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Synthesis, characterization and bioevaluation of irinotecan- collagen hybrid materials for biomedical applications as drug delivery systems in tumoral treatments

Research Article

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Abstract: The purpose of the present study is the preparation and characterization of collagen/antitumor drug hybrids as drug delivery systems. Materials used for obtaining collagen-based drug delivery systems were collagen type I (Coll) as matrix and irinotecan (I) as hydrophilic active substances. After incorporation of I into Coll in differing ratios, the obtained hybrid materials (Coll/I) could be used according to our results as potential drug delivery systems in medicine for the topical (local) treatment of cancerous tissues or bone.

The released amount of I varies with amount of Coll from hybrid materials: the higher, the slower the release amount of irinotecan transferred is in the first 6 hours.

The *in vitro* citotoxicity demonstrates an antitumoral activity of the obtained hybrid materials and their potential use for biomedical applications as drug delivery systems in tumoral treatments.

Keywords: *Collagen • Irinotecan • Hybrid materials • Drug delivery system*

1. Introduction

Coll is an adequate support for drugs' transfer, offering the advantage of being a natural biomaterial with hemostatic and wound healing properties. The positive effects of Coll on tissue regeneration and its interaction with cells are the main causes of increased interest in the local treatment of affected tissue or in the formation of new tissues such as bone, skin or nerves [1-8].

Coll hydrogels are three-dimensional networks of fibrils formed randomly, which include large amounts of fluids [9,10]. Water retention is due to hydrophilic groups: amine, carboxyl and hydroxyl, which also allow hydrogen bond formation and ionic and hydrophobic interactions, which ensures their consistency.

The mechanical properties are determined by their structural integrity and ease of fibrillar fluid displacement through the fibrils which form the network [9-15]. They are improved by reticulation with chemical or physical agents.

Chemical changes of Coll are performed in order to obtain new materials with superior properties to native biopolymers, in strict correlation with desired applications. This goal can be achieved using the following methods polymer-analogous transformations (reactions to functional groups of natural polymers with low-molecular compounds, from which new polymers are produced), grafting reactions (reactions to the main biopolymer chain involving the participation of a low-molecular reactive polymerisable and synthesis of

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configurations branched type), cross-linking (reactions from which are obtained three-dimensional structures).

Irinotecan (I) is a hydrophilic compound; it is a topoisomerase I inhibitor, which prevents DNA from unwinding, chemically being a semi-synthetic analogue of the natural alkaloid camptothecin. It is a relatively new antitumoral drug, used to treat many types of cancer (rectal and colon cancer, ovarian and small-cell lung cancer and malignant glioma), generally used in combination with other chemotherapeutic drugs, but also in the terminal stages of cancer of various types. Currently it is administered intravenously, in shock doses, in different concentrations depending on the treatment plan and it produces disastrous side effects such as myelosuppression, cardiotoxicity, alopecia, stomatitis, tissue damage, venal sclerosis [16-19].

Controlled release is another way in treating affected tissues. The term refers to the capacity of the system to maintain for a long period of time the desired drug concentration in the blood - in the case of transdermal systems or affected tissue - for topical systems, the transfer is being made with controlled speed [1,6-8,20-23]. Kinetics of drug delivery systems is affected by several factors: characteristics of drug dissolution/diffusion, its distribution in matrix or hydrogel, drug/polymer ratio, swelling and erosion of the support and surface geometry [1,6-8,10,20-23]. Collagen based drug delivery systems are well known in the literature for many skin and bone applications because collagen is one of the main component of these tissues. It is also worth mentioning that collagen is recommended as a support for drug delivery because it assures faster wound skin and bone healing [24,25]. An extensive review published by Friess [5] presents the main application forms of collagen and collagen based materials for the treatment of different diseases by using various drugs from antibiotics and cytostastics to vitamins and hormones.

The production of drug delivery systems, having in composition collagen (Coll) and antitumoral drugs for chemotherapy, may contribute to the improvement of human health by reducing the incidence of surgical treatment and stages of cancer development. Most devices used in local drugs release have polymeric support, the ideal vehicles for release being biodegradable polymers [20-23].

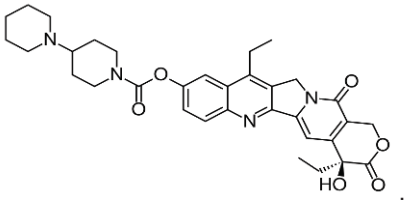
Taking into account the side effects of I, this present study aims at preparing and characterizing collagen/irinotecan (Coll/I) hybrid materials. We aimed to achieve the synthesis and analysis of Coll/I hybrid materials, for a possible use as a drug delivery system of I in the treatment of various cancer types. This controlled drug delivery system could be transdermally used in the treatment of skin cancer. The obtained Coll/I hybrid materials were characterized by means of X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR), UV-Vis spectroscopy and scanning electron microscopy (SEM). The *in vitro* profile reveals the potential of the obtained hybrid materials for biomedical applications as drug delivery systems in tumoral treatments.

2. Experimental procedure

2.1. Materials

The materials used in this study were: type I fibrilar bovine collagen (MW=300 000Da, 2.54% and pH=7) from National Institute of Textiles Leather Research & Development, Branch Leather and Footwear Research Institute located in Bucharest obtained through chemical extraction was used; irinotecan (Sigma Aldrich; purity 99.9%); glutaraldehyde solution for the cross-linking of the collagen (Sigma Aldrich; 1%).

The irinotecan structure is:



2.2. Preparation of the hybrid materials

The Coll/I hybrid materials were obtained starting from Coll gel and I powder as can be seen in Fig. 1. It was used different concentrations of Coll (0.5%, 1%, 1.5%) and of I (1%, 3%, 5%), as presented in Table 1.

Collagen cross-linking was performed with 1% glutaraldehyde solution (glutaraldehyde was 0.5% compared to Coll dry) to produce stabilization of Coll molecules, by maintaining this stability during use and by increasing the time of transfer, stability which is also necessary in the freeze drying process. Chemical cross-linking is essential for ensuring the stability of matrix in contact with the physiological environment and it is responsible for the changes of the physico-chemical and morphological properties, for the drug transfer and their biological properties [1-3,5,9]. Obtaining hybrid materials as porous /cell by keeping the gel structure was achieved by drying the gels using freeze drying technique. The freeze-drying parameters were freezing at -55°C (2 h), followed by manual main drying at 0.1 mbar (12 h at -55°C followed by 8 h at 0°C and 16 h at 35°C).

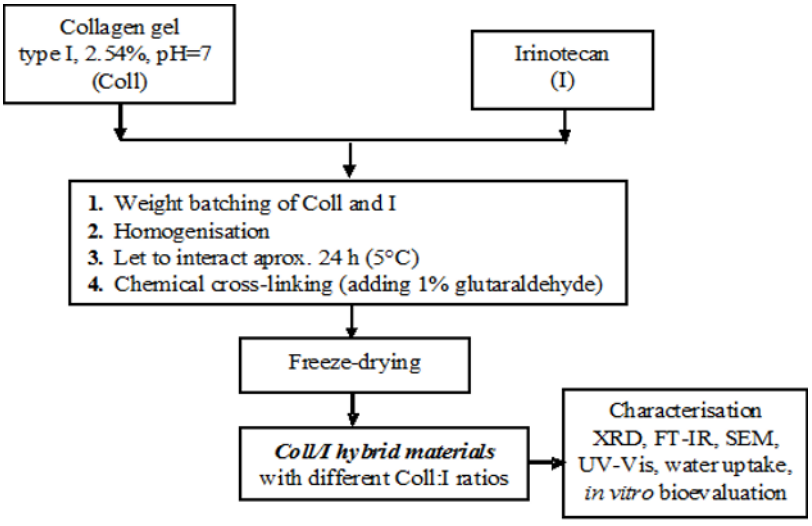


Figure 1. Scheme of the synthesis of Coll/I hybrid materials with desired morphology and biological activity.

Table 1. Composition of synthesized hybrid materials.

Symbol hybrid material	Concentration of I* (%)	Final concentration of Coll* (%)
A1	1	1.5
A2	1	1
A3	1	0.5
B1	3	1.5
B2	3	1
B3	3	0.5
C1	5	1.5
C2	5	1
C3	5	0.5

**these materials were weighting as dried components*

2.3. Characterization

For the identification of crystalline phases of powders and to gather information on their degree of crystallinity, X-ray diffraction analysis was carried out on a Shimadzu diffractometer XRD 6000 - Ni-filtered CuKα (λ = 1.5406 Å) radiation, scanning speed of 2°/min in 2θ range of 3 - 40°.

Additional information regarding the structure and the bonds between chemical species present in the analyzed materials was obtained by IR using a Nicolet 6700 FT-IR with ATR. The spectra were recorded over the wave number range 400–4000 cm^{-1} with a resolution of 2 cm^{-1}.

Morpho-textural study of the synthesized materials was carried out using a scanning electron microscope Hitachi S 2600 N.

The water uptake capacity was carried out using phosphate buffer pH 7.4 as immersion medium (n = 3). Pieces which contain collagen matrices of approx. 2 cm^2 area were weighed (W_d) and then immersed in phosphate buffer pH 7.4. At established time intervals, the hydrated scaffolds were weighed (W_w) and water uptake was calculated using the following equation [12,26]:

$$\text{Water uptake} = (W_w - W_d)/Wd \ (g\ g^{-1}),$$

where W_w represents the weight of wet matrices at immersion time t and W_d denotes the weight of dry scaffolds.

In order to highlight the ability of a controlled release of the drug from hybrid materials similar to those synthesized one, there are particularly used spectrometric methods such as UV-Vis technique. Kinetics and the transfer mechanism of the I and also the quantitative determination of drug released from Coll was performed through an UV-Vis at a wavelength of 370 nm using UV obtained on product (0.025 g I/1L distillated water). For these was used a Thermo Evolution 300 spectrophotometer.

For in vitro citotoxicity of the obtained hybrid materials to assess the antitumor activity, 1×10^5 HCT8 cells (ATCC CCL 244™) were seeded in wells with 1 cm diameter and maintained in RPMI 1640 (Gibco, NY, SUA) supplemented with 10% heat-inactivated bovine serum and penicillin/streptomycin, at 37°C with 5% CO_2 and humid atmosphere. After 24 hours, into each well was added one sponge that was cut to the size 0.35 cm^2. After another 24 hours, the effects were

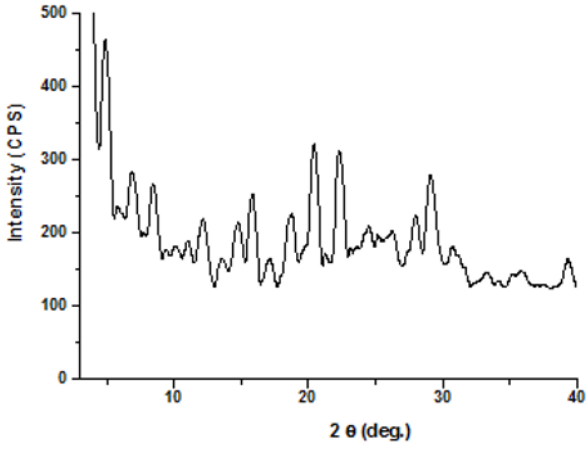


Figure 2. XRD pattern of irinotecan.

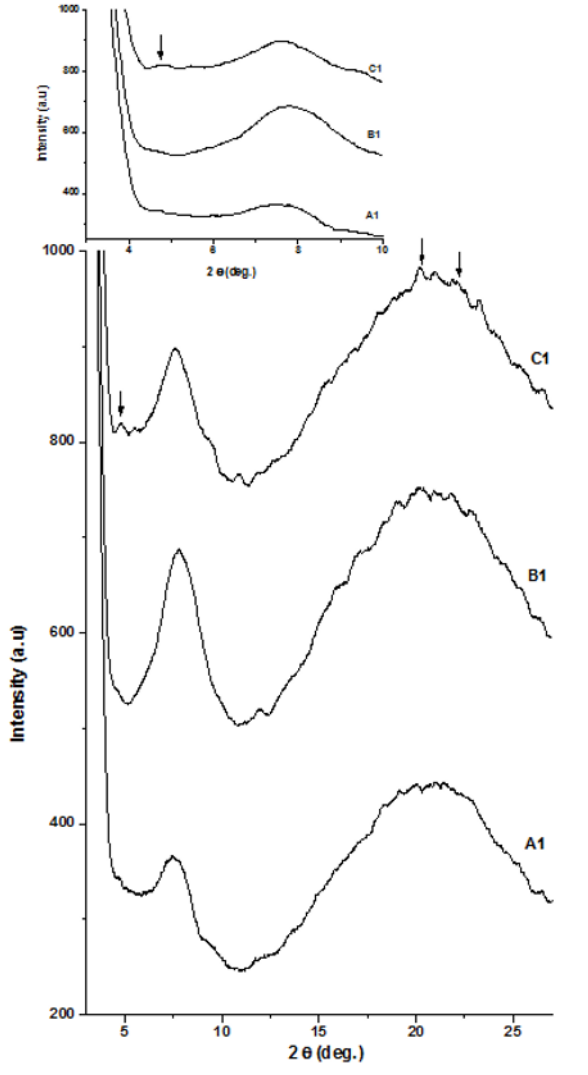


Figure 3. XRD patterns of A1, B1 and C1 obtained hybrid materials.

evaluated using fluorescein diacetate and propidium iodide. In order to evaluate the dead cells (red) and the viable ones (green), fluorescence on the surface was quantified using Observer.D1 Carl Zeiss microscope.

3. Results and discussion

3.1. X-ray diffraction

In Fig. 2 is shown the specific diffractogram of drug I, observing a low degree of its crystallinity through its low intensity of diffraction interferences and the halo of the small angle.

Fig. 3 shows that hybrid materials have a low degree of crystallinity, mainly by Coll (halos of 5-10 deg. and 13-27 deg. fields). For sample C1, which has the highest drug concentration (5%) it can be observed a low intensity interference feature of I at 4.81 deg. (see arrow). The detection of this interference in A1 and B1 masses is not observed due to the low concentration of drug in the sample, below the detection limit of the device.

3.2. FT-IR spectroscopy

In Fig. 4 are shown the FTIR spectra for samples A, B and C at different concentrations of drug (1-5% irinotecan). In the case of Coll/I hybrid systems, FT-IR spectra confirm the characteristic peaks of both the drug and the support material. In addition to the characteristic bands of Coll (1626, 1540, 1230 cm^{-1}) it also highlights the most important band characteristic of the 1715cm^{-1}, which shows the drug incorporation into the Coll matrix.

These are in according to the literature data [15,27-30] which showed Coll characteristic absorption bands are located at 3297 cm^{-1} (amide A), 2931 cm^{-1} (amide B), 1632 cm^{-1} (amide I), 1547 cm^{-1} (amide II) and 1240 cm^{-1} (amide III). The most important band for studying higher order structures of proteins is the amide I, 1650 cm^{-1}, sensitive to the secondary structures its shape being representative for the secondary structure of Coll.

The broad band at 3297 cm^{-1}, amide A, is due to the NH stretching vibration. It is also due to the OH component, which confirms the active participation of water in the collagen molecule [31].

The amide I band appears in the range 1632 cm^{-1}. It is produced mainly by the peptide bond C=O stretching vibration. This band is used for secondary-structure analysis of the polypeptide [32]. Amide II absorption arise from amide N–H bending vibrations and C–N stretching vibrations [32,33]. The amide III peak is complex, consisting of the components from C–N stretching and

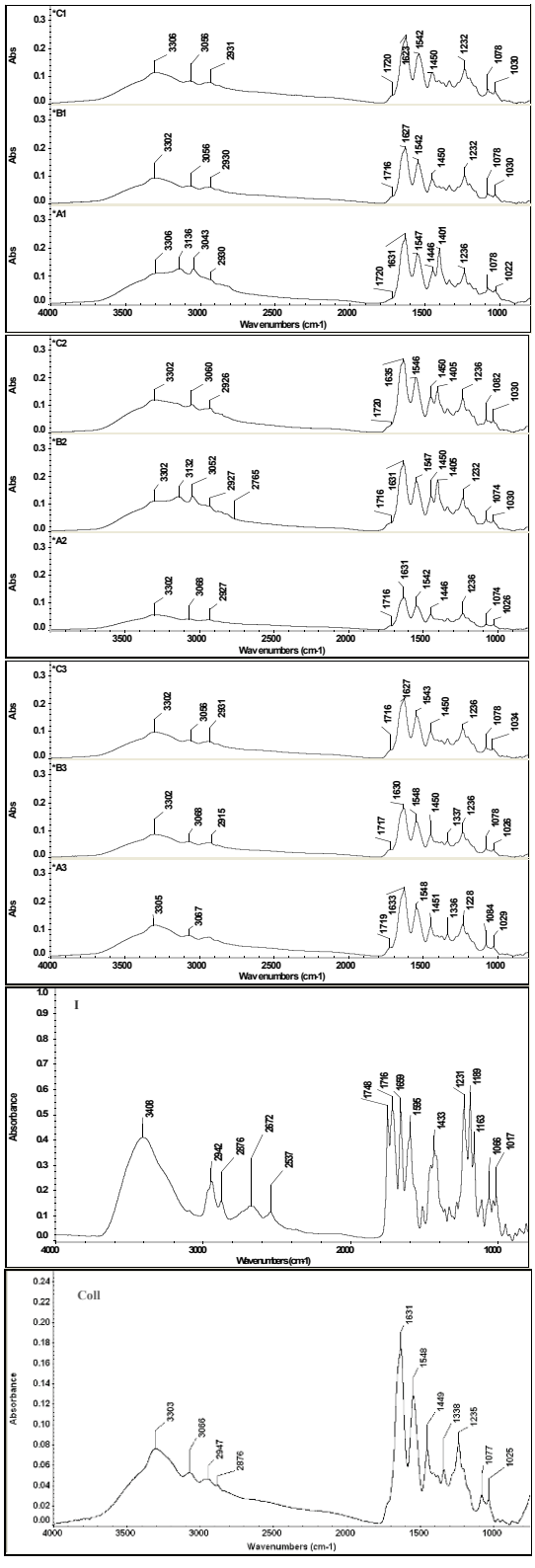


Figure 4. FT-IR spectra of the collagen (Coll), irinotecan (I) and of A, B and C obtained hybrid materials, where: A - with 1% I, B - with 3% I, C - with 5% I; 1 – 1.5% Coll; 2 - 1% Coll; 3 - 0.5% Coll.

N–H in plane bending from amide linkages, as well as the absorptions arising from wagging vibrations from CH_2 groups in the glycine backbone and proline sidechains [33,34].

Since I has several groups capable of forming bonds, there were temporary bands assignments to the spectrum of its functional groups. Thus, the group C = O bond vibration occurs in the region 1870-1540 cm^{-1} (1748, 1715 and 1657 cm^{-1} from lactone, carbamate and pyridone, respectively) [23,35,36]. The most important bands of I can be observed at 1715 cm^{-1} and 1657 cm^{-1}. The carbonyl group at 1715 cm^{-1} is the one assigned to quinoline from the product structure and at 1657 cm^{-1} in the I spectrum at pH 7.0 was given the rest of the pyridone carbonyl group. The stretching vibration of aromatic C-C and the bending vibration of C=N appear both at 1515 cm^{-1}. The bands at ~1620 cm^{-1} and 1234 cm^{-1} correspond to aromatic C=C and to C-CH_3 stretching vibrations. Esther C-O has a stretching vibration peak at 1150 cm^{-1} and the broad band found at 3431 cm^{-1} is due to associated OH groups.

3.3. Scanning electron microscopy (SEM)

Coll matrices are porous structures with pores of different sizes, shapes and orientations. The pore characteristics are induced by the colagen gel concentration, by processing route as well as by the presence of other components [37,38]. Based on these, it is expected that increasing the amount of irinothecan will induce morphological changes. The morphological structure of Coll matrices influences drug diffusion through network, degradation properties and interaction with cells. The effect of the interaction between Coll and the incorporated drug on matrix morphology was revealed through pore size using SEM.

In Fig. 5 are presented SEM images for the Coll/I obtained hybrid materials and it can be observed that increasing drug concentration leads to thickening of fibers for constant Coll content (compare A1 with B1 and C1), and at different concentrations of Coll and constant content of drug notes that with the decreasing concentration of Coll matrix is composed mainly of fibrils, being a more porous microstructure.

As a general rule, all the samples exhibit a special suprastructure, collagen mollecules being assembled in interconnected fibrils. The layered suprastrastructure of collagenous materials are known in literature [39], the layers being mainly composed by plates while fibrils act as spacing agents between the layers. Without any other components, the morphology of these materials is far from that obtained e.g. in the presence of polyvinyl alcohol but able to induce different delivery of irinothecan, as presented below.

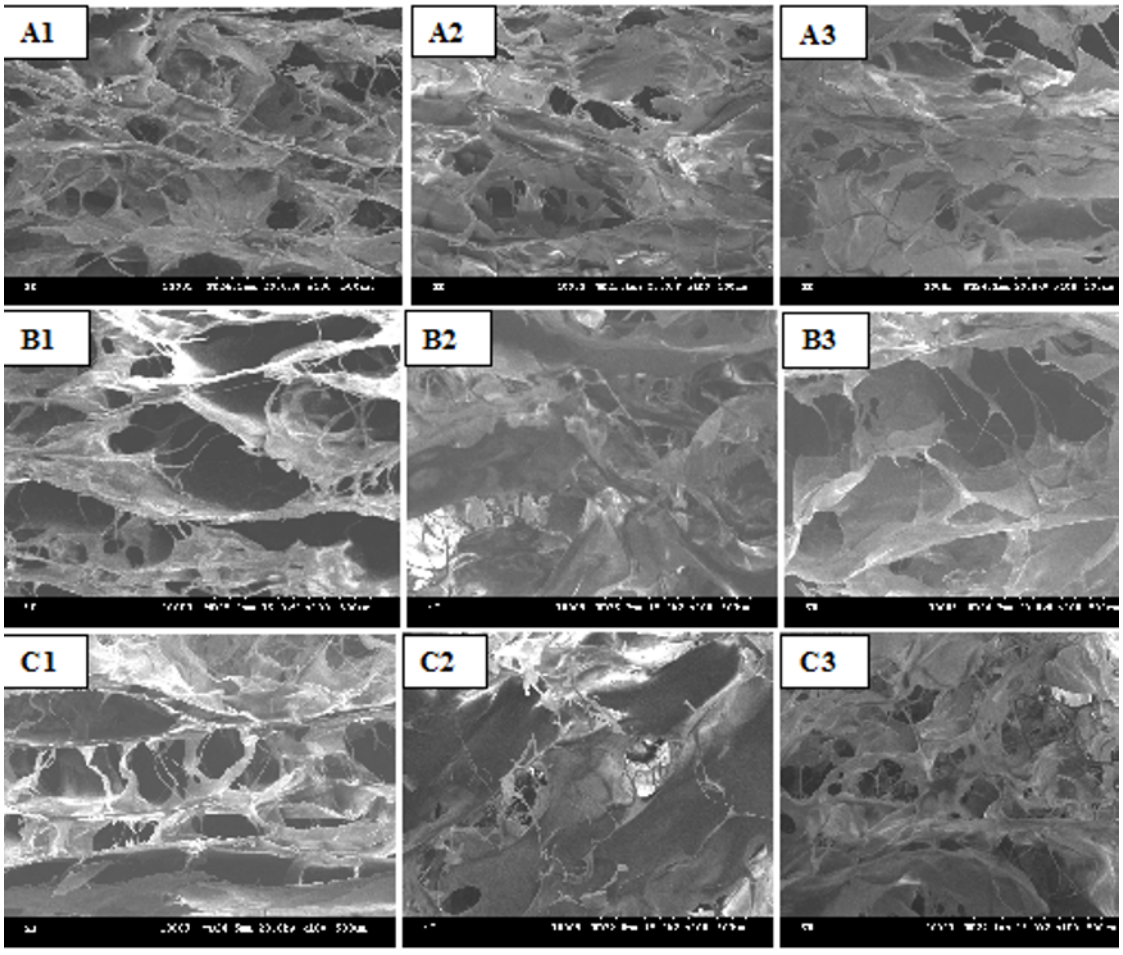


Figure 5. SEM images of A, B and C obtained hybrid materials, where: A - with 1% I, B - with 3% I, C - with 5% I; 1 – 1.5% Coll; 2 - 1% Coll; 3 - 0.5% Coll.

The matrix A1 has regular structure, lamellar, zigzag, with elongated pores, interconnected through Coll fibers, A2 material has porous structure with circular and elongated pores, interconnected through fiber, while the A3 material presents a more disordered structure with thin blades, and even transparent and with interconnected pores with a multitude of fibrils which considerably increase in size. Compared with materials A, B and C hybrid materials have similar structures, which differ depending on the concentration of Coll (its decreasing leads to the formation of some disorganized structures with bigger and unordered pores and thin layers).

3.4. Determination of water uptake capacity of collagen matrix from Coll/I hybrid materials

In order to establish a hydrophilic feature of the synthesized hybrid materials and thus to obtain information on their porosity water uptake was performed. Depending on the material hydrophilicity it can be appreciated its integration/acceptance in the body; experimentally it was demonstrated that due to the increase hydrophilicity it can more easily integrate/be accepted in the body. The obtained water uptake curves for synthesized materials are presented in Fig. 6.

It is noted that the water uptake values are high for all synthesized hybrid material which highlights a strong hydrophilic character. Also, for the masses with a constant content of drug but a variable content of Coll it is observed that the absorption is higher for the masses with a lower content of Coll. In this case the amount of water uptake is explained in addition to the hydrophilic nature of the hybrid material also by a higher porosity. Water uptake values recommend the synthesized hybrid materials as potential materials that can be used in contact with living tissue.

3.5. UV spectrophotometry

From literature data it is known that the kinetics of drug release from a controlled release system has the specific

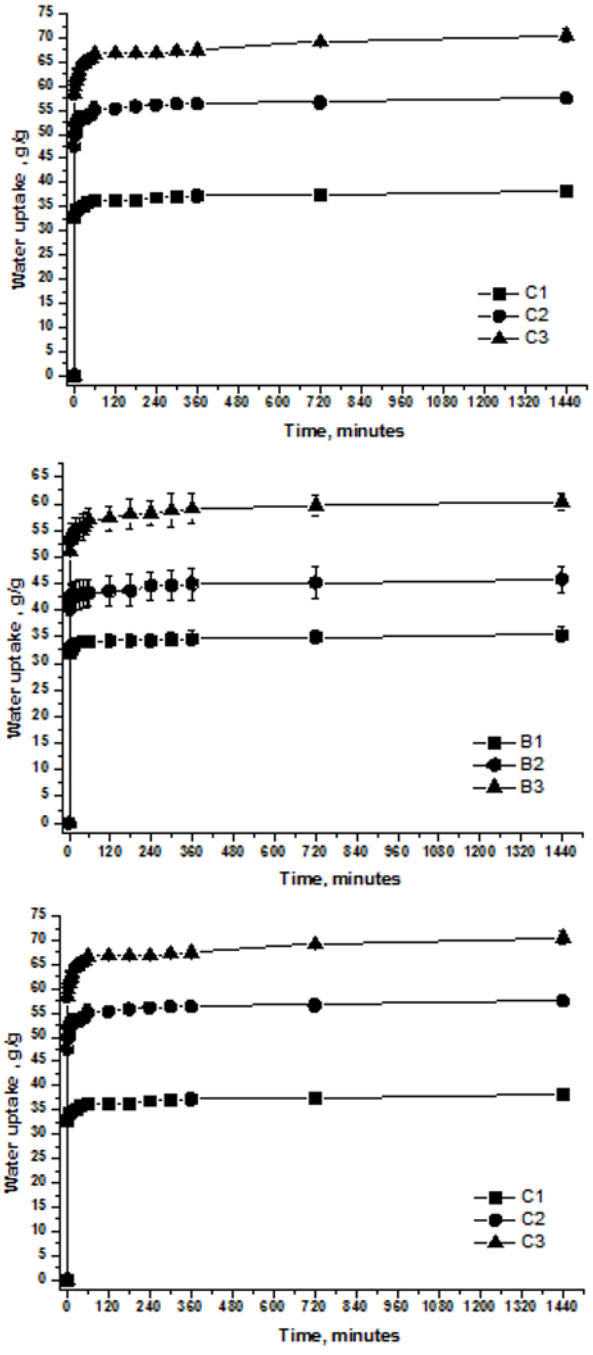


Figure 6. Water uptake curves of A, B and C hybrid materials, where: A - with 1% I, B - with 3% I, C - with 5% I; 1 – 1.5% Coll; 2 - 1% Coll; 3 - 0.5% Coll.

graphical representation [37]. Kinetics and the transfer mechanism of I and also the quantitative determination of drug released from Coll was performed through an UV spectrophotometry. The release of I is presented in Fig. 7, with detailed release within 6 hours.

While analyzing the kinetic release plots it was found that in the first period of time (about 4 hours) there was a rapid increase in the amount of drug released in all materials involved. In the following period of time there was an observed slower release. This phenomenon is explained by the fact that the drug initially broadcasts from the superficial layers of the material, specifically from its pores and only then, from the meshes of the interpenetrate network.

Table 2. The release kinetic of synthesized hybrid materials by fitting data with the Peppas equation.

Sample	Peppas – Power law	
	Order	R^2
A1	0.0860	0.9619
B1	-	-
C1	-	-
A2	0.2980	0.4132
B2	0.1280	0.9417
C2	0.0560	09616
A3	0.0382	0.9835
B3	0.1026	0.8959
C3	0.0162	0.9961

Also, it can be observed that the time destinated for the release of the drug during the first 4 hours depends on the concentration of the drug, the amount of drug that was released being proportional to the concentration of the irinotecan from the samples. Thus, by comparing the absorbances corresponding to material B with the absorbances corresponding to material A a 3:1 ratio was determined, and by comparing the absorbances corresponding to material C with the absorbances corresponding to material A a 5:1 ratio was determined. This shows an optimal retention of the drug into the Coll matrix with controlled release kinetics.

Comparing the obtained curves in Fig. 7 with the theoretical statistical curves from literature data [40] shows that these hybrid materials can be used as potential systems of controlled drug release.

Based on the in vitro data, the release kinetic was studied by fitting these data with the Peppas equation as well as the logarithmic one – Table 2. Due to the very complex release mechanism the Peppas model is used [24]. The release data obtained in the case of B1 and C1 do not fit to the Peppas equation. The regression factor (R^2) for the sample A2 is only 0.4132 which mean that the release of irinotecan is not controlled by Peppas mechanism. The delivery of the other systems can be considered to be controlled by Peppas mechanism. Based on experimental order, it can conclude that the delivery rate is strongly influenced by support (especially collagen concentration) and degree of loading (irinotecan concentration). Peppas equation:

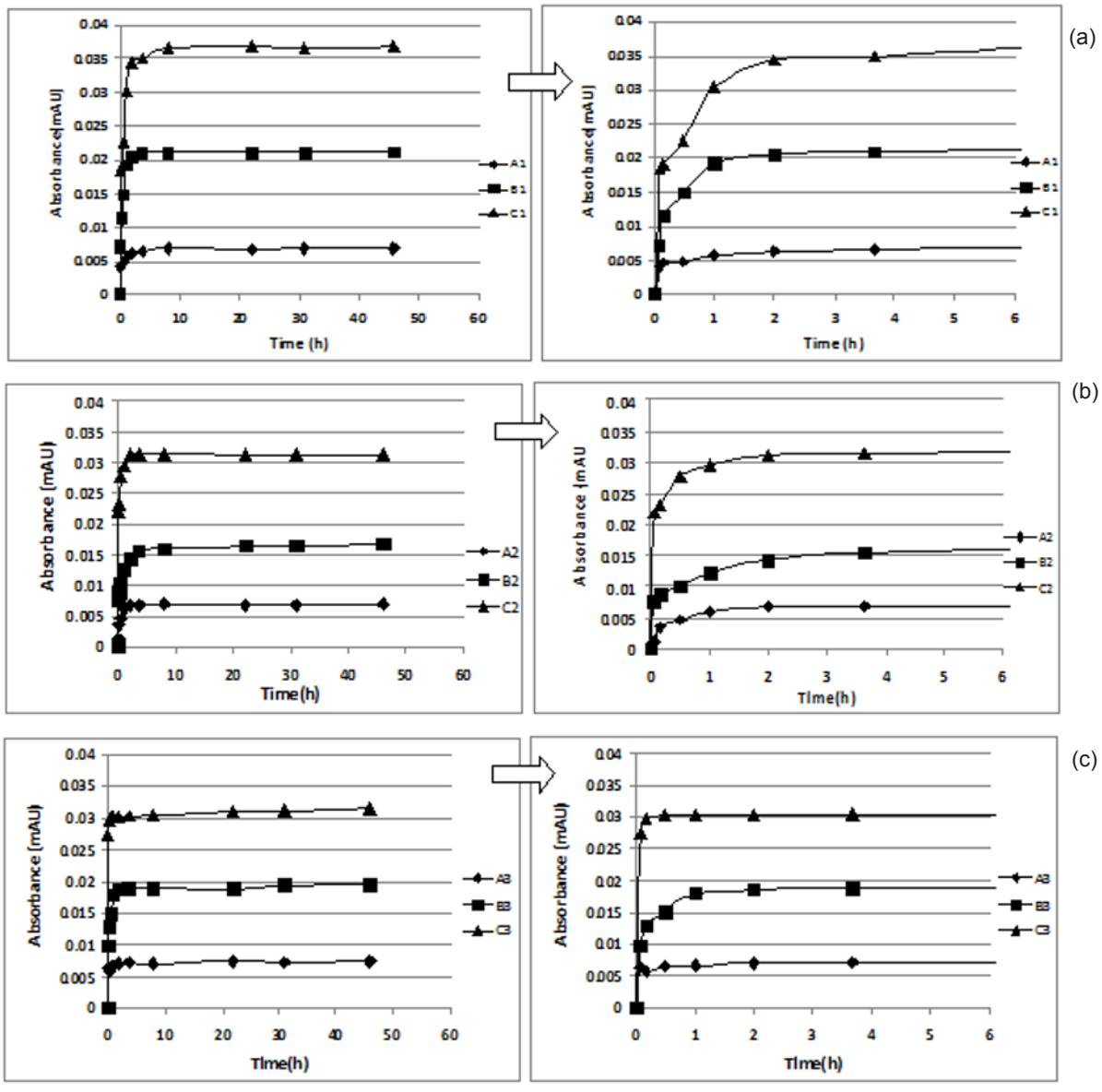


Figure 7. In vitro transfer profile of I from Coll/I hybrid materials; A, B and C hybrid materials, where: A - with 1% I, B - with 3% I, C - with 5% I; 1 – 1.5% Coll; 2 - 1% Coll; 3 - 0.5% Coll.

$M_t/M_\infty = k \cdot t^n$, where M_t/M_∞ is the fraction of drug released after time t in respect to amount of drug released at infinite time, k is the rate constant and n is the diffusion exponent.

3.6. Citotoxicity

In Fig. 8 are presented fluorescent microscopy images which show a lower cytotoxicity level in the A1, B1, C1 and increased cytotoxicity in series: A<B<C and A1(B1, C1) <A2(B2, C2) <A3(B3, C3), simultaneous with an increase of I content and decrease of Coll concentration (viable cell - green and dead cells – red). So, the *in vitro* citotoxicity proves an antitumoral activity of the obtained materials and their potential use for biomedical applications as drug delivery systems.

4. Conclusions

Synthesis and the properties of hybrid materials were detected by XRD, FT-IR, UV-Vis and SEM. The *in vitro* citotoxicity proves that novel obtained hybrid materials could be used for biomedical applications, especially as drug delivery systems in the tumoral treatments.

For the treatment of various cancerous tissue diseases requiring cytotoxic substances administered

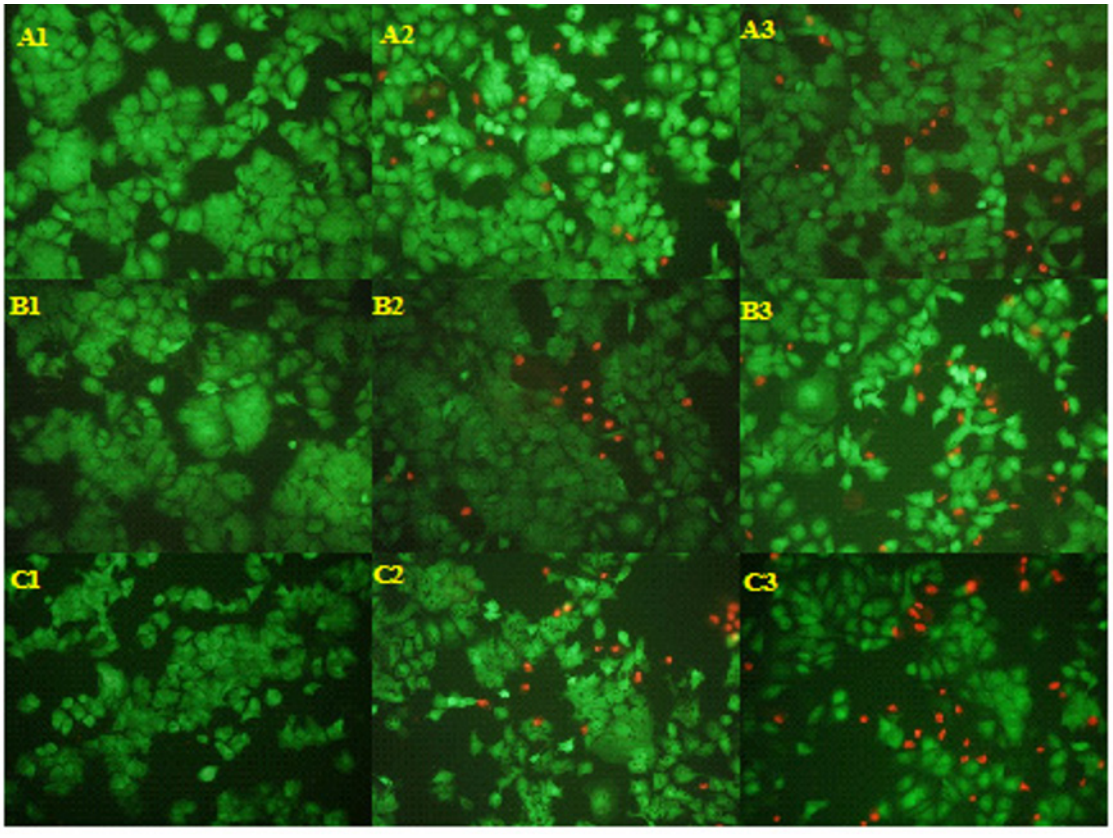


Figure 8. Fluorescent microscopy images of A, B and C hybrid materials (IF 200x), where: A - with 1% I, B - with 3% I, C - with 5% I; 1 – 1.5% Coll; 2 - 1% Coll; 3 - 0.5% Coll.

transdermally, a controlled release system containing I and Coll, in any of the concentrations presented during this study could be used, depending on the stage of development of the disease. A major advantage is that the Coll structure is not distorted, matrices are easily recognized by the body in the process throughout the tissue repair process.

As perspective, other collagen based drug delivery systems will be obtained and tested for the treatment of bone and skin cancer.

Acknowledgements

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