrier-salicylic acid (T_SA) and both salicylic acid and fluocinolone acetonide (T_SA+FA) (B).

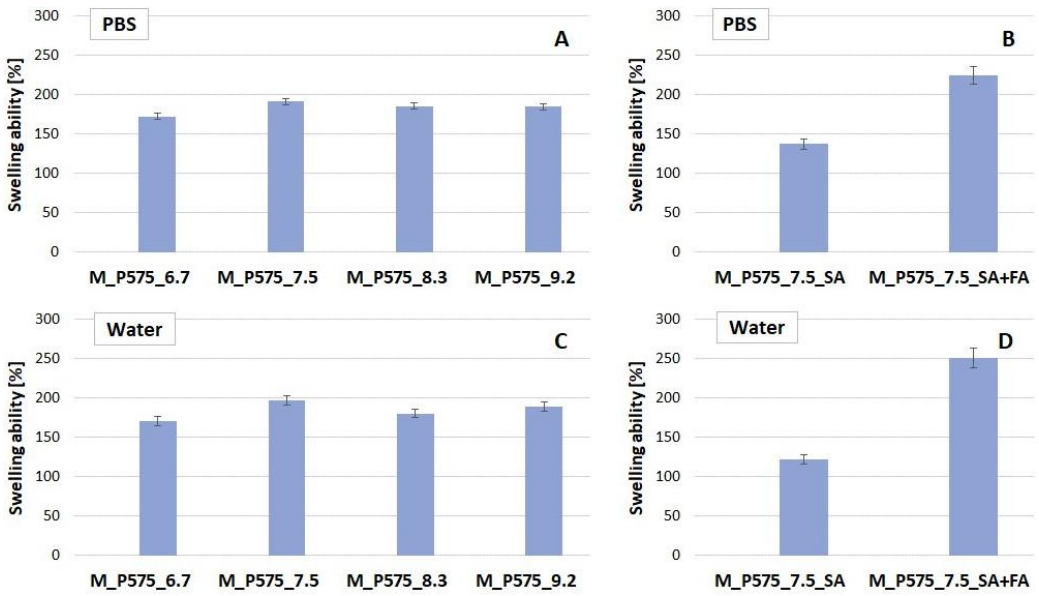

Figure 5. Swelling abilities (%) of hydrogels crosslinked with different amounts of PEGDA 575 and bio-hybrid hydrogels containing the system of thermosensitive nanocarrier-salicylic acid (T_SA) and both salicylic acid and fluocinolone acetonide (T_SA+FA) after tests in PBS (A, B) and distilled water (C, D).

the parameter for the M_P575_6.7 sample in both incubation fluids is approximately 170%. The degree of swelling for the other hydrogels containing the cross-linking agent in the amount of 7.5; 8.3 and 9.2% remains at a similar level, which is less than 200%. The introduction of active substances into the selected base matrix – M_P575_7.5, significantly affects the value of the swelling degree of the obtained bio-hybrid hydrogels. For the matrix containing only salicylic acid (M_P575_7.5_SA), a significant reduction in the value of the analyzed parameter was observed up to 138% in PBS and 122% in distilled water. However, in the case of the matrix containing both salicylic acid and fluocinolone acetonide (M_P575_7.5_SA+FA), the swelling ratio increased to 225% in PBS and 250% in distilled water. The increase in the value of the parameter is associated with a reduced value of the gel fraction compared to the base matrix and a more developed porous structure of the material compared to the hydrogel containing only one drug. A similar tendency was observed in previous studies on bio-hybrid hydrogels cross-linked with PEGDA 700 (Bialik-Wąs et al., 2022a).

3.3. FT-IR analysis

FT-IR spectra of hydrogels containing different amounts of PEGDA 575, but with the highest gel fractions, were compared in Fig. 6, while Fig 7 presents FT-IR spectra of bio-hybrid hydrogels.

The FT-IR spectra of the base matrices with the content of the cross-linking agent equal to 7.5; 8.3 and 9.2% do not show significant differences and overlap to a large extent. The most significant difference is the variable intensity of the band

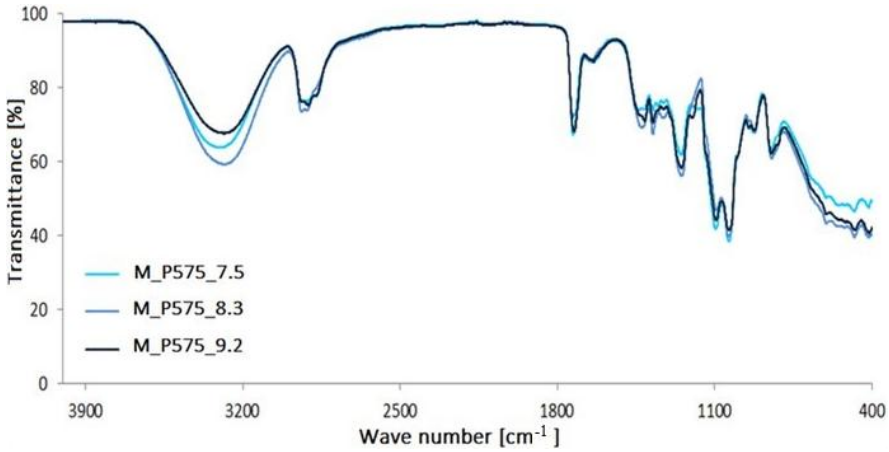

Figure 6. FT-IR spectra of hydrogels containing different amounts of PEGDA 575.

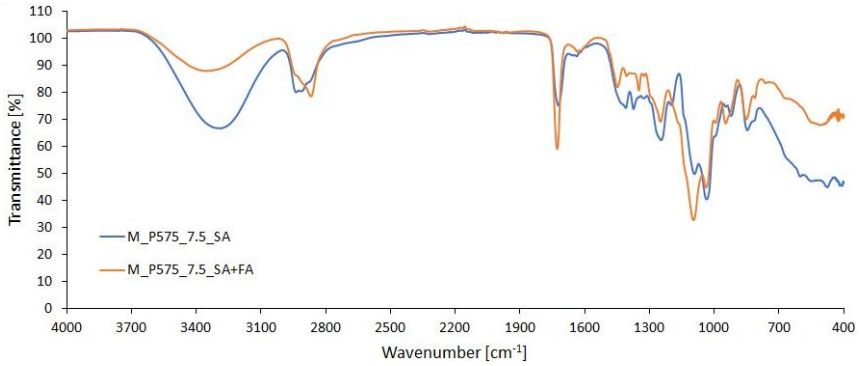

Figure 7. FT-IR spectra of bio-hybrid hydrogels.

at the wavenumber of $\sim$ 3300 cm $^{-1}$. This band corresponds to the vibrations of stretching –OH bonds, which are present in the basic components of the matrix, such as poly(vinyl alcohol), sodium alginate, and *Aloe vera*. The increase in the intensity of the bands in this range can be interpreted as an increase in the share of hydrogen interactions in the matrix structure. In addition, the bands at wavelengths of 1720, 1090 and 1035 cm $^{-1}$ correspond to C=O stretching vibrations and C–O–C stretch vibration present in the structure of the cross-linking agent – PEGDA 575 (Bialik-Wąs et al., 2022b; Silverstein et al., 2005; Socrates, 2001).

The incorporation of the system of thermosensitive nanocarrier-drugs to the base matrix influenced the course of the FT-IR spectrum, especially in the case of the sample containing two-drugs (M_P575_7.5_SA+FA). In relation to the matrix with one drug (M_P575_7.5_SA) a decrease in the intensity of the band at the wavenumber $\sim$ 3300 cm^{-1} is visible, which corresponds to the strength of hydrogen interactions in bio-hybrid hydrogel. In addition, the same sample is characterized by an increase in the intensity of the bands characteristic of the bonds present in the structure of the cross-linking agent (1720 and 1090 cm^{-1}) and a decrease in the intensity of the bands corresponding to the C–O–C group derived from glycosidic bonds (1250 and 1035 cm^{-1}). In addition, the bands present at a wavelength of 1410 cm^{-1} for the M_P575_7.5_SA sample and 850 cm^{-1} for both bio-hybrid hydrogels correspond to the C=C bonds present in the structure of unreacted PEGDA 575 cross-linker. The presence of the mentioned bands confirms the lower gel fraction of bio-hybrid hydrogels compared to the base matrix (Bialik-Wąs et al., 2022b; Silverstein et al., 2005; Socrates, 2001).

3.4. SEM analysis

SEM images were made for bio-hybrid hydrogels, taking into account both the surface morphology (Fig. 8) and cross-section (Fig. 9) of analyzed samples.

The surface morphology of analyzed bio-hybrid hydrogels, in the case of M_P575_7.5_SA, exhibits less porosity and the structure is more dense, which confirms also the cross-section

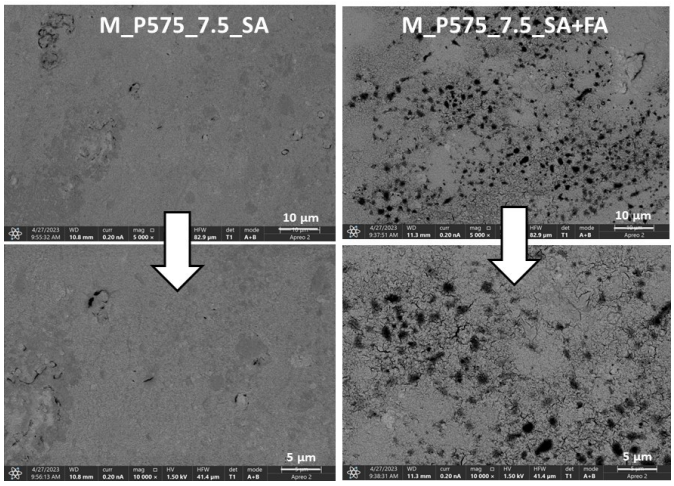

Figure 8. The surface morphology of bio-hybrid hydrogels.

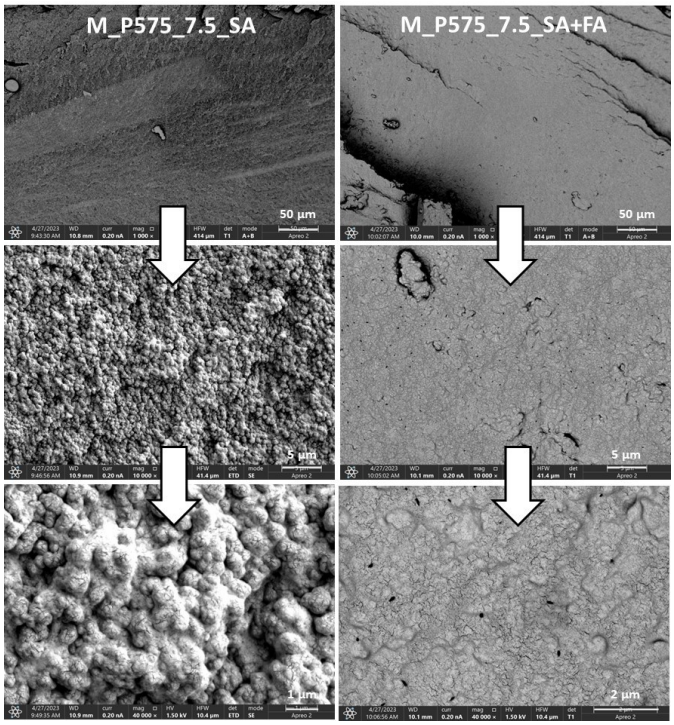

Figure 9. The cross-section of bio-hybrid hydrogels.

of this sample. The result is similar to hydrogel crosslinked with PEGDA 700 (Bialik-Wąs et al., 2022a; Bialik-Wąs et al., 2022b). However, the introduction of two drugs (salicylic acid and fluocinolone acetonide) into matrix crosslinked with 7.5% of PEGDA 575 causing the surface to have more pores, which is visible also in its cross-section. Probably, it is associated with differences in the gel fraction of bio-hybrid hydrogels. The matrix – M_P575_7.5_SA was characterized by higher gel fraction, which was about 63%. Hence, stronger interactions between sodium alginate, poly(vinyl alcohol), *Aloe vera* and PEGDA occurred in this hydrogel, which confirmed also FT-IR analysis. The three-dimensional network of M_P575_7.5_SA consists of densely intertwined polymer chains, which has a huge impact on the release profile of active substances from the matrix.

3.5. *In vitro* release of salicylic acid and fluocinolone acetonide using USP4 method

Generally, taking into account the drug release profiles from polymeric matrix or other polymeric carriers, you can distinguish more often three phases, rarely two – mainly in the case of micro- and nanoparticles (Kamaly et al., 2016). Obviously, the release of active components depends on many parameters (acceptor media, temperature, release method) and carrier properties (porosity, gel fraction, swelling ability, degradation possibility). In this research double release system consisting of thermosensitive nanocarrier and hydrogel matrix, was used to avoid the "burst effect", which was observed in our previous studies (Bialik-Wąs et al., 2022a). Release profiles of salicylic acid and fluocinolone acetonide from bio-hybrid hydrogels using the flow-through cell method (USP4) are presented in Figs. 10 and 11.

Results of this study clearly indicate that the salicylic acid is much faster released from the M_P575_7.5_SA+FA matrix. The first phase can be specified as "lag time", when drug is gradually released. Importantly, no burst effect was observed during the first measurements. The maximum concentration of the drug in the acceptor media, such as $Q = 54\%$, appeared after 24h of the starting analysis. Subsequently, the second increase of released drug was observed after 120h. Although, release profile of salicylic acid from the M_P575_7.5_SA matrix is similar, "lag time" is shifted and prolonged and the maximum concentration of drug ($Q = 61\%$) appeared after 96h. It may be caused by the differences in physicochemical and morphological properties of bio-hybrid hydrogels. In the case of M_P575_7.5_SA+FA, matrix is more porous (SEM images) due to the fact that acceptor media molecules penetrate rapidly inside the three-dimensional structure, which was confirmed also in swelling results. Moreover, this sample is characterized by lower gel fraction, which indicates that polymeric chains are loosely in-

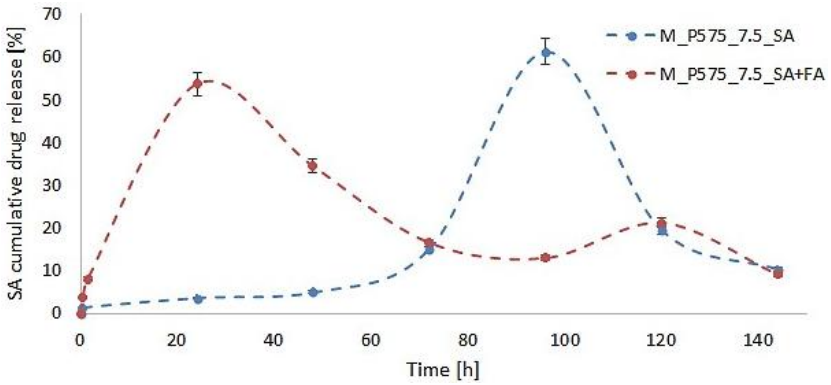

Figure 10. Release profiles of salicylic acid from bio-hybrid hydrogels: M_P575_7.5_SA and M_P575_7.5_SA+FA.

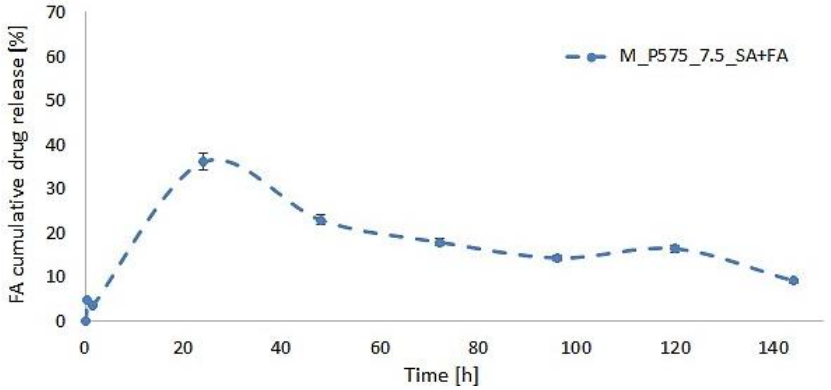

Figure 11. Release profiles of fluocinolone acetonide from the bio-hybrid hydrogel: M_P575_7.5_SA+FA.

tertwined. However, the structure of M_P575_7.5_SA matrix is more dense and the gel fraction is higher. That is why the drug can be longer inside of the hydrogel. Furthermore, depending on the kind and amount of crosslinking agents, it can design different polymeric carriers, which allows to release active components within the desired time (Bialik-Wąs et al., 2022a). Whereas, in the first phase of the release of fluocinolone acetonide, the situation is similar to salicylic acid release from M_P575_7.5_SA+FA. It means that also after 24 h of analysis, the concentration of drug was the highest and it was about 36%. However, next the amount of released drug decreased slightly and then it remained at a quite constant level until 120 h.

4. CONCLUSIONS

To sum up, the flow-through cell USP4 method seems to be a good alternative to release active substances, especially poorly-water soluble, because it allows to get better correlation of *in vitro* data with *in vivo* situations. Moreover, this method can be used in the case of advanced carriers, such as: hybrid hydrogels, polymeric matrix, implants or stents. The determination of gel fractions and swelling degrees as well as structural (FT-IR spectra) and morphological (SEM images) analysis allowed to explain the differences in the release of poorly water-soluble compounds from bio-hybrid hydrogels. The M_P575_7.5_SA sample, characterized by a higher gel fraction, a slightly lower degree of swelling and more dense structure, indicated prolonged release with shifted "lag time" of salicylic acid (Q_{max} = 61%, after 96 h) compared to the matrix containing the system of thermosensitive nanocarrier with two drugs Ms_P575_7.5_SA+FA (Q_{max} = 54%, after 24 h). These release profiles were possible to achieve thanks to the double drug delivery system, such as polymeric nanocarriers and hydrogel matrix. It can be concluded that by controlling the degree of cross-linking of the hydrogel matrix and its physicochemical properties, it can design carriers with the desired release profile depending on the application.

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