The evaluation of the antimicrobial properties of biodegradable prodrugs with chlorphenesin

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Abstract: Spheres of prodrug of polylactide (PLA) or polycaprolactone (PCL) or a copolymer thereof with chlorphenesin (CF) were obtained. Furthermore spheres with an active substance additionally dispersed in the prodrug matrix – hybrid spheres – were prepared. The antimicrobial properties of the prodrug forms obtained were investigated towards bacteria, yeast, and filamentous fungi to verify whether the CF activity of the new formulations maintains. This research shows a wide spectrum of antimicrobiological application, especially when using CF as a preservative.

Keywords: antimicrobials activity, preservatives, fungi, biodegradation, biopolymers.

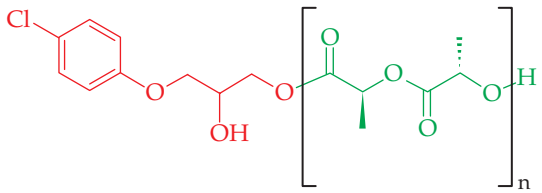
Ocena właściwości antymikrobiologicznych proleków chlorofenezyny

Streszczenie: Otrzymano sfery z proleku chlorofenezyny (CF) i polilaktydu (PLA), polikaprolaktonu (PCL) lub ich kopolimeru, a także sfery hybrydowe z substancją aktywną dodatkowo rozproszoną w matrycy proleku. W celu sprawdzenia, czy aktywność CF została zachowana, zbadano właściwości antymikrobiologiczne wszystkich otrzymanych form proleku wobec bakterii, drożdży i grzybów strzępkowych. Uzyskane wyniki wskazują na możliwe szerokie spektrum aplikacji tych form leku, szczególnie w charakterze konserwantu.

Słowa kluczowe: aktywność antymikrobiologiczna, konserwanty, grzyby, biodegradacja, biopolimery.

Apart from the search for new active pharmaceutical ingredients (APIs), current studies focus also on the methods of their effective and safe use. An increase in chronic disease incidence has made it necessary to develop the controlled release systems, so called drug delivery system (DDS) [1]. One of the diseases which afflicts the society is dermatomycosis. Its treatment is tedious and long-lasting, and what is more, once acquired, dermatomycosis tends to recur.

Chlorphenesin (CF), [3-(4-chlorophenoxy)-1,2-propanediol, Formula (I) – a chemical structure of polylactide (green) prodrug of chlorphenesin (orange)], is an antifungal drug which acts in an inhibiting manner on Gram(+) and Gram(–) bacteria and yeasts [2]. Within the European Union, it is approved as a preservative in skin cosmetics in the amount of less than 0.3 wt % [3], and it is commonly used. It can be administered orally or dermally. Upon oral administration, it is readily absorbed from the digestive system and metabolized in liver with the half-life of 4 h. The greatest disadvantage of CF is its complete elimination from the human organism within 24 h. As much as 85 % of CF is removed with urine in the form of glucuronides [4]. In case of CF administered dermally, the absorption half-life is 126 h [2], while the elimination half-life is only 22 h. This leads to the necessity to increase the dose which is required in order to maintain the therapeutic effect, increasing the costs and the risk of adverse events, *e.g.*, using high concentrations of CF can cause irritation in sensitive skin [5]. Maintaining the therapeutic effect with a minimized risk of side effects can be achieved by administration of CF in the form of DDS.



Formula (I)

DDS include, among others, polymeric nano- and microspheres, as well as macromolecular prodrugs [see Formula (I)] [6]. A prodrug is a compound, which either

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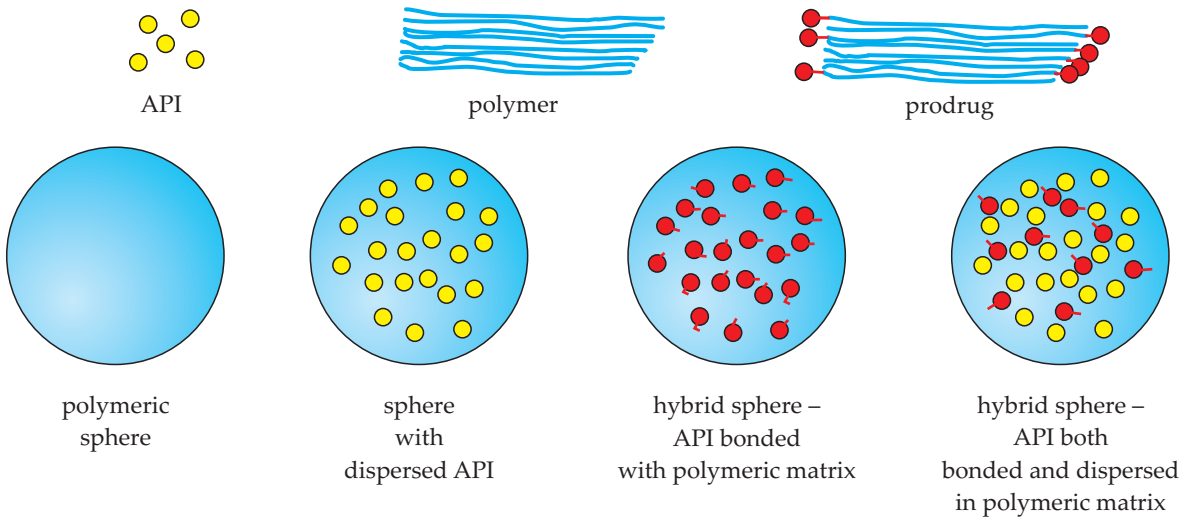

Fig. 1. Different structures of various drug delivery systems

does not show therapeutic activity or shows one much lower than that of an active substance. The biological function appears only as a result of metabolic changes. In macromolecular prodrugs, the therapeutic effect is present only upon the release of an API molecule from a polymer chain, as a result of hydrolysis. Macromolecular prodrugs can be administered also in the form of micro- and nanospheres. Usually, spheres are made of biodegradable polymers which, in the course of the matrix degradation, release the API dispersed therein. Examples of such polymers are polycaprolactone, polyglycolide and polylactide [7, 8]. A combination of these two systems gives hybrid spheres with a matrix made of a macromolecular prodrug and, additionally, an active substance dispersed therein (Fig. 1).

The major advantage of DDS in comparison with traditional drug forms is that, in the organism, the effective API concentration is maintained for much longer, therefore, the dosing is less frequent and drugs are safer and more effective. Controlled release drug systems are still the area of intensive research, yet some of them, as well as cosmetics with the use thereof, are already available commercially.

The aim of this study was to obtain different forms of DDS with chlorphenesin and to test their antimicrobial properties. CF prodrug spheres of polylactide (PLA-CF), polycaprolactone (PCL-CF), and a block copolymer thereof (PCLA-CF) were obtained, as well as hybrid spheres with additionally dispersed CF. Antimicrobial tests were performed towards bacteria, yeasts, and filamentous fungi. Test results were compared with the activity of chlorphenesin, empty polylactide spheres and polylactide spheres with dispersed CF.

EXPERIMENTAL PART

Materials

To obtain the spheres, prodrugs of chlorphenesin and polylactide (PLA-CF) (M_w = 3400 g/mol, *PDI* = 1.1), polycaprolactone (PCL-CF) (M_w = 4400 g/mol, *PDI* = 1.4), and the block copolymer thereof (PCLA-CF) (M_w = 4400 g/mol, *PDI* = 1.2) were used, obtained in-house by the L-lactide and/or ε-caprolactone ring-opening polymerization method with zinc 2-ethylhexanoate and 5 mol % of the active substance [9]. The active substance, chlorphenesin, 3-(4-chlorophenoxy)-1,2-propanediol, was provided by Alfa Aesar, and zinc 2-ethylhexanoate was from in-house production. As an emulsion stabilizer, poly(vinyl alcohol) (PVA), Sigma Aldrich Mowiol 4-88 (M_w = 30 000 g/mol, degree of hydrolysis of 88 %) or Mowiol 18-88 (M_w = 130 000 g/mol, degree of hydrolysis of 88 %) was used. For comparative purposes, polylactide Nature Works 2003D (M_w = 146 000 g/mol, *PDI* = 1.7) was used.

In antimicrobial tests, strains from the collection of the Plant Breeding and Acclimatization Institute of National Research Institute (IHAR-PIB) in Młochów and from American Type Culture Collection (ATCC) were used, as well as the following media: Mueller-Hinton Broth (MHB), Yeast Extract - Peptone - Dextrose Broth (1 % yeast extract, 2 % peptone, 2 % glucose) (YPD), and Potato Dextrose Agar (PDA), Merck, Biocorp or BioShop. Antibiotics with well-defined microbiological activity, BioShop amphotericin B for yeast and filamentous fungi, and ampicillin sodium salt for bacteria were used as controls. The following solvents were used: dichloromethane and DMSO (dimethyl sulfoxide), analytical grade, POCH, and demineralized water from in-house production. Crude materials available commercially were used without purification.

General procedure for the preparation of spheres of macromolecular CF prodrugs

All spheres with CF prodrugs were obtained according to the previously developed procedure for PLA-CF [10]. To a 250 cm^3 round flask with a magnetic stirrer, placed in a water bath, 100 cm^3 of 0.1 g/cm^3 PVA aqueous solution was added. Within 15 min, 5 cm^3 of 1 % solution

of polymer with chlorphenesin prodrug in was dropped into the flask. The process was conducted at 25 °C, while simultaneously stirring the solution at 600 or 1200 rpm. Subsequently, the flask was left open for about 1 hour for the organic solvent to evaporate, next the water solution of spheres was filtered off using a filter crucible with 3G porosity, and the filtrate was collected to test the size of spheres by dynamic light scattering method.

General procedure for the preparation of hybrid spheres

The procedure was as for the prodrugs, except that to the 250 cm^3 round flask with magnetic stirrer 100 cm^3 of 0.1 % (w/v) PVA aqueous solution and 0.025 g of chlorphenesin were added, and 5 cm^3 of 1 % (w/v) prodrug solution (PLA-CF, PCL-CF or PCLA-CF) in dichloromethane (CF : prodrug 1 : 2 w/w) was added dropwise.

General procedure for the preparation of polylactide spheres (without API)

The procedure was as for the prodrugs, except that 5 cm^3 of 1 % polylactide solution in dichloromethane was added dropwise.

General procedure for the preparation of polylactide spheres with dispersed API

The procedure was as for the prodrugs, except that to the 250 cm^3 round flask with magnetic stirrer 100 cm^3 of 0.1 % (w/v) PVA aqueous solution and 0.025 g of chlorphenesin were added, and 5 cm^3 of 1 % (w/v) polylactide solution in dichloromethane (CF : PLA 1 : 2 w/w) was added dropwise.

Procedure for testing antimicrobial activity towards bacteria and yeast

The following bacteria: *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Salmonella typhimurium* ATCC 14028, and yeast *Candida albicans* ATCC 10231 were cultured in a liquid medium. A loopful of material from single colonies of bacteria or yeast was used to inoculate 10 cm^3 of MHB or YPD liquid medium in a 100 cm^3 Erlenmeyer flask. Cultures were incubated overnight (about 18 h) at 37 °C on a shaker with stirring at 200 rpm. Subsequently, they were diluted with a fresh medium to obtain the required concentration of 10^5 units per cm^3 (cfu/cm^3, cfu – colony-forming unit) based on the previously obtained growth curve for each microorganism.

Tests were performed using 96-well plates for bacterial suspension cultures. 100 mm^3 of prepared media (bacteria – MHB, yeast – YPD) mixed with 20 mm^3 of tested preparations (solution of spheres with the prodrug, spheres with dispersed CF, hybrid spheres and spheres without API) was plated, and subsequently 100 mm^3 of microbial suspension was added. Also, a control was prepared without the addition of the tested preparations, with chlorphenesin aqueous solution (20 mg/cm^3), and with the addition of antibiotics with well-defined microbiological activity – ampicillin and amphotericin (20 mg/cm^3 in DMSO). The plates were incubated at 35 °C for 24 h with stirring at 200 rpm. Subsequently, after 24 h of incubation, the optical density of tested samples was measured at $\lambda = 600$ nm.

All samples were prepared in three repetitions and the standard deviations were determined.

Procedure for testing antimicrobial activity towards filamentous fungi

The following filamentous fungi: *Aspergillus niger* ATCC 16404, *Colletotrichum coccodes* MC 1, *Fusarium oxysporum* MF 5 and *Fusarium sambucinum* MF 1 were cultured on a solid medium PDA. 1 cm^3 of the tested preparation (solution of spheres with the prodrug, spheres with dispersed CF, hybrid spheres and empty spheres) was added to 100 cm^3 of dissolved PDA and was poured into Petri dishes to solidify. A control was prepared without the addition of tested preparation as well as with chlorphenesin aqueous solution (20 mg/cm^3). Subsequently, 6 mm of mycelium, cut from the cultured microorganism mycelium, was placed on agar plates [11]. Petri dishes were incubated at 25 °C for 3–5 days, depending on the strain growth rate. Following the incubation, the fungal growth inhibition zone was measured with a ruler.

All samples were prepared in three repetitions and the standard deviations were determined.

Methods of testing

The size of spheres was determined by the dynamic light scattering (DLS) method with a Zetasizer Nano ZS apparatus, Malvern Instruments. Microorganisms were cultured in a Benchtop SI-600R shaker, Lab Companion. Optical density tests were performed with the use of a Synergy H4 plate reader, BioTek.

RESULTS AND DISCUSSION

Prodrug spheres PLA-CF, PCL-CF and PCLA-CF, and hybrid spheres were obtained by the emulsion method with the evaporation of the organic solvent [12–17]. As controls, spheres of polylactide (PLA) and of polylactide with dispersed chlorphenesin (PLA + CF) were prepared. The process conditions were selected to obtain a comparable size of spheres, close to 100 nm. Antimicrobial activity tests were performed towards bacteria, yeasts, and filamentous fungi.

Activity towards bacteria and yeasts

The tests were performed in a liquid medium towards the following bacteria: *E. coli*, *S. aureus*, *B. subti-*

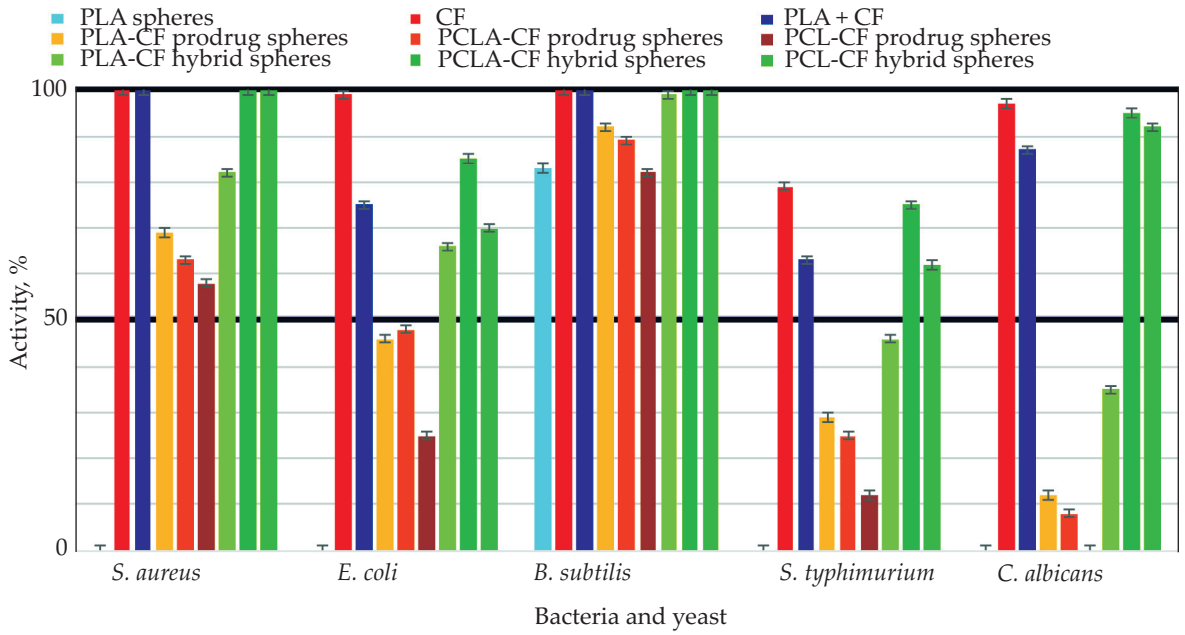

Fig. 2. Antimicrobial activity of tested formulations against bacteria and yeast; PLA spheres bar occurs only for *B. subtilis*

lis, *S. typhimurium*, and yeast *C. albicans*. The activity of prodrug spheres prepared from chlorphenesin and polylactide (PLA-CF), chlorphenesin and polycaprolactone (PCL-CF), and chlorphenesin and lactide-caprolactone block copolymer (PCLA-CF), as well as of hybrid spheres therefrom, was tested. Additionally, as references, the microorganisms were tested with free API, spheres made of PLA (without chlorphenesin), and spheres made of PLA with dispersed chlorphenesin (PLA + CF). The antimicrobial activity was calculated from the following formula:

$$\text{Activity} = (ODH/ODH_{control}) \cdot 100\ \% \qquad (1)$$

where: ODH – optical density of the microorganism culture with the addition of spheres, $ODH_{control}$ – optical density of the microorganism culture without the addition of API in any form.

The formulation is considered antimicrobiologically active if the activity is above 50 %.

The bacterial strain which is the most resistant towards chlorphenesin is *S. typhimurium* (Fig. 2). It should be noted that CF is mainly an antifungal drug, hence it does not inhibit the growth of some bacterial strains fully.

Spheres from polylactide without CF caused over 80 % growth inhibition in *B. subtilis*, which could be explained by the environment acidification caused by the release of lactic acid during the degradation of PLA.

The activity of spheres obtained from prodrugs is much lower than that of hybrid spheres with dispersed chlorphenesin. In case of *E. coli*, *S. typhimurium*, and *C. albicans*, they lost their therapeutic properties completely. API from spheres of this kind first has to be released from the polymer chain. This stage is conditioned by time and requires an action from a hydrolyzing agent. Comparing prodrugs which contain different matrices, the lowest activity is observed for PCL-CF, because of the degradation time for polycaprolactone being the longest.

The activity of hybrid spheres is much higher than of spheres from prodrugs, and almost in all of the cases the test result is positive. The activity dropped below 50 % only in hybrid spheres prepared from PLA-CF prodrug towards bacteria *S. typhimurium* and yeast *C. albicans*. The highest activity is shown by spheres made of PCLA-CF copolymer, and the lowest of PLA-CF.

Activity towards filamentous fungi

Tests were performed towards the following filamentous fungi: *A. niger*, *C. coccodis*, *F. oxysporum*, and F. *sambucinum* on a solid medium.

The activity of prodrug spheres obtained from PLA-CF, PCL-CF and PCLA-CF, as well as hybrid spheres was tested. Analogously to bacteria, as references, the fungi were tested with API, spheres made of PLA (without chlorphenesin), and spheres made of PLA with dispersed chlorphenesin.

The activity of specific preparations was determined by measuring the fungal growth zone using the formula:

$$\text{Activity} = [(d_{control} - d)/d_{control}] \cdot 100\ \% \qquad (2)$$

where: d – diameter (mm) of the fungal growth zone regarding the tested preparation, $d_{control}$ – diameter (mm) of the fungal growth zone without the addition of inhibiting agents.

The formulation is considered antimicrobiologically active if the activity is above 50 %. In all cases, the preparations tested show antifungal activity (Fig. 3). The highest activity decrease was observed for PCL-CF prodrug spheres, because of longer degradation time. In case of hybrid spheres, the activity was usually higher than for

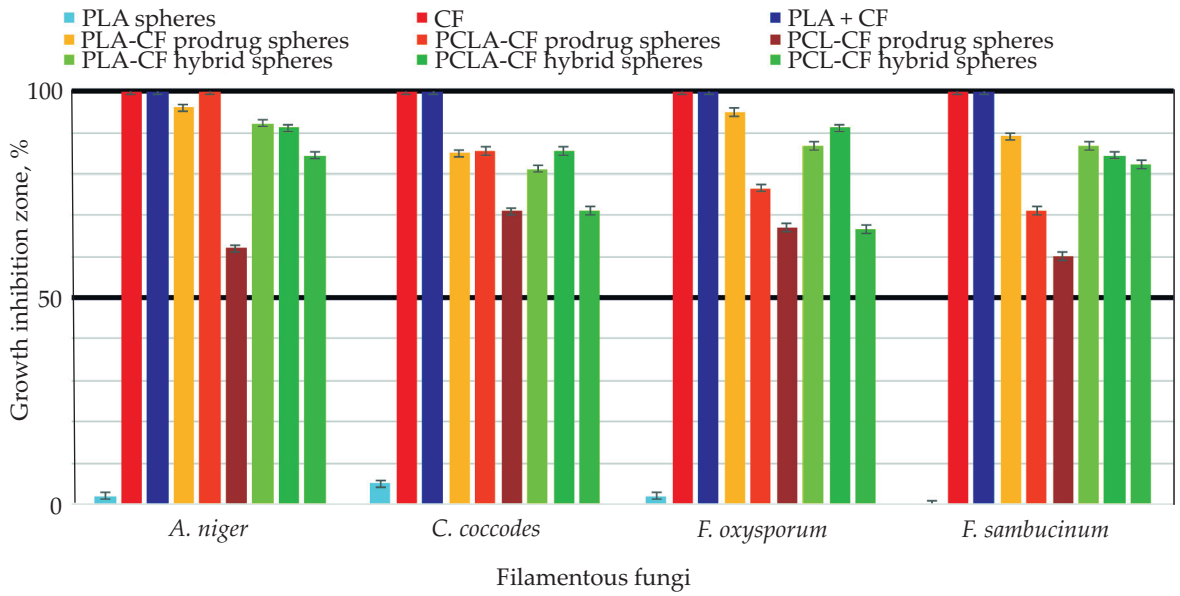

Fig. 3. Antimicrobial activity of tested formulations against filamentous fungi

corresponding prodrug spheres, the exceptions being PLA-CF or PCLA-CF spheres in *A. niger* (still, these are high level values, above 80 %). This could be related to the type of microbial culture. The culture on a solid medium, agar, causes problems with CF migration, and therefore it released from the polymer matrix with more difficulty.

CONCLUSIONS

Controlled release drug systems were obtained in the form of PLA-CF, PCL-CF, and PCLA-CF prodrug spheres, as well as hybrid spheres, and subsequently these were compared in terms of antimicrobial properties.

It has been demonstrated that hybrid spheres have significantly higher antimicrobial activity towards bacteria and yeast than prodrug spheres without dispersed API. Lower activity of prodrug spheres results from the fact that first the matrix-API binding has to be hydrolyzed in order to pharmaceutically activate the prodrug. In case of prodrug spheres, the lowest activity is shown by PCL-CF, due to the longest degradation time for this polymer. In case of hybrid spheres, such a clear trend is not observed. As for the antimicrobiological activity towards filamentous fungi, it was fully maintained without significant differences between these drug forms and without a clear trend.

Using chlorphenesin in the form of hybrid spheres improves its antibacterial and antiyeast properties, without significantly decreasing its antifungal properties. This provides a wide spectrum of antimicrobiological application, especially when using CF as a preservative. It has been found that both prodrug spheres and hybrid spheres can be checked as drug systems with controlled release of chlorphenesin. This positive result gives scientific grounds for further research for example release kinetic of API. Such formulation could be administered on skin in form of cream or gel.

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