ABSTRACT
In addition to its well-known anti-inflammatory and analgesic effects, indomethacin (IMC) has been shown to reduce the proliferation rate and induce apoptosis in several cancer cell lines. In this study we investigated the biological activity of IMC and poly(vinyl acetate) nanoparticles with indomethacin (IMC-pVAc) in three cell lines. Treatment with IMC-pVAc caused prominent morphologic alterations of serum-free McCoy-Plovdiv and mouse lymphoma L5178Y cells. At least for the McCoy-Plovdiv line we showed that IMC doubtlessly induces apoptosis in the cells. The mouse lymphoma cells were more sensitive to IMC treatment which can be due to the different physiology of cancer cells and their shorter doubling time. In both cell lines, however, a clear concentration- and time-dependent effect of IMC treatment was demonstrated. Interestingly, IMC-pVAc always led to higher mortality of cells than IMC alone. This was not due to a cytotoxic activity of the nanoparticles. pVAc nanoparticles were shown to be biocompatible with respect to cell cultures and did not affect the proliferation rate and normal development of cells in vitro. Thus, the greater effect of IMC-pVAc could be explained with the prolonged release of the active substance, included in the polymer carrier.
Keywords: Indomethacin, indomethacin-loaded nanoparticles, apoptosis, biocompatibility, cell cultures.

INTRODUCTION
In recent years there has been a constantly growing interest in the wide range of nanocarriers that can be used as drug delivery systems.[1] Nanoparticles (NPs), liposomes, nanosuspensions and nanoemulsions have been studied as drug delivery and drug releasing systems for different formulations. [2-5] Nanocarriers have been used to increase the drug solubility[1-4] and the drug stability both in storage and in a biological environment. These effects lead to an increase in drug bioavailability [1, 4] and reduction of the dose, drug toxicity and side effects.[6] Different nanocarriers have also been studied as systems for targeted delivery and controlled drug release. [2, 3, 7]

The nature of the used drug, the type of nanocarrier in which the drug is included, and its parameters such as size distribution, surface, zeta-potential etc. determine the effect of the nano-system on cells, tissues and the animal or human organism. [7]

Indomethacin (IMC), ((1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-yl)-acetic acid) is a nonsteroidal anti-inflammatory drug, used in ophthalmology in the form of topical eye drops for prevention of miosis during cataract surgery, cystoid macular edema and conjunctivitis. [8, 9] Its use in liquid formulations is limited due to its insolubility in water, low bioavailability and ocular mucosa irritation. In our previous studies we have demonstrated the possibility of in-situ inclusion of IMC in poly(vinyl acetate) (pVAc) and poly(styrene) NPs via emulsifier free radical polymerisation. [10] and we have proved the sustained drug release. [10, 11] These studies were made due to our wish to include the obtained IMC-NPs in an ophthalmic formulation. There was not information in the available literature about the interaction between IMC and the used monomers and initiators of polymerization, as well as about the IMC influence on the stability of monomer and polymer dispersions in water. The preliminary experiments allowed us to choose the emulsion polymerization conditions, excluding chemical modification and degradation of the IMC molecule. [10, 11, 12] On the other hand, the IMC concentration (1% (w/v)) led to minimum coagulate formations during the polymerization with high yield of NPs. [13] Even more, stable polymer latexes with included IMC in nanosized latex particles, were produced without usage of surfactants – an important advantage of this method for drug formulation. The challenge was to easily find available and feasible technological parameters for the effective control of IMC release from the polymer
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NPs. It was achieved by changing the composition of the mixture of the compatible polymers (pVAc, poly(3-dimethyl(methacryloyloxyethyl) ammonium propane sulfonyl) (pDMAPS), Carbopol®, p(VA-co-DMAPS) and chitosan) from which the NPs with included IMC were prepared. The obtained results confirm the efficiency of these approaches for the control of the IMC degree of loading, encapsulation efficiency, its release degree and also rate of release. [13]

There are ample evidences in the available literature that IMC causes a number of cellular responses in a wide range of concentrations. According to Kralj et al. IMC reduces the proliferation rate of CaCo-2 colon cancer cells (up to 60% at a concentration of 4 x 10^{-4} M), alters their morphology and induces cell death by apoptosis. [14] IMC reduces the proliferation rate and induces apoptosis in CaCo-2 colon cancer cells through enhanced expression of c-myc, p53, and p27 proteins.

Kapitanović et al. showed the effect of IMC on growth inhibition, induction of apoptosis, and gene expression of several genes involved in Wnt signalling in HT-29 colon cancer cells. [15] Their results suggest that the antiproliferative effect of IMC may contribute to enhanced cell adhesion through increased expression of E-cadherin and translocation of β-catenin from the nucleus to the cell membrane.

In another study Carrasco-Pozo et al. showed that in Caco-2 cells IMC promoted Ca^{2+} efflux from the endoplasmic reticulum (ER), resulting in an early, but transient, increment of cytosolic Ca^{2+}, followed by a subsequent increment of intra-mitochondrial Ca^{2+}. IMC also induced cytotoxicity, apoptosis, and increased caspase activities and cytochrome c release, probably through the activation of IP3R and RyR receptors. [16]

On the other hand, Bogdanffy showed that vinyl acetate induced intracellular acidification in rat hepatocytes. [17] Maintenance of pH within a critical range is of paramount importance to the normal function of cellular machinery and the changes in pH values of the environment may be relevant for risk assessment. The aim of the present study was to investigate the biological activity of indomethacin-poly(vinyl acetate) (IMC-pVAc) NPs on serum-free McCoy-Plovdiv cells, mouse lymphoma L5178Y, and retinoblastoma WERI-Rb-1 cells. The reason to use the serum-free McCoy-Plovdiv cells was based on the high sensitivity of these cell cultures, compared to the serum-containing ones. [18] Mouse lymphoma and retinoblastoma cells were used for comparison as suspension-type systems and cancer cells.
MATERIALS AND METHODS

Chemicals/ reagents
Indomethacin (IMC), vinyl acetate (VAc) and ammonium persulfate were purchased from FLUKA (Buchs, Switzerland). Potassium dihydrogen phosphate and di-sodium hydrogen phosphate were purchased from MERCK (Darmstadt, Germany). Fetal bovine serum (FBS), EDTA, dimethyl sulfoxide (DMSO), sodium bicarbonate, Dulbecco’s Minimal Essential Medium (DMEM), Ham’s 12, RPMI-1640 and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from SIGMA-ALDRICH (St. Louis, MO, USA); penicillin and streptomycin – from ANTIBIOTIC-CO (Razgrad, Bulgaria). Trypsin was acquired from DIFCO (Detroit, USA). Membrane filters (0.2 µm) were purchased from Millipore (Billerica, MA, USA).

Preparation of IMC-loaded nanocarriers
IMC-pVAc NPs were obtained by an emulsifier-free radical polymerization of monomers (v/v), in the presence of IMC 1% (w/v). The polymerization was conducted in a nitrogen atmosphere and at a temperature of 55°C, for 90 min under ultrasonic impact (Ultrasonicator Siel UST7.8-200, Gabrovo, Bulgaria). 1% ammonium persulphate was used as an initiator. The latex was exposed to dialysis through a membrane with MWCO 8000 Da for 9 hours to eliminate the low molecular weight compounds (e.g. the initiator of the process, residual monomers or free IMC) from the primary latex, and then the samples were freeze-dried. TEM and DLS were used to observe the microstructure and determine the particle size. XRD-, FTIR-, UV-spectroscopy and simultaneous DTA-TG analysis were applied for the determination of the IMC inclusion and in vitro release characteristics.

Cell cultures
The adherent McCoy-Plovdiv cells were isolated and stabilized as a serum-free cell line from human synovial cells McCoy. The cells were cultivated in DMEM:Ham’s F-12 - 1:1 medium according to Draganov et al. The mouse lymphoma cells L5178Y were obtained from the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC, Sofia, Bulgaria) and were cultivated according to the instructions of Arlett & Cole. The human retinoblastoma cell line WERI-Rb-1 was obtained from ATCC (American Type Cultural Collection, Manassas, VA, USA) and was cultured according to the ATCC protocol. Both cell lines (L5178Y and WERI-Rb-1) were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 UI/ml penicillin and 100 µg/ml streptomycin in 25 cm² tissue culture
flasks (Greiner, SIGMA-ALDRICH). All cells were cultivated at 37°C, 5% CO₂ and high humidity in a thermostat. The morphology of the cells and the state of the cell monolayer were observed and documented with an inverted phase contrast microscope (Nikon Eclipse TS100, NIKON, Tokyo, Japan).

**Cell viability**
Cell viability was determined with the colorimetric MTT assay [21] or with the trypan blue exclusion test. *McCoy* cells were cultured for 24 hours in 96-well plates with a seeding density of 4 x 10⁴ cells/well. The cell medium was replaced with fresh medium containing IMC or IMC-pVAc NPs at the following concentrations – 100, 80, 75, 60, 50, 40, 25, 12.5, 10 µg/ml. 5 replicates were performed for each of the studied concentrations together with controls containing cells treated with PBS. After 24 hours the culture medium was changed with fresh medium containing MTT (500 µg/ml). After a 3-hour incubation cell viability was measured according to the procedures described in the INVITTOX protocol No 17. [22] Quantitative colorimetric determination was carried out at 620 nm on the ELISA reader Multiskan MCC (MTX Labsystems, Vienna, VA, USA). The L5178Y cell suspension was diluted to a concentration of 1 x 10⁵ cells/ml. 2 ml of the suspension were placed in a 50 ml conical centrifuge tube and centrifuged at 200 g for 10 min. The supernatant was removed and the sediment was resuspended in 1 ml fresh growth medium, which contained IMC or IMC-pVAc NPs at concentrations of 100, 80, 75, 60, 50, 40, 25, 12.5, 10 µg/ml. The cells were incubated in 24-well plates for 24 hours. Cell viability was determined with the trypan blue exclusion test 48 hours from the start of the treatment. Cell numbers were determined with a haemocytometer (Bürker).

**Apoptosis detection**
Serum-free *McCoy-Plovdiv* cells were cultured in 10-well microscopic slides (25 µl/well) (concentration of the cell suspension: 4-5 x 10⁵ cells/ml) for 24 hours. The medium was removed and replaced with fresh medium (25 µl/well), which contained 100 µg/ml IMC or IMC-pVAc. After 15-18 hours the cells were washed and stained with anti-Annexin V antibody, conjugated to FITC. Apoptosis was detected with the Annexin V-FITC Fluorescence Microscopy Kit (BD Pharmingen, Franklin Lakes, NJ, USA). The visual assessment of cell morphology was carried out on an invert microscope (Nikon Eclipse TS100).
Calculation of IC\textsubscript{50} values

The cells were cultivated and treated with IMC and IMC-pVAc NPs at all concentrations, described above. The concentration of the test compound at which the cells were inhibited by 50% was calculated theoretically according to the INVITOX protocol No 3b.\textsuperscript{[23]}

Biocompatibility testing

Serum-free McCoy-Plovdiv and retinoblastoma WERI-Rb-1 cell lines were cultured in 24-well plates with a seeding density of 2x10\textsuperscript{5} cells/ml and treated with IMC-pVAc and pVAc (without IMC) dry NPs for 18, 48 and 168 hours. The used concentrations for both NPs were selected so as to ensure a high mortality rate of the cells (150, 250, 350, 450 and 550 µg/well). The visual assessment of cell morphology was carried out on an invert microscope (Nikon Eclipse TS100).

RESULTS

The model IMC-pVAc NPs were obtained and characterized in our previous study\textsuperscript{[13]}. The spherical shape of the NPs was observed with TEM and DLS determined the particle size (128.10 ± 3.4 nm), its polydispersity index (0.133) and zeta-potential (-31.5 ± 1.2) mV. A monomodal particle size distribution was observed for the NPs. Drug loading assessments showed higher values for drug loading (7.67±0.32), encapsulation efficiency (82.92±1.01) and NP yield (98.32±1.33) for this model. Results of release kinetic analyses showed that the release of IMC from IMC-pVAc NPs followed the first kinetics order.\textsuperscript{[13]}

IMC causes morphologic alterations and apoptosis in McCoy-Plovdiv and L5178Y cells

Serum-free McCoy-Plovdiv cells were treated for 24 hours with solutions of IMC and IMC-pVAc, both at a concentration of 100 µg/ml (Fig. 1A and 1B). The synovial cells developed as a culture of adherent cells which was well seen in the control cells (Fig. 1C and 1D). The morphology of the cells was typical of cells which connect and spread over the substrate with well-defined polygonal outlines and visible nucleus and nucleolus. The cells formed a monolayer covering the entire surface of the culture flask. Cells in different stages of mitosis were also observed.

In contrast, formation of a cell monolayer was not detected in cells treated with either IMC (Fig. 1A) or IMC-pVAc (Fig. 1B). Single cells with elongated bipolar shape were attached to the substrate. They possessed vacuolated cytoplasm and indiscernible nuclei and nucleoli (Fig. 1A and 1B). Rounded cells dominated which were either weakly bound to the substrate
or completely separated from it. Interestingly, these cells were not dead and were not stained with trypan blue. This can be explained by the preserved membrane permeability which does not allow the diffusion of the vital dye in the cytoplasm and thus hinders the staining of cells. High cell viability was also recorded in the MTT and NR assays (data not shown).

Figure 1: McCoy-Plovdiv cells treated with 100 μg/ml IMC (A) and 100 μg/ml IMC-pVAc (B) for 24 hours. Control cells were treated with PBS (C, D). Images were taken with an inverted phase contrast microscope (20x objective).

Figure 2: Fluorescence of apoptotic cells in McCoy-Plovdiv cell culture. The arrows point at cells positively stained for apoptosis. Prior to testing serum-free cells were treated with IMC 100 μg/ml for 18 hours. The image is taken with a fluorescent microscope (40x objective).
A lower concentration of IMC and IMC-pVAc (75 µg/ml) also affected the monolayer structure and morphology of cells but to a lesser extent (data not shown). Cells treated with even lower concentrations of IMC (50, 25 and 12.5 µg/ml) resembled the cells in the control samples treated with PBS. Using a standard kit, we demonstrated that IMC induces apoptosis in *McCoy-Plovdiv* cells (Fig. 2).

![Image of cell cultures](image)

**Figure 3:** *L5178Y* cells treated with 50 µg/ml IMC (A) and 50 µg/ml IMC-pVAc (B) for 24 hours. Control cells were treated with PBS (C, D). Images were taken with an inverted phase contrast microscope (20x objective).

The mouse lymphoma cells (*L5178Y*) were strongly altered after 24 hours of incubation with IMC and IMC-pVAc at a concentration of 50 µg/ml (Fig. 3A and 3B). In the cultures treated with lower concentrations of the studied substances (25 and 12.5 µg/ml) both damaged and normal cells were observed which looked like the cells in the control samples (Fig. 3C and 3D).

**IMC reduces cell viability in a concentration- and time-dependent manner**

The results of the MTT test in *McCoy-Plovdiv* cells clearly show that the increase in IMC concentration leads to reduced cell viability (Fig. 4). Moreover, for all studied concentrations the treatment with IMC-pVAc had a stronger effect on the number of living cells than the treatment with IMC alone. 39.5% of cells survived after a 48-hour treatment with IMC at a concentration of 60 µg/ml, whereas only 27.4% of living cells were detected for the same concentration of IMC-pVAc. In the mouse lymphoma cell line (*L5178Y*) cell viability was
perturbed to an even greater extent (Fig. 5). For high concentrations of the studied substances (100 µg/ml and 80 µg/ml) no living cells were detected. For a concentration of 40 µg/ml the cells, which survived after treatment with IMC-pVAc, were more than twice as few as in the samples treated with IMC alone (Fig. 5 – 5.4% vs. 11.6%).

Figure 4: A graph of the survival of McCoy-Plovdiv cells, treated with IMC (black bars) and IMC-pVAc (grey bars). Control cells were treated with PBS. Cell number was calculated with the MTT test, 48 hours from the start of the treatment. The results are mean values, obtained from three independent experiments, each performed in three replicates.

Figure 5. A graph of the survival of L5178Y cells, treated with IMC (black bars) and IMC-pVAc (grey bars). Control cells were treated with PBS. Cell number was recorded in a haemocytometer using the trypan blue exclusion test 48 hours from the start of the treatment. The results are mean values, obtained from three independent experiments, each one performed in three replicates.
Increasing IMC concentration led to a decrease in the number of surviving *McCoy-Plovdiv* cells (Fig. 6). This concentration-dependent effect is observed for the two periods of treatment – 24 hours and 48 hours. No living cells were detected after a 48-hour incubation with 100 μg/ml IMC. IMC treatment for 2 and 4 hours did not influence cell viability (data not shown) even at high concentrations. Cell density and vitality corresponded to those of the control sample.

![Figure 6: Dynamics of the cell viability in the *McCoy-Plovdiv* culture, depending on the concentration of IMC and the duration of the treatment – 24 hours (black bars) and 48 hours (grey bars).](image)

**pVAc nanoparticles do not affect the state and growing rate of cells**

For a direct visualization of the activity of IMC-pVAc and pVAc-only particles (without IMC), an experiment was conducted, in which NPs and their agglomerates were placed in 24-well plates before addition of *McCoy-Plovdiv* cells. The used concentrations were selected so as to ensure a high mortality rate of the cells (150, 250, 350, 450 and 550 μg/well). Figure 7 shows that after 18 hours incubation only the cells treated with 150 μg IMC-pVAc were damaged and rounded (Fig. 7B). A stable cell monolayer was formed in the well with pVAc NPs 18 hours after seeding the cells (Fig. 7F). The cells were outspread with well-defined nucleus and nucleoli much as the cells in the control sample (Fig. 7D). There were many dividing cells. The cells were microscopically observed in the following days of incubation and images were obtained at 48 hours and 168 hours after seeding (Fig. 8). pVAc NPs without IMC did not change the state and growing rate of the cultivated cells. The late post-confluent changes of the cell monolayer were similar to those observed in the control cells.
They were characterized by a decrease in the cell size, accumulation of cells in height and a small number of dividing cells. At this stage, in the cultures treated with pVAc NPs a cell overgrowth of small particles was observed.

The results were confirmed in retinoblastoma cells WERI-Rb-1 (Fig. 9). The cells did not form a monolayer but grew as large clusters (Fig. 9D). All cells died after 18 hours in the sample treated with IMC-pVAc (Fig. 9F) in comparison to the control treated with pVAc NPs without IMC (Fig. 9B). Microscopic evaluation of the state of the cultures in the following days showed no visible negative impact on the development of those cultures in which pVAc NPs were present in the range of the tested concentrations.

![Figure 7](image_url)

Figure 7: McCoy-Plovdiv cells, plated with a seeding density of 2x10⁵ cells/ml. A, B: a well with pVAc particles (400μg) (indicated with thick arrows) at the time of seeding (A) and 18 hours later (B); C, D: the control well at the time of seeding (C) and 18 hours later (D); E, F: a well with IMC-pVAc particles (150μg), (indicated with thin arrows) at the time of seeding (E) and 18 hours later (F). A, C, E: images are taken with 10x objective; B, D, F: images are taken with the 20x objective.
Figure 8: McCoy-Plovdiv cells, plated with a seeding density of $2 \times 10^5$ cells/ml. A, B: a well with pVAc particles (400μg) (indicated with thick arrows) 48 hours after seeding (A) and 168 hours after seeding (B); C, D: the control well 48 hours after seeding (C) and 168 hours after seeding (D); E, F: a well with IMC-pVAc particles (150μg), (indicated with thin arrows) 48 hours after seeding (E) and 168 hours after seeding (F). A, C, E: images are taken with 10x objective; B, D, F: images are taken with the 20x objective.
Figure 9: WERI-Rb-1 cells, plated with a seeding density of $2 \times 10^5$ cells/ml. (A) - a well A, B: a well with pVAc particles (400μg) (indicated with thick arrows) 48 hours after seeding (A) and 168 hours after seeding (B); C, D: the control well 48 hours after seeding (C) and 168 hours after seeding (D); E, F: a well with IMC-pVAc particles (150μg), (indicated with thin arrows) 48 hours after seeding (E) and 168 hours after seeding (F). A, C, E: images are taken with 10x objective; B, D, F: images are taken with the 20x objective.

DISCUSSION
The results of our study showed that IMC induced morphological and physiological changes, characteristic of apoptosis, in McCoy-Plovdiv and L5178Y cells. This was proven with a fluorescent test which detects early apoptotic changes such as the translocation of the membrane lipid phosphatidylserine from the cytosolic to the exoplasmic face of the cell membrane. The microscopic observations allowed the changes in cell morphology to be determined very early, a few hours after treatment, especially when IMC was used at a concentration of 100 μg/ml (Fig. 3). For the suspension cell lines L5178Y and WERI-Rb-1 no apoptosis-specific test was used, but the observed changes in cell morphology as well as the preserved cell membrane prompted the assumption that cell death occurred through
apoptosis-dependent mechanisms. Although the cell changes were visible and well emphasized the concentration-dependent effect, we could not prove them with viability dyes such as MTT, NR or trypan blue even at 24 hours of treatment. On the 48th hour of treatment, however, a MTT test was carried out in the serum-free McCoy-Plovdiv cells (Fig. 4) and a trypan blue exclusion test in the mouse lymphoma L5178Y cells (Fig. 5). This is due to the fact that cells, in which induced cell death-related processes occur, have a preserved cell membrane and there is no reduction in cell density and/or vitality.

For both cell lines (McCoy-Plovdiv and L5178Y) a concentration-dependent effect was established. Reducing the concentration of IMC led to an increase in the percentage of surviving cells (Fig. 4 and Fig. 5). The lymphoma cells showed a higher sensitivity towards treatment with IMC than McCoy-Plovdiv cells. This was also confirmed by comparing their IC50-values. For McCoy-Plovdiv IC50 was 65.8 μg/ml and for L5178Y IC50 was 26.8 μg/ml. The stronger cytotoxic effect of IMC in lymphoma cells could be due to the physiology of cancer cells and the shorter doubling time – 10-12 hours for lymphoma cells[20] versus 20-24 hours for McCoy-Plovdiv cells.[19]

In addition to the concentration-dependent effect, a time-dependent effect of IMC treatment was also observed. Longer IMC incubation times led to a greater decrease in the number of surviving cells. Very short periods of IMC treatment (2-4 hours) did not affect cell viability and more than 80% of the cells survived even at an IMC concentration of 100 μg/ml. The same concentration led to a very small number of living cells after 24 hours of treatment (6.5%). After 48 hours no living cells were detected (Fig. 6).

The IMC-pVAc NPs showed a higher activity for all of the applied concentrations than the IMC solution and led to a greater decrease in the number of living cells. We ascribe this effect to the slow release of IMC included in the NPs which greatly reduces cell vitality, especially at 48 hours of treatment.

To exclude that pVAc carriers cause cell death per se, we performed an experiment in both cell lines McCoy-Plovid and WERI-Rb-1 (Fig. 7 – 9). The results clearly confirmed that pVAc NPs (without IMC) did not cause any inhibitory or cytotoxic effects, which could prevent cell adhesion and normal development of serum-free McCoy-Plovdiv and human retinoblastoma WERI-Rb-1 cells.
CONCLUSIONS
Indomethacin perturbs the viability of cells in vitro through apoptosis-related mechanisms. Furthermore, IMC affects cells in a concentration- and time-dependent manner. IMC-pVAc nanoparticles exert a greater cytotoxic effect on cells than IMC alone which is attributable to the delayed release of the active substance, included in the polymeric carrier. The pVAc NPs are biocompatible with respect to cell cultures and do not alter the proliferation and development of cells.

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