Vol. 8, No. 4, 2014 Chemistry

***Liudmyla Makeieva, Iryna Gladyr, Rita Rozhnova and Nataliia Galatenko***

# SYNTHESIS OF BIOACTIVE FOLATE-FERROCENE CONJUGATE

*Institute of Macromolecular Chemistry of the National Academy of Sciences of Ukraine*
*48, Kharkivske Shosse, 02160 Kyiv, Ukraine; lyudmila.makeeva@gmail.com*

*Received: June 17, 2013 / Revised: July 07, 2013 / Accepted: January 16, 2014*



**Abstract.** Through the stage of ferrocene carboxylic acid synthesis folate-ferrocene conjugate was first synthesized. The structure of the conjugate was confirmed by IR and $^1$H NMR-spectroscopy. A study of the biological activity of folate-ferrocene conjugate by method of tissue culture was undertaken.

**Keywords:** ferrocene carboxylic acid, folate-ferrocene conjugate, folic acid, biological activity, method of tissue culture.

## 1. Introduction

Ferrocene is one of the most well-known metal-organic compounds, which is used in catalysis, organic synthesis, and industry [1, 2]. Thermostability, chemical resistance, and physical properties of ferrocene determine its use in many fields of science. Its properties related to biological activity, namely the antitumor activity by immune system stimulating, attract special interest [3].

Since folate receptors are expressed in some tumors, receiving of folate-ferrocene conjugate as magnetosensitive material in malignant tumors hyperthermia is topical [4-6]. Conjugation of folic acid will secure efficient selective transport of magne-tosensitive particles to cancer cells in the presence of normal cells, thereby improving the efficiency of guided local hyperthermia of cancer.

Conjugation of folic acid to the ferrocene will allow to receive a new material that can be used for both direct delivery and the development of new bioactive polymer implantation materials.

For this purpose we have developed a method of folate-ferrocene conjugate synthesis and studied the biological activity of the synthesized materials using tissue culture.

## 2. Experimental

### 2.1. Materials

For acquisition of folate-ferrocene conjugate (FFC) folic acid (FA) (Acros, 96.6 %) – N-pteroyl-L-glutamic acid was used. Empirical formula $C_{19}H_{19}N_7O_6$. $T_{resolve}$ = = 523 K. It was dried before synthesis at the temperature of 393 K to constant weight.

Ferrocene (Ukrorgsyntez Research and Production Enterprise). Empirical formula $C_{10}H_{10}Fe$. $T_m$ = 445 K. It was used without further purification.

Dimethyl sulfoxide (U.S. Pharm., 99.8 %) previously dried over NaOH (48 h) and then distilled in a dry argon flow at boiling point 316–318 K/3 mm Hg [7].

### 2.2. Techniques

Chemical structure of the synthesized compounds was investigated by IR- and $^1$H NMR spectroscopy. IR-spectra were recorded by «Bruker» IR-spectrometer with the Fourier transform «Tensor-37» in 400–4000 cm$^{-1}$ range. Samples were produced by tableting powders with KBr. The assignment of absorption bands was performed according to the data of the research [8].

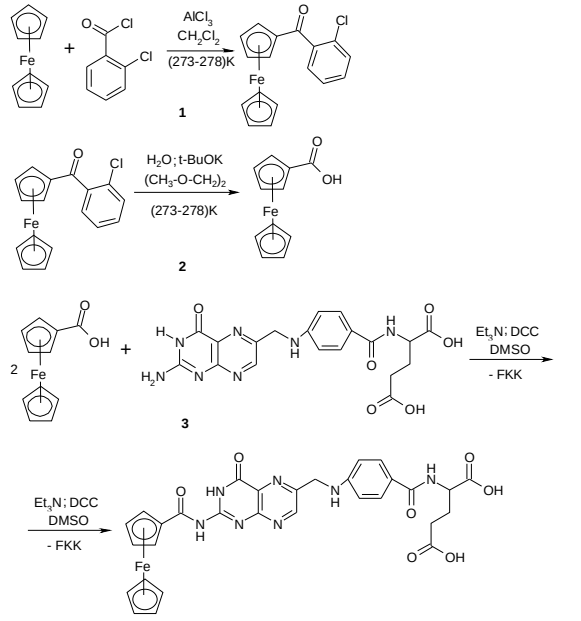
$^1$H NMR spectra were recorded by “Varian VXR-300” spectrometer in the solution of fully deuterated DMSO-d6, internal standard – tetramethylsilane. The identity of the absorption bands was done according to the data of the research [9].

To determine the melting temperature Kofler microheating table (Reichert, Austria) was used.

Ultimate analysis of ferrocene carboxylic acid for the content of carbon and hydrogen were carried out gravimetrically.

Biological activity of folate-ferrocene conjugate was investigated by tissue culture [10].

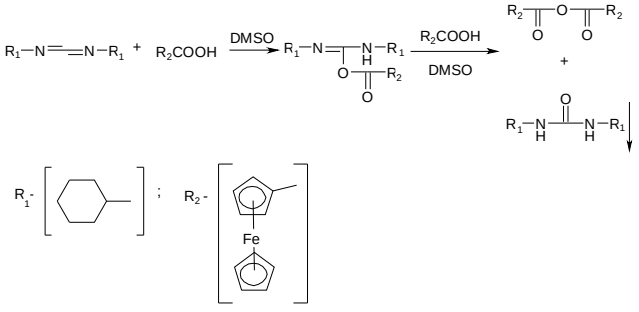
*Synthesis of FFC* was carried out through the stage of ferrocene carboxylic acid (FCA) synthesis in accordance to the scheme:



**Scheme 1.** Synthesis of folate-ferrocene conjugate

The first and the second stages illustrate synthesis of FCA [11]. FCA was derived as a yellow powder, soluble in organic solvents (DMSO, DMF, DMA) at $T_m$ = 455–471 K, Mr = 230.04. Found (%): C 56.92, H 4.33. Calcd. (%): C 57.42, H 4.38. $^1$H NMR ($\delta$, ppm): 4.4 (2H, t, $Cp_1$), 4.6–4.7 (2H, t, $Cp_1$), 4.3 (5H, s, $Cp_2$), 12.1 (1H, s, –COOH). FCA was recrystallized from toluene. The yield was 52.4 %.

The third stage represents synthesis of folate-ferrocene conjugate. In three-necked reactor, equipped with a stirrer, a thermometer, and a system for argon supply, 0.5 g (0.00113 mol) of folic acid was dissolved under stirring in 10 ml of anhydrous dimethyl sulfoxide at 338–343 K. After complete dissolution of folic acid, the reaction mixture was cooled to the temperature of 293–295 K. To block the acid groups of folic acid 0.229 g (0.00226 mol) of triethylamine was added [12]. The course of reactions was controlled by IR spectroscopy. After 30 min, 0.474 g (0.00226 mol) of ferrocene carboxylic acid was added and 0.466 g (0.00226 mol) of *N*,*N*'-dicyclohexylcarbodiimide was added as a reaction initiator [4]. Conjugation of folic acid to ferrocene carboxylic acid is going through the stage of formation of anhydride of ferrocene carboxylic acid [13]:



**Scheme 2.** The formation of ferrocene carboxylic acid anhydride

The reaction mixture was kept under stirring at 293–295 K for 24 h. The precipitate (by-product) which is formed by the reaction was filtered and the conjugate was isolated from the solution by eliminating into diethyl ether, washed with distilled water and dried in the drying chamber at 343 K to constant weight.

The reaction product, isolated from diethyl ether and washed with water, was obtained as a brown powder with $T_m$ = 449–455 K, with practical yield of 67.2 %. $^1$H NMR ($\delta$, ppm): 4.4 (2H, t, $Cp_1$), 4.6–4.7 (2H, t, $Cp_1$), 4.3 (5H, s, $Cp_2$), 6.5–6.6 (2H, d, $CH_{Ar}$), 7.5–7.6 (2H, d, $CH_{Ar}$), 8.1–8.2 (1H, d, –NH–C(O)–), 6.9–7.0 (1H, t, –NH–$CH_2$–), 4.4–4.5 (2H, d, –NH–$CH_2$–), 4.3–4.4 (1H, m, –CH–), 1.91–2.03 (2H, dm, –$CH_2$–), 2.2–2.4 (2H, t, –$CH_2$–), 8.6–8.7 (1H, s, pteroyil surplus), 6.8–7.0 (1H, s, –NH–).

## 3. Results and Discussion

The chemical structure of the reaction products was confirmed by infrared method and $^1$H NMR-spectroscopy. IR spectra of folic acid, ferrocene carboxylic acid, and folate-ferrocene conjugate are presented in Fig. 1.

In the IR-spectrum of the reaction product (Fig. 1) the disappearance of the vibration bands is observed at 2626 and 2548 cm$^{-1}$, which characterize the fluctuations of ionized group of FCA acid residue. There are new bands of amide groups ($\delta_{NH}$ 1655 cm$^{-1}$ (amide I), $\delta_{C=O}$ 1701 cm$^{-1}$ (amide I) and $\delta_{NH}$ 1530 cm$^{-1}$ (amide II)), and there is a new $\upsilon_{NH}$ band at 3246 cm$^{-1}$, indicating that the additional formation of an amide bond appeared.

To establish the biological activity of folate-ferrocene conjugate a study of this compound was performed by means of tissue culture.

As the main mode of cultivation the method of explantation in plasma clot (solid phase) in Carrel vials with nutritional mixture (liquid phase) adding was used. Subcutaneous tissue of white laboratory rats, males of three month age, served as the explantation material. The cultures were incubated at 310 K. The replacement of the liquid phase of nutrient medium was performed every 3 days.

Observations on tissue cultures have shown that in most cases the latency period (the time it takes to adapt to the conditions of culturing for explants prior to cell migration) lasted on average 2–2.5 days. On the third day after explantation the migration of rare fibroblastic elements, that were mainly fusiform, started and in some areas bands composed of 3–4 cells were formed (Fig. 2a). During this period there was also quite active migration to the internal medium of isolated macrophage items presented by cells bigger than fibroblasts, of mostly

irregular polygonal shape. Some have shifted to a considerable distance from the explants, while others were placed closer to it. Typically, the growth and migration of fibroblasts and migration of histocytic elements occurred with approximately the same intensity along the entire perimeter of the explants. However, in some cases, the growth of cellular elements was observed only from one side of the cultivated tissue.

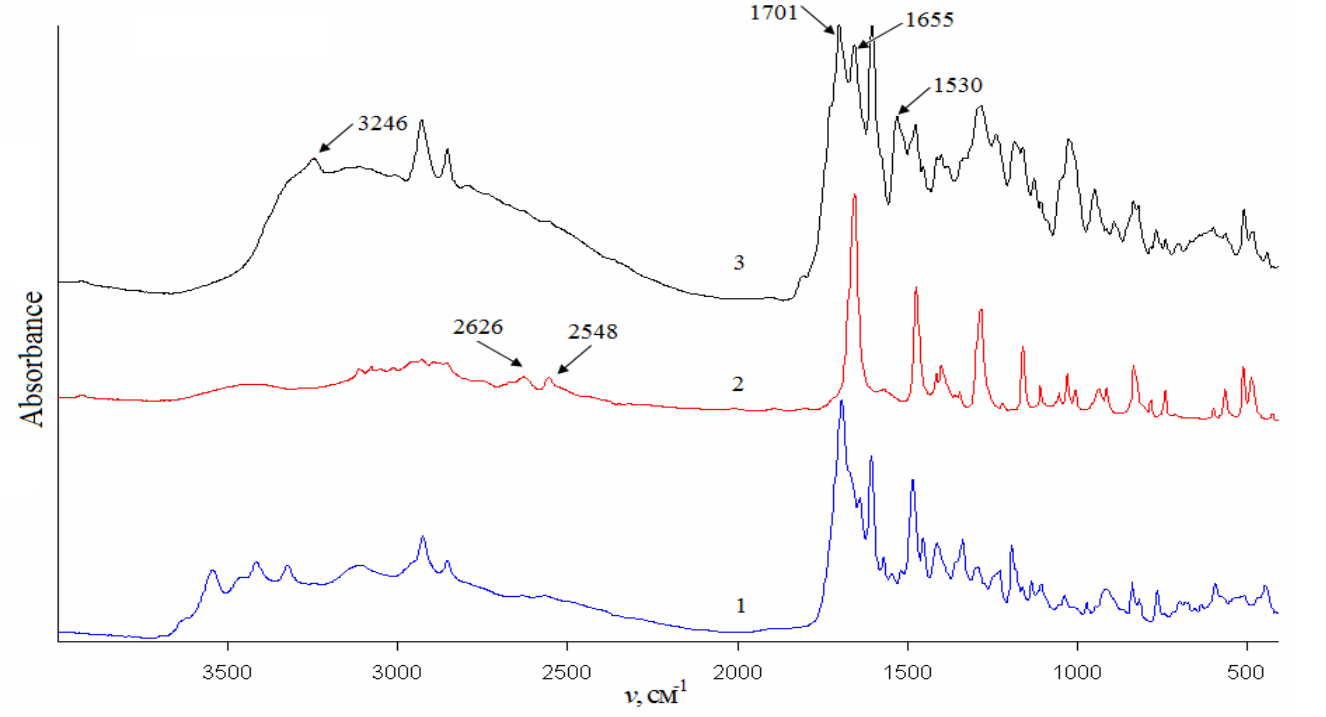
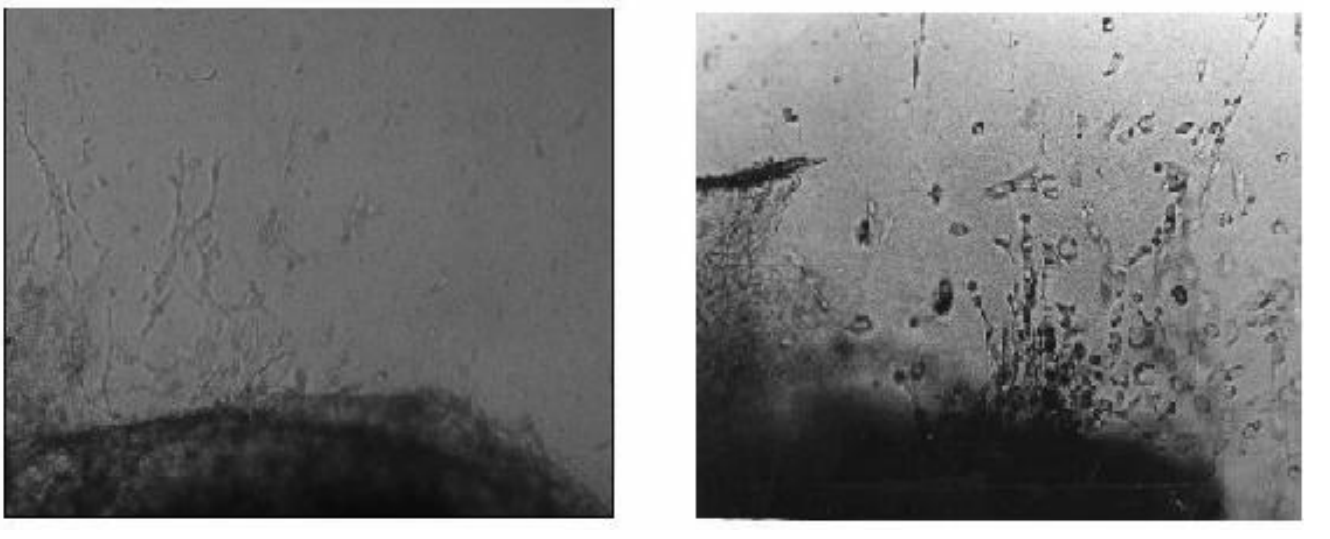
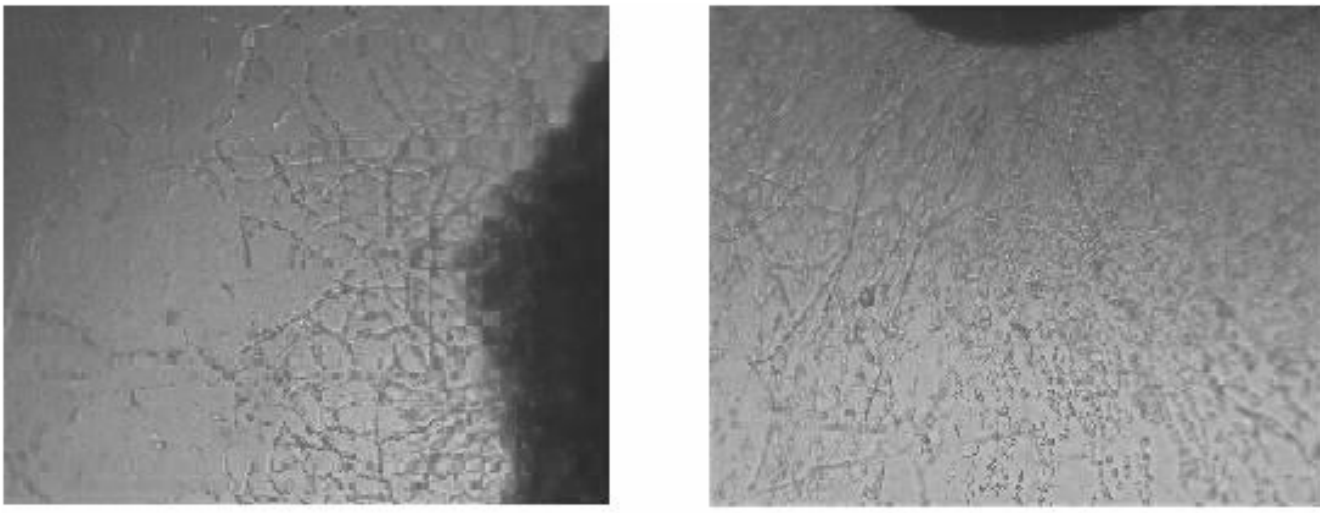



**Fig. 1.** IR spectra: folic acid (1); ferrocene carboxylic acid (2) and folate-ferrocene conjugate (3)



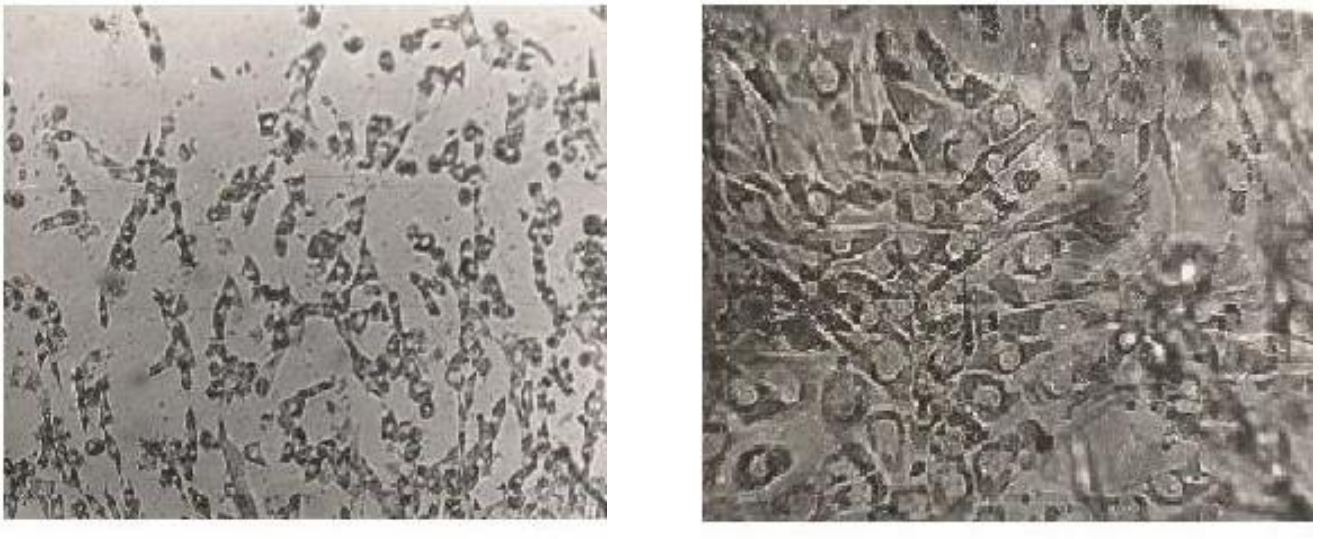
a) b)

**Fig. 2.** Migration of fibroblastic elements on the third day of cultivation in control, magnification of 100x (a) and with the introduction of folate-ferrocene conjugate to the medium, magnification of 100x (b)



a) b)

**Fig. 3.** Compact and reticular growth zones of fibroblastic elements in control on 5th day of cultivation, magnification of 100x (a) and tissue-like cell growth with the introduction of folate-ferrocene conjugate to the internal medium, magnification of 100x (b)

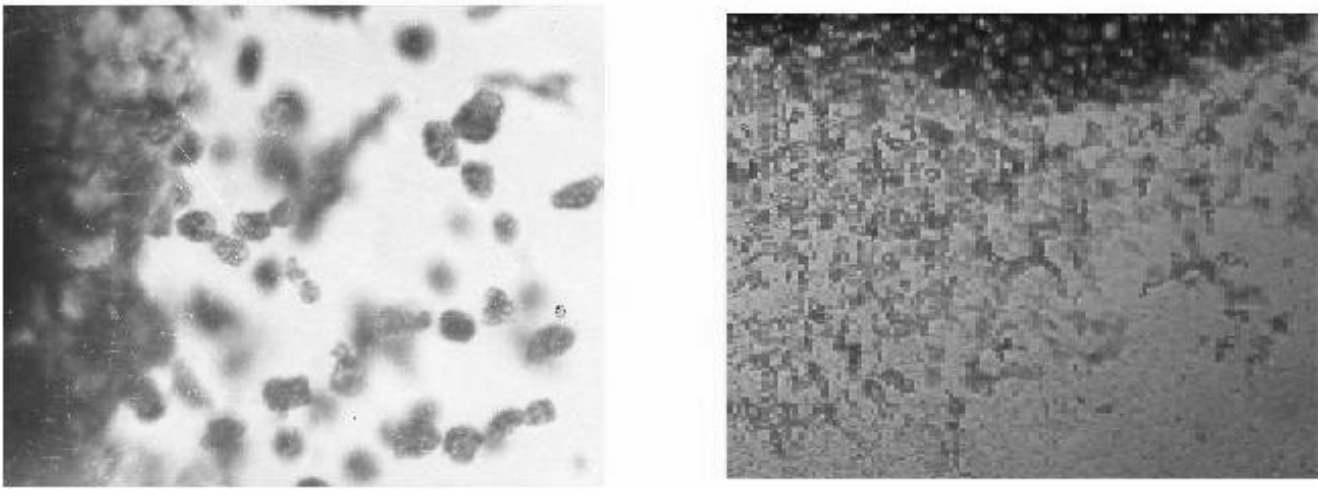


a) b)

**Fig. 4.** The division of cells of reticular growth area of fibroblastic elements in control, magnification of 150x (a) and compact growth area of fibroblast-like cells of polygonal shape with the introduction of FFC to the internal medium, magnification of 200x (b)

On 5th day the distribution of growth zones in 2 parts (compact and reticular) was observed around the explants. Compact zone consisted of cells of spindle and polygonal forms tight to each other. In addition to fibroblastic elements fibroblast cells of larger size and irregular shape were also observed. Under the compact zone clusters and strands of cells with reticular location were formed. There was a significant number of cells that were being divided (Fig. 3a).

On 7th day, the growth area is clearly divided into three: compact, reticular, and area of single migrating cells. The structure of the first two has not significantly changed till that time, only the number of cells has increased. Unlike in the previous period most cells that were being divided were observed in the third area. On 10th day after explantation in all three areas the signs of degenerative changes of fibroblastic elements as well as fibroblast cells were observed. These changes appear in the form of enhanced vacuolization of the cytoplasm, rounded cell bodies with the processes disappearance, resulting in division of cells of reticular area. Degenerative changes were more expressed in the compact area (Fig. 4a).



a) b)

**Fig. 5.** Grainy degeneration of cytoplasm of the cells on 14th day in control, magnification of 150x (a) and the beginning of degenerative changes in the compact growth area at the introduction of folate-ferrocene conjugate to the medium, magnification of 100x (b)

On 14th day, the cell population entered a stage of severe degeneration, which appears in acute vacuolization of cytoplasm and its granular degeneration (Fig. 5a). The number of histocytic elements that have lost their normal structure has significantly increased. Besides, architectonic of compact and reticular growth area was finally disturbed.

Available data on the nature of growth and transformation of cellular elements of subcutaneous connective tissue of white outbred rats is generally similar to results obtained by other authors by using of other variants of nutrient media, cultivation conditions and animals of all ages [14].

Folate-ferrocene conjugate was contributed to the liquid phase of culture media during explantation at early cultivation in the amount of 0.1 %. Observations on tissue cultures with FFC showed that migration of fibroblastic elements, the same as in control, begins from the third day in the form of strands and single cells located at the base perpendicular to the surface of explants. It is worth noting the greater variability of cell forms from spindle to polygonal, and bigger amount of isolated cells of polygonal shape that lie in the distance from the explants (Fig. 2b).

On 5th–7th day of cultivation in Carrel vials, as well as in control, three areas of growth are formed: compact, reticular, and individual migrating cells. So-called tissue-like growth is also present (Fig. 3b). Growth area of individual migrating cells is broader than in control and differs by bigger variety of cell forms.

On the 10th day of degenerative changes study the calls are not observed in the compact and reticular growth areas. There is a significant increase in the area of tissue-like cell growth of polygonal shape (Fig. 4b). The area of single cells growth expands as well.

On the 14th day of cultivation the growth areas of cellular elements in vials with folate-ferrocene conjugate significantly differ from control cultures. Growth areas are represented by wide fields of cells of polygonal shape mainly macrophage elements. Growth area of individual migrating cells in the form of fibroblasts of fusiform is marked.

After 21 days there is complete degradation of growth areas in the control, while in the experimental vials degradation phase occurs only in the compact growth area, in parallel with the migration of fibroblastic elements in the growth area of single migrating cells (Fig. 5b).

## 4. Conclusions

Thus, as a result of the studies folate-ferrocene conjugate was synthesized through the stage of ferrocene carboxylic acid synthesis. The conjugate was characterized by IR and $^1$H NMR spectroscopy. The studies of biological activity evidence that folate-ferrocene conjugate, which was introduced in tissue culture, stimulates the growth of fibroblastic and histocytic elements at all stages of cultivation. In the later stages of cultivation a significant growth of macrophage and histocytic elements occurs, confirming the biological activity of the synthesized compounds in tissue culture.

## References

[1] Togni A. and Halterman R.: Metallocenes. Wiley-VCH, Weinheim 1998.
[2] Gomez Arrayas R., Adrio J. and Carretero J.: Angewandte Chemie – Int. Edn., 2006, **45**, 7674.
[3] Jaouen G.: Bioorganometallics. Wiley-VCH, Weinheim 2006.
[4] Min-Hua Chen, Chung-King Hsu, Feng-Huei Lin *et al.*: Adv. Sci. Techn., 2006, **53**, 50.
[5] Zhang J., Rana S., Srivastava R. and Misra R.: Acta Biomaterialia, 2008, **4**, 40.
[6] Orel V., Shevchenko A., Dzyatkovska I., Nikolov M. *et al.*: Dopovidi Nats. Akad. Nauk Ukrainy, 2013, **2**, 177.
[7] Gordon A. and Ford R.: Sputnik Khimika. Mir, Moskwa 1976.
[8] Bellami L.: Infrakrasnye Spektry Molekul. Goskhimizdat, Moskwa 1957.
[9] Prech E., Byulmann F. and Affoler K.: Opredilenie Stroeniya Organicheskikh Soedineniy. Tablicy Spektralnykh Danykh. Mir, Moskwa 2006.
[10] Yacenko V.,Galatenko N., Pchakadze G. *et al.*: Cytologiya i Genetika, 1984, **4**, 280.
[11] Reeves P.: Organic Syntheses, 1988, **6**, 625.
[12] Pasut G., Canal F., Dalla Via L. *et al.*: J. Contr. Release, 2008, **127**, 239.
[13] Bocharov B.: Uspechi Khimii, 1965, **34**, 488.
[14] Freshny R.: Kultury Zhyvotnych Kletok. Metody. Mir, Moskwa 1989.

## СИНТЕЗ БІОЛОГІЧНО АКТИВНОГО ФОЛАТ-КОН'ЮГОВАНОГО ФЕРОЦЕНУ

***Анотація.*** *Через стадію синтезу фероценкарбонової кислоти вперше синтезовано фолат-кон'югований фероцен. Будову кон'югата підтверджено методами ІЧ- та $^1$Н ЯМР-спектроскопії. Проведено дослідження біологічної активності фолат-кон'югованого фероцену методом культури тканин.*

***Ключові слова:*** *фероценкарбонова кислота, фолат-кон'югований фероцен, фолієва кислота, біологічна активність, метод культури тканин.*