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Colony-Forming and Cell Viability Assay to Assess Nanotoxicity of Maleic Acid and N-vinyl Caprolactam-Based Nanoarchitectures

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Abstract

Organo-montmorillonite (Org-MMT) is a widely used silicate in polymer nanotechnology, enhancing the durability of nanocomposites by improving polymer strength and thermal stability. This study evaluates the anticancer effects of poly(maleic anhydride-alt-N-vinyl caprolactam) [poly(MA-alt-VCL)]-Org-MMT nanocomposites synthesized with varying clay concentrations. The cytotoxicity of Org-MMT, poly(MA-alt-VCL), and their nanocomposites were tested on HeLa (cervical carcinoma), A549 (lung cancer), and HDF (human dermal fibroblast) cells using MTT and colony formation assays. Our results indicate that cell viability is significantly inhibited in both cancer cell types with an IC50 value of 2 mg/mL especially in A549 cells, while 10 mg/mL in HDF cells. Nanocomposites significantly inhibited colony formation in both cancer cell lines, particularly in the HeLa cell line. The data indicated an inverse correlation between clay content in the copolymer complex and cell viability. The copolymer complex without clay had no negative impact on the cells. These findings suggest organo-clay nanocomposites as promising candidates for anticancer drug research.

Keywords: Organo-clay; Montmorillonite; poly(MA-*alt*-VCL); Anticancer; Drug delivery

Introduction

Nanocomposites constitute a class of biocompatible materials. These materials are created by combining biomaterials, polymeric, and biodegradable matrix structures with nano-sized fillers [1]. Material dimensions on the nanometer scale enable the formation of interaction phase interfaces, allowing the material properties to be improved [2]. Polymer nanocomposites have properties such as high thermal stability, high resistance to abrasion, and low gas permeability [2]. Among the nanocomposites produced, especially polymer/clay nanocomposites have attracted great attention in recent years due to their high mechanical and thermal resistance [3].

Clay ratios are used in various biomedical applications because they are natural and safe. Nanomodifications consist of minerals with different chemical contents and different morphologies [4]. Although montmorillonite is a frequently used clay, its use with some organic polymers is limited due to its extremely hydrophilic nature [5]. Therefore, formed by organic modification Organo-montmorillonite (Org-MMT) is a type of clay frequently used in polymer nanotechnology [6]. The reason why this clay is frequently used both in laboratory research and in industry is its low cost and good polymer compatibility.

Polymers are being studied intensively for the development of drug delivery systems. Among these polymers, polymers that are classified as smart polymers and can change their microstructure from hydrophilic form to hydrophobic form in response to changes in environmental conditions attract a lot of attention [7].

Poly (N-vinyl caprolactam) is a temperature-sensitive and highly biocompatible polymer. They exhibit a very stable behavior towards hydrolysis. Poly(N-vinyl caprolactam) is a very important polymer since the use of thermally sensitive polymers in biomedical applications constitutes [8]. Maleic anhydride (MA) is an unsaturated and non-polymerizable dicarboxylic anhydride with different functional groups [9]. Anhydride-containing copolymers constitute a wide field of research with areas of use such as solubility-enhanc-

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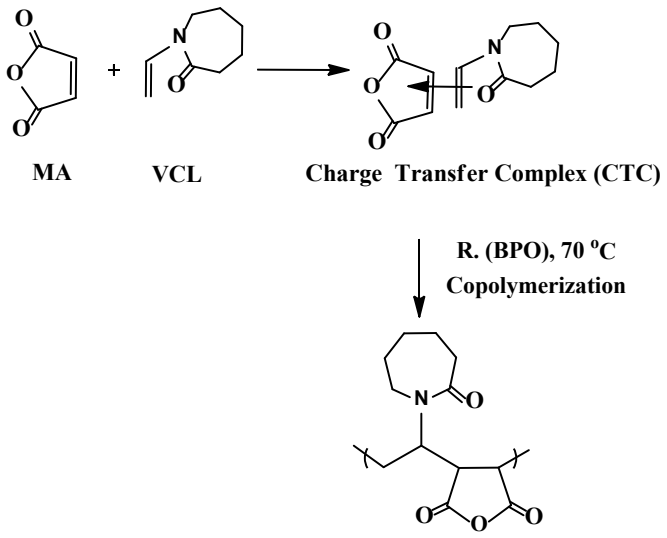
ing components, chemicals that can perform surface modification, and drug delivery systems [10]. For example, in a study, the chemotherapeutic effectiveness of nanoparticle drug carrier systems coated with Poly(N-vinyl caprolactam) and loaded with doxorubicin was evaluated on A549 lung cancer [9]. In another study, the cytotoxic effects were investigated by using Poly(N-vinyl caprolactam) coated graphene oxide as a nanocargo to deliver a water-insoluble anticancer drug, camptothecin, to KB cells [11]. While the produced nanocargo did not have a toxic effect on the cells alone, a toxic effect was observed on the cells when loaded with medicine. At the same time, temperature-dependent drug release was achieved thanks to Poly(N-vinyl caprolactam) [9]. We aimed to evaluate the anticancer properties of copolymers of N-vinyl caprolactam and maleic anhydride, along with copolymer-clay nanocomposites on different cancer cell lines and healthy cell lines. We used HeLa and A549 cells to evaluate the anticancer effects of the materials, while we used HDF cells for the healthy control group. MTT cell viability assay, colony formation assay, and AO/PI live/dead cell staining assays were performed to evaluate anticancer activity.



Scheme 1. Diagrammatic depiction of the copolymerization process of maleic anhydride (MA) and N-vinyl caprolactam (VCL) [12, 13].

Materials-Methods

Chemicals

The chemicals were used for experiments as follows, Maleic anhydride monomer (MA) (Sigma-Aldrich, Germany), N-vinyl caprolactam (VCL) monomer (Fluka, Switzerland), the benzoyl peroxide (BPO) (Fluka) , Octadecyl amine-modified montmorillonite (Org-MMT or O-MMT) (Istanbul, Turkey). MA was subjected to purification by vacuum distillation, whereas the VCL was purified through recrystallization. BPO was recrystallized twice from a methanol solution to ensure maximal quality and purity. All the other solvents and reagents used for experiments were of analytical grade and were utilized without further purification.

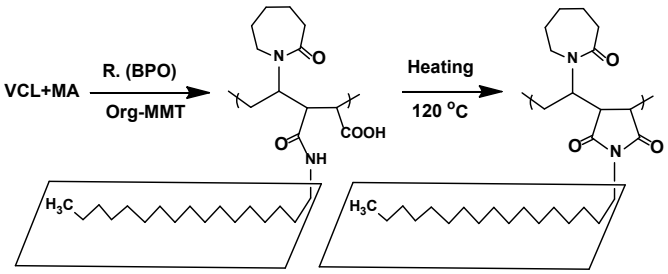
Cell viability, Acridine Orange/Propidium Iodide staining, and colony formation experiments were done using; Tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyl tetrazolium bromide (Cat no: M5655, Sigma Aldrich, Germany), DMEM: Ham's F12 (DMEM/F12) or DMEM High Glucose medium with 2 mM L-glutamine, 10% fetal bovine serum, and 100 IU/mL Penicillin–Streptomycin (Cat no: L0092, Cat no: S0615, Cat no: L0022 Cat no: S0615, Cat no: L0022; Biowest, USA), Crystal violet (Cat no: 101408, Merck).

Copolymerization

Org-MMT (Organo-montmorillonite), H1 Org-MMT (%0.5 Org-MMT 8h), H2 Org-MMT (%5.0 Org-MMT 8h), H3 Org-MMT (%0.5 Org-MMT 24h), H4 Org-MMT (%5.0 Org-MMT 24h), H1-BULK (Copolymer 8h), H2-BULK (Copolymer 24h) all copolymer and its nanocomposites were synthesized by our team in previous studies (Scheme 1). [12, 13]. The process of creating poly(MA-*alt*-VCL) *involved combining* MA and VCL monomers in a 50:50 ratio and initiating the reaction with 0.5% BPO at 70°C in a nitrogen atmosphere using carousel-type Pyrex glass microreactors equipped with a thermostated heater and magnetic mixer. The resulting copolymers were separated from the reaction mixture by precipitation with diethyl ether, washed with several portions of benzene, and then dried at 50°C under vacuum [12, 13].

Interlamellar Copolymerization

Polymer/clay nanocomposites were synthesized under consistent copolymerization conditions, varying the amounts of octadecyl amine-modified montmorillonite (Org-MMT) and reaction times. MA and VCL monomers, along with octadecyl amine-MMT, were suspended in a 0.5% to 5.0% molar ratio and subjected to intensive mixing at room temperature for 4 hours to form the intercalated complex of monomer and clay, without the addition of an initiator. Subsequently, BPO was introduced as the initiator at a concentration of 0.5%, and copolymerization was carried out at 70°C under a nitrogen atmosphere for reaction times of 8 hours and 24 hours, using a 1:1 molar ratio of the initial monomers." (Scheme 2). The prepared mixture was subsequently treated with a large volume of diethyl ether, followed by washing with multiple portions of benzene, and then dried under vacuum. The resulting nanocomposites were post-cured in an oven at 120°C for 8 hours to ensure the complete transformation of the amide linkages into imide forms and to produce polymer hybrids with a more compact nanostructure [12, 13].



Scheme 2. Diagrammatic depiction of intercalative *in situ* bulk copolymerization of *N*-vinyl caprolactam (VCL) with maleic anhydride (MA) [12, 13].

Cell viability test

Tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphnyltetrazolium bromide (MTT) assay was used for cell viability experiments. The materials prepared 0,2 mg/mL, 2 mg/mL, 5 mg/mL, and 10 mg/mL concentrations and sterilized by filtration using 0.22 µm syringe filters.

HeLa, A549, and HDF cells were seeded at a density of 1×10^4 cells/well in 96-well plates and incubated at 37 °C and under 5% CO_2 in air. After 24 h of seeding, the cells were treated with 0,2 mg/mL, 2 mg/mL, 5 mg/mL, and 10 mg/mL concentrations of materials for 24 h, and an MTT assay was performed described our previous work [3]. Briefly, culture medium containing MTT was added to each well and incubated for 4 h. The medium containing MTT was aspirated after incubation and 100 µL of acidic isopropanol (0.05 N HCl in absolute isopropanol) added to each well. Absorbance (OD) was measured at 570 nm using a microplate reader connected to a UV-visible spectrophotometer (EZ Read 400 Microplate Reader, Biochrom, UK). Morphological changes of the cells were examined using an inverted microscope (IX70 Olympus, Japan). All experiments were performed in triplicate.

Acridine Orange/Propidium Iodide Staining

Acridine orange (AO)/propidium iodide (PI) staining was used to assess cell morphology and viable/dead cells. In this technique, live cells stained with AO/PI emit green fluorescence under dark-field microscopy, whereas nonviable cells emit orange fluorescence [13]. After 24h of treatment with the materials the medium on the cells was removed and the cells were stained for 20 seconds with AO/PI (Sigma-Aldrich, Germany) (600 mg/mL AO, 600 mg/mL PI, ratio 1:1). The cells were then washed twice with PBS (Sigma-Aldrich, Germany) and examined under fluorescence microscopy (IX70; Olympus, Tokyo, Japan) and photographed with a digital camera (DP71; Olympus, Tokyo, Japan) under 10X magnification [14]. The viable (green nucleus) cells were counted using Image J software (Bio-Rad) and the percentage of cells was given as graphs.

Colony Formation Assay

A colony formation assay was done to assess the effects of the materials on colony formation ability of cancer cells. Cancer cells seeded at a density of 600 cells/well in 6-well plates. After 24 hours of incubation, cells were treated with Org-MMT, poly(MA-*alt*-VCL) and poly(MA-*alt*-VCL)/Org-MMT polymer clay materials at concentrations of 0.2 mg/mL and incubated for 10 days. After 10 days of incubation, the medium was removed and the plate was placed on ice. Cells were washed with cold PBS and incubated with methanol for 10 minutes. The methanol was removed, and 0.5% crystal violet (containing 25% methanol) was added and incubated for 5 minutes at RT. After 5 minutes, the wells were washed until no color remained and the wells were photographed, and colonies were counted using Image J software (Bio-Rad).

Statistical Analysis

All treatment groups data were compared with the control group using t-test. The analyses of collected data were done using Graph Pad Prism (Graph Pad Software, version 8, USA). $p<0.05$ were considered significant. All experiments were performed in triplicate.

Results

Polymer clay nanocomposites hold significant potential for advancing biomedical applications, including diagnostic and therapeutic devices, tissue regeneration, drug delivery systems, and various biotechnologies inspired by biological processes but with only indirect biomedical connections.

Water-soluble anhydride-containing copolymers, utilized as polyanions, and their functional derivatives exhibit significant biological and physiological activity, notably including antimicrobial and antitumor properties. In this regard; our group defined synthetic approaches by *in situ* solution charge transfer complex copolymerization (CTC) allowing the preparation of polymer/clay nanocomposites which facilitates the design and synthesis of functional alternating copolymers and nanocomposites. Functional copolymers, featuring a combination of rigid and flexible linkages and the capability to form complexes with the interlayer surfaces of organo-silicate materials, along with their nanocomposites, have been synthesized via interlamellar complex-radical copolymerization. This process involves the use of intercalated monomer complexes of MA and VCL with org-MMT and/or several monomer mixtures. The poly(maleic anhydride-alt-N-vinyl caprolactam) poly(MA-alt-VCL) pristine copolymer and poly(MA-alt-VCL)/organically modified MMT (Org-MMT) nanocomposites were characterized by Elemental Analysis, FTIR, XRD, DMA, and TEM methods and published by Kaplan Can [12-13].

This study will enlighten the anticancer effects of characterized copolymer and its nanocomposites. We used HeLa and A549 cells to evaluate the anticancer effects of the materials, while we used HDF cells for the healthy control group. MTT cell viability assay, colony formation assay, and AO/PI live/dead cell staining assays were performed to evaluate anticancer activity.

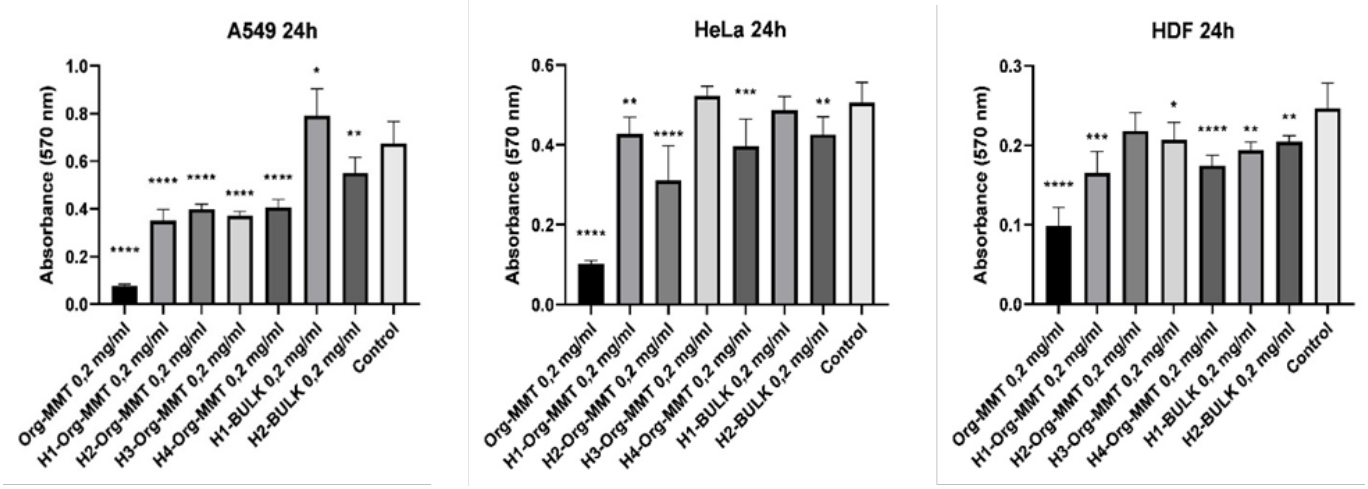


Figure 1. Cell viability after 0,2 mg/mL treatment. Cytotoxicity evaluation of poly(MA-*alt*-VCL), and poly(MA-*alt*-VCL)Org-MMT nanocomposites materials after 24 h of incubation for 0,2 mg/mL concentrations in HeLa, A549, and HDF cells. Cell viability differed in poly(MA-*alt*-VCL) and poly(MA-*alt*-VCL)/Org-MMT polymer clay materials groups. Cell viability was significantly reduced in the poly(MA-*alt*-VCL)/Org-MMT polymer clay materials group, especially in A549 cells. Org-MMT (clay) shows high toxicity in all cell lines. Additionally, data from the other groups indicate that an increase in clay content and polymerization time leads to a decrease in cell viability. Graph Pad Prism (Graph Pad Software, V8, USA) used for data analysis (* p<0,05 / **p<0,009 / ***p<0,0005 / ****p<0,0001).

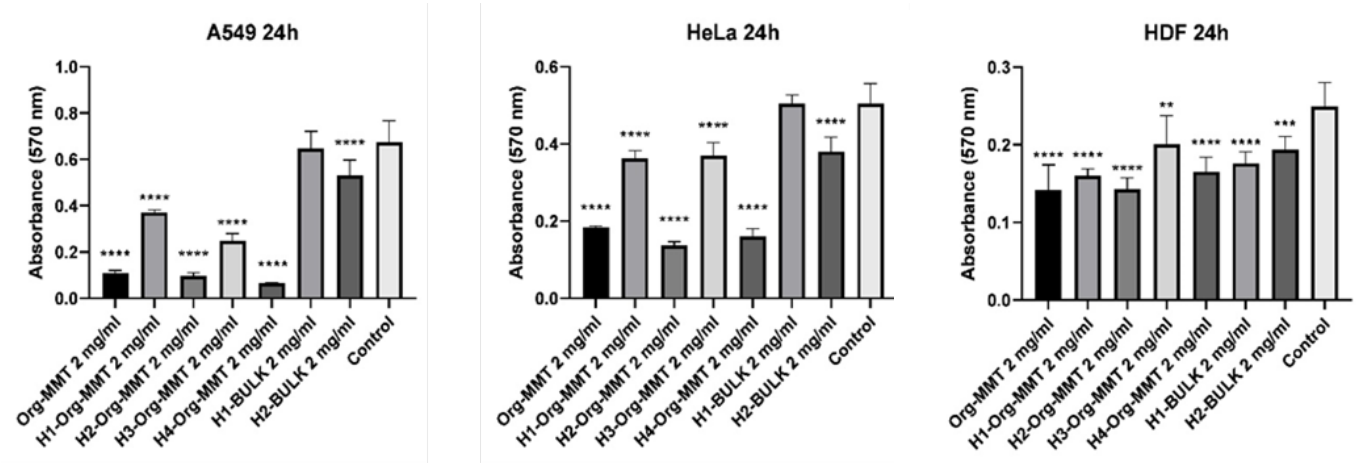


Figure 2. Cell viability after of 2 mg/mL treatment. Cytotoxicity evaluation of poly(MA-*alt*-VCL) and poly(MA-*alt*-VCL)/Org-MMT polymer clay materials at 2 mg/mL concentrations in HeLa, A549, and HDF cells after 24 h incubation. Cell viability was different in poly(MA-*alt*-VCL) and poly (MA-*alt*-VCL)/Org-MMT polymer clay material groups. Cell viability was significantly decreased in the poly(MA-*alt*-VCL)/Org-MMT polymer clay materials group, especially in A549 cells. Figure 2 shows that there was high toxicity in the clay-containing groups. In addition, the increase in the applied concentration led to a significant decrease in cell viability. When the healthy control group, HDF cell line and cancer cells are compared, it can be said that nanocomposites have less toxic effect on healthy cells. Graph Pad Prism (Graph Pad Software, V8, USA) was used for data analysis. (* p<0,05 / **p<0,009 / ***p<0,0005 / ****p<0,0001).

Cell Viability

Our results showed that a concentration of 2 mg/mL gave an IC50 value for cancer cells and that the materials were especially more effective in the A549 cell line, while this value was 10 mg/mL in healthy cells (Fig 1 and Fig 2). Cell viability decreased significantly in the poly (MA-*alt*-VCL)/Org-MMT polymer clay materials group, especially in A549 cells. Org-MMT, the clay-containing group, had the highest effect on cell viability for each cell type (p<0.0001). Significant decreases in cell viability were observed in copolymer complexes in which polymers and clay contents were used together, especially when the polymerization time was increased from 8 to 24 hours. Decrease in

cell viability was observed very clearly, especially in the A549 cell line. While Org-MMT was the most effective compound in this cell line, H1-Org-MMT, H2-Org MMT, H3-Org-MMT and H4-Org-MMT compounds also significantly reduced cell viability compared to the control and p values $p<0.0001$ were found for each compound at 0.2 and 2mg concentrations. No substantial reduction in cell viability was observed in copolymer complexes composed solely of polymers, as compared to those incorporating clay ($p<0.009$). Considering these results, we can say that the toxic effect of clay content on cells is high, but this toxic effect is not seen in polymer complexes. The data showed an inverse correlation between clay content in the copolymer complex and cell viability. The clay-free copolymer complex had no adverse effect on the cells.

Acridine Orange/Propidium Iodide

AO/PI staining showed morphological changes in both A549 cells and HeLa cells. The both cells normally have epitheli-

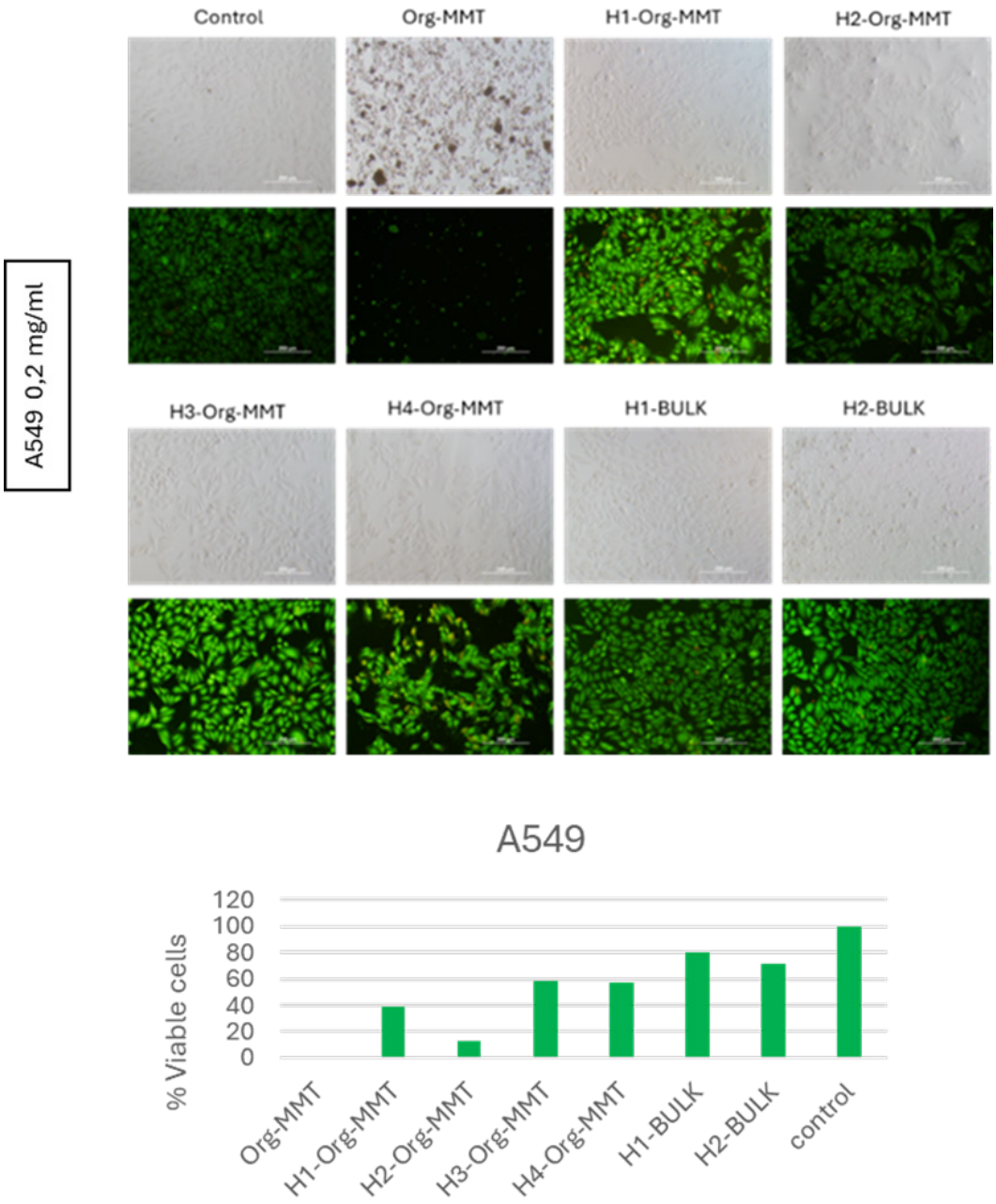


Figure 3. AO/PI live/dead cell staining images of A549 cells at a concentration of 0.2 mg/mL; Epithelial-like morphology was preserved in the control group, a major change in the morphology of the cells was observed in the treated groups, especially in the Org-MMT group. Similarly, changes in the morphology of the cells were also observed after H2-Org-MMT treatment. Accordingly, the number of viable cells decreased especially in Org-MMT, H1-Org-MMT, H2-Org-MMT, and H4-Org-MMT groups (10X magnification, DP71; Olympus, Tokyo, Japan).

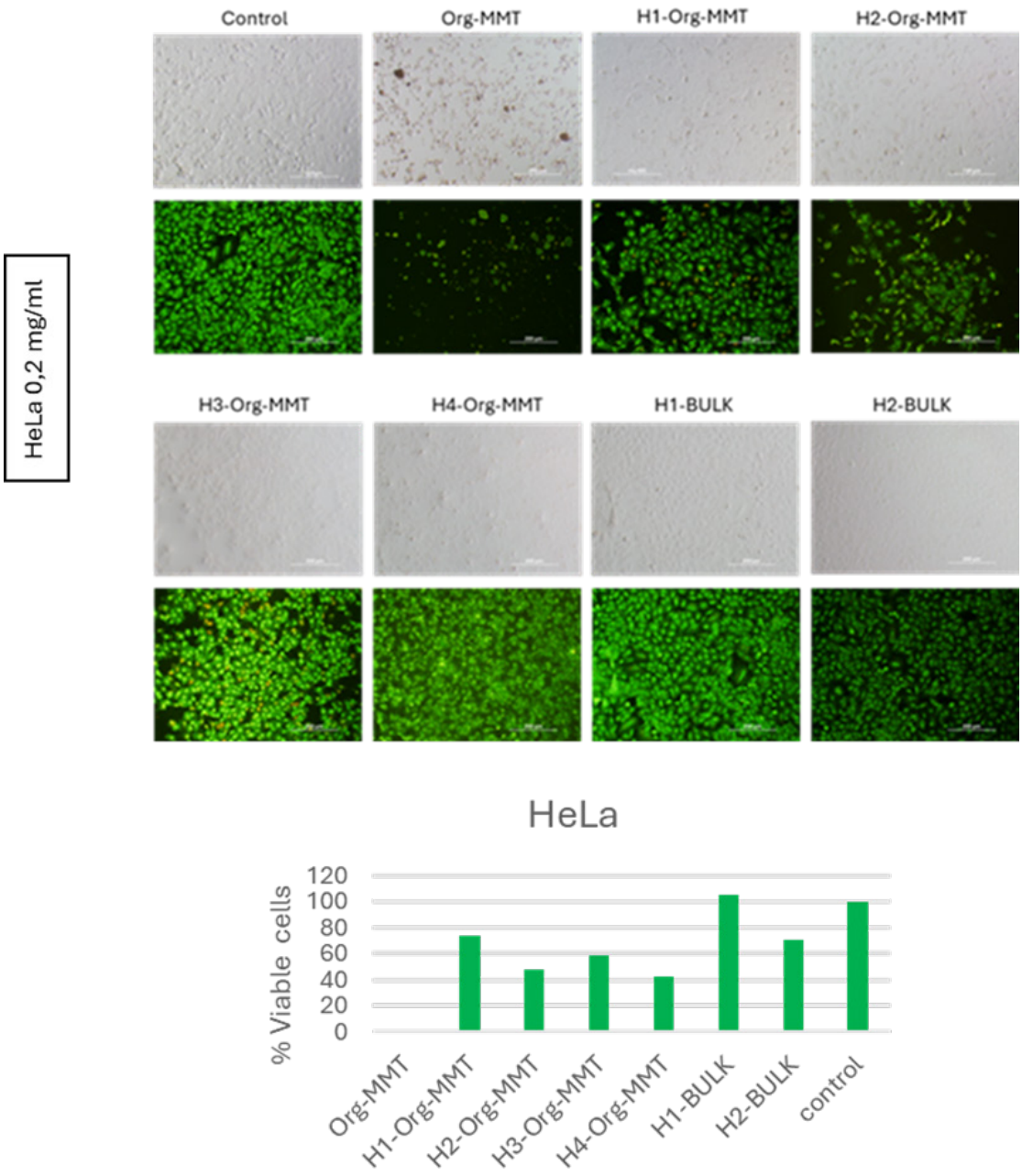


Figure 4. AO/PI live/dead cell staining images of HeLa cells at a concentration of 0.2 mg/mL; Among the treated groups, especially in Org-MMT, H1-Org-MMT, H2-Org-MMT, and H4-Org-MMT groups, deterioration in cell morphology and decreases in cell viability were observed compared to the control (10X magnification, DP71; Olympus, Tokyo, Japan).

al-like morphology. While this epithelial-like morphology was maintained in the control group, a major change was observed in the morphology of the cells in the treated groups, especially in the Org-MMT group (Fig 3 and Fig 4). These cells lost their natural morphology and changed to a round form after Org-MMT application. Among these groups, it was observed that the number of live cells decreased compared to the control, especially in the Org-MMT, H1-Org-MMT, and H2-Org-MMT groups. The results obtained from AO/PI staining support the MTT results, and the efficacy of the materials is higher in the A549 cell line. The percentage of the viable cells (green nucleus) were counted, and results showed that the Org-MMT, H1-Org-MMT, and H2-Org-MMT groups showed the lowest living cell ratio (Fig 3 and Fig 4).

Colony Formation Assay

The results of the colony formation assay align with the findings from the cell viability and AO/PI staining experiments at a concentration of 0.2 mg/mL. Polymer clay materials suppress colony formation in cancer cells. In particular, a decrease in the ability of cancer cells to form colonies was observed with increasing clay content. These results showed that the clay content was highly effective in suppressing colony formation (Fig 5). When we look at Figures 5.1 and 5.2, it is seen that the ma-

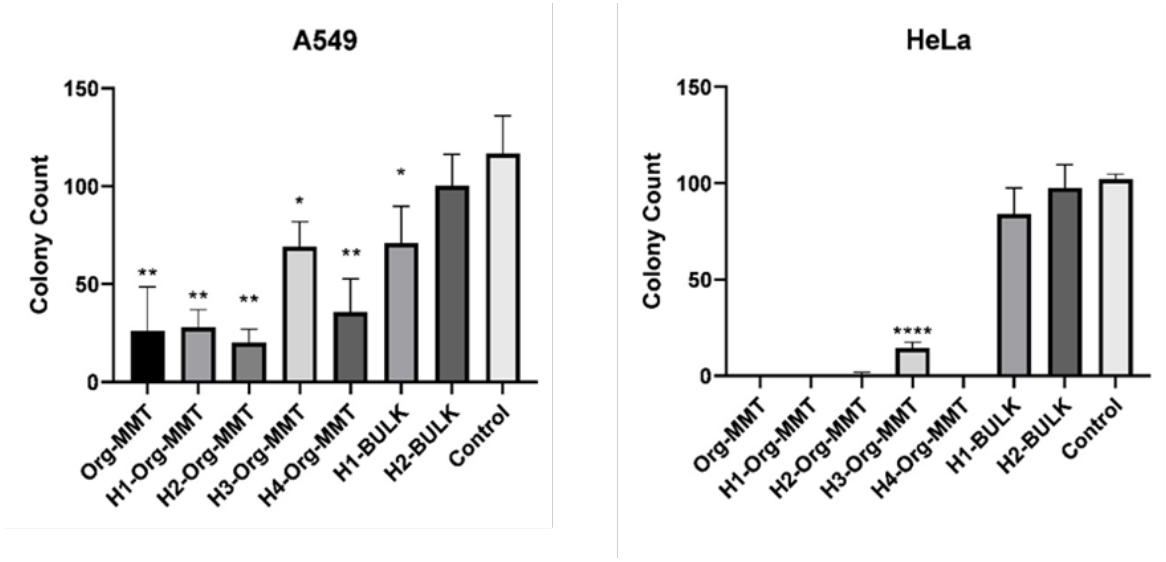


Figure 5. Colony formation assay results. In the graphs generated according to the number of colonies, a significant decrease in the number of colonies was observed in the treated groups compared to the control group. As a result of the colony suppression experiment on cells and A549 cells, no negative effect of H1-BULK and H2-BULK polymer groups on colony formation was observed. On the other hand, colony formation was significantly suppressed in the groups containing clay. Especially in HeLa cells, colony formation was almost completely suppressed in all groups containing clay. Graph Pad Prism (Graph Pad Software, V8, USA) was used for data analysis. (* p<0,05 / **p<0,009 / ***p<0,0005 / ****p<0,0001).

terials, which were found to be more effective in the A549 cell line in MTT cell viability analysis and AO/PI staining results, showed the different effect in colony formation and were more effective in HeLa cells.

Cancer is a global health problem characterized by the uncontrolled growth of cells and their spread throughout the body [15]. According to data from the National Agency for Cancer Research, cancer remains one of the leading causes of death worldwide [16]. While current treatment options include surgery, radiation therapy, chemotherapy, hormone therapy, and immunotherapy, the associated side effects highlight the necessity for new therapeutic alternatives [17]. Researchers are exploring the potential of biocompatible polymers and clay materials, particularly in the development of drug carrier systems, as a promising avenue for further investigation [18].

Nanocomposites synthesized by combining various polymers with montmorillonite clay have been researched for their potential in drug delivery, cancer treatment, antibacterial, and anti-inflammatory activities [19]. Poly (N-vinyl caprolactam) is a remarkable polymer that responds to changes in temperature. As the temperature rises above 35°C, it transitions from being hydrophilic to hydrophobic in nature, and this change is reversible, making it a perfect candidate for targeting hyperthermia in tumor cells [8]. Additionally, maleic anhydride, a pH-sensitive polymer, can also be used to target pH changes in tumor cells [7]. These unique properties make both polymers and their copolymer complexes highly promising for drug research for the treatment of cancer. Although there are studies on N-vinyl caprolactam, maleic anhydride polymers, and montmorillonite clay in the literature, studies using these three materials together are limited. In this perspective, we assess the anticancer effects of these nanocomposites, whose anticancer effects have not been evaluated when synthesized together before.

Poly(N-vinyl caprolactam) microgels prepared by Etchenausia et al. were tested on HeLa and RAW cells, and cell viability was found to decrease by 70% after applying this compound [20]. Rahmani et al. showed that Poly(ε-caprolactone)-co-poly maleic anhydride micelles were suppressed MDA-MB-231 breast cancer cell line viability whereas the micelles did not show toxicity when applied as empty [21]. Connolly et al. evaluated Org-MMT toxicity in HaCaT cells, C3A cells, and J774.1 cells and found that the presence of organic modifiers increased the cytotoxic effects of clay [22]. Our studies on compounds synthesized by copolymerization of N-vinyl caprolactam and maleic anhydride polymers and nanocomposites formed by adding Org-MMT to these compounds gave similar results to these studies in the literature. In the polymer groups, cell viability showed a profile close to the control. However, the increase in clay content led to a decrease in cell viability.

In our previous study, AO/PI results showed no morphological change in cells after poly(maleic anhydride-*alt*-vinyl acetate) treatment of cells, even at the highest concentration [23]. Parallel to these results, we showed that maleic anhydride and N-vinyl caprolactam copolymers had no cytotoxic effects on cells. Karimi-Soflou et al. evaluated the effects of Org-MMT-containing scaffold on cell viability and morphology on bone marrow-derived mesenchymal stem cells, their results showed that clay did not cause any changes in cell viability [24]. On the contrary, in our experiments, Org-MMT caused

significant disruptions in cancer cell morphology while healthy cells showed higher cell viability for the same concentration.

Colony formation assay is a method used to measure the colony-forming potential of cells and is preferred to assess the ability of cancer cells to form colonies [25, 26]. Yang et al. performed a colony formation assay with HeLa cells to evaluate the anticancer potential of the Garcinone-E compound and reported a dose-dependent decrease in the number of colonies [27]. Choudhury et al. performed a colony formation assay after applying the synthesized nanocomposites to HeLa and A549 cells and reported that colony formation was suppressed in both cell lines [28]. We performed a colony formation assay with HeLa and A549 cells to evaluate the ability of poly(MA-*alt*-VCL) and poly(MA-*alt*-VCL)/Org-MMT polymer clay materials to suppress colony formation in cancer cells. The data showed that the colony forming ability of HeLa cells was strongly suppressed and colony formation was suppressed in both cell lines due to the increase in clay content. In contrast, copolymer complexes containing only polymer did not show any negative effect on colony formation in both cell lines. Based on these results, we propose that poly(MA-*alt*-VCL) nanocomposite exhibits a highly biocompatible structure and the addition of Org-MMT clay exhibits anticancer potential by inducing cytotoxic effect in cancer cells. Potential limitations of this study include the necessity for comprehensive molecular analyses across various cancer cell lines and apoptotic assays to further elucidate the mechanisms underlying cell death. Also, in vivo studies with animal models could be conducted to validate the *in vitro* findings.

Conclusion

Based on several experimental parameters, our data suggested that clay-free polymer structures do not have a toxic effect on healthy cells and therefore the use of N-vinyl caprolactam-maleic anhydride copolymers in drug delivery systems seems promising. We also found that increased clay content in polymer-clay nanocomposite structures leads to an increase in the toxic effect on cancer cells. Therefore, it can be concluded that the synthesized nanocomposites demonstrate promising anticancer activity. Possible limitations of this study include the need for detailed molecular analysis, the use of different cancer cell lines, and in *vivo* studies. Clay nanocomposites may serve as promising candidates for targeted drug delivery systems in cancer therapy. Their ability to control drug release, protect drugs from degradation, and achieve site-specific delivery makes them valuable tools for drug research.

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Conflicts of Interest

All the authors declare that they do not have any potential competing interests. This article does not contain no study with human or animal subject performed by any of the authors.

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