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ORIGINAL PAPER

Antiproliferative effect of organoclay, poly(NCA) and their nanocomposites on HeLa cell line

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Abstract

In this study, the synthesis, characterization and thermal properties of poly(*N*-cyclohexylacrylamide) polymer/organoclay-based nanocomposites were investigated by in situ polymerization. FTIR, XRD, SEM and TGA techniques have been used in the characterization of nanomaterials and whether they are exfoliated or intercalated has been investigated. It was determined from XRD and SEM measurements that the morphology of nanocomposites was exfoliated when the clay content in the polymer matrix was kept at 3% and 5%. From thermal analysis, a positive correlation was observed between the clay ratio and thermal stability of nanomaterials. Furthermore, in vitro anticancer efficacy against HeLa cell has been studied and reported by 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide assay. According to the findings, nanocomposites have been successfully synthesized and characterized and their cytotoxic properties have been proven by in vitro study. However, the potential of these nanocomposites as a drug delivery system is needed to be validated and proved in vivo studies in further research.

Keywords *N*-Cyclohexylacrylamide (NCA) · Polymer/organoclay nanocomposite · Organoclay · Thermal stability · In situ polymerization · HeLa cell line · Antiproliferative activity

Introduction

Organoclay consists of the replacement of cations in clay minerals with organic substances such as alkylammonium, dialkylammonium, quaternary ammonium cations [1, 2]. When ion exchange takes place in the clay, organoclay is formed,

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so the surface energy of the clay decreases, the physicochemical properties of the surfaces change, the interaction characteristics with the monomer/polymer structure improve and as a result, the distance between the layers can be opened up to approximately 1 nm. This expansion facilitates the diffusion of polymer chains between the organoclay layers in subsequent steps. In addition to the diffusion of large molecular weight compounds between layers, this feature also allows cations between layers to be replaced with large molecular weight compounds. Thus, the clay surface can be modified by adding hydrophobic properties. There are two consequences in the reaction of converting clay into organoclay. I: Organic cations settle in the range of clay layers, reducing the surface energy of the clay and widen the layer intervals of the clay. II: By changing the surface properties of the clay, the hydrophilic structure turns into a hydrophobic or organophilic structure [2, 3]. The type, chemical structure, amount and hydrophobic character of the clay-modifying organic agent also change the characteristics and the behavior of the organoclay. Thus, these organic agents provide functional groups with which the polymer matrix and reagent can interact [4–6].

In the drug development, the use of clay minerals has some limitations because of the strong interactions between clay mineral and drugs. The strong interactions between the drug and clay minerals can affect the release rate inside the cell. To overcome this handicap, the most successful strategy is to coat clay minerals with polymers to slow down the drug release and provide an administration of the drug for a long time [7]. Also, polymer/clay nanocomposites are now an active area of research because of their enhanced physical, mechanical and chemical properties when compared with those of pure polymer. Research interest in the surface modification of clay minerals has grown significantly in recent years. Polymer/clay nanocomposites, together with their chemical design, are capable of being loaded/bound with various substances including antibiotics, chemotherapeutics, growth factors, RNA and DNA and can be used as a vehicle for drug transportation by being released in a continuous/controlled way [8–12].

In cancer treatment, polymer/clay systems might be successful by the controlled release of the drug [13, 14]. In this present work, synthesis and characterization of amide-containing polymer/clay nanocomposites were made, morphological properties were investigated and found to be exfoliated. Then, the investigation of the cytotoxicity properties of C10A organoclay, *N*-cyclohexylacrylamide homopolymer (polyNCA) and their nanocomposites in HeLa cells was performed. Since one of the most commonly used human cell lines on medical research is the HeLa cancer cell line, HeLa cancer cell lines were used to determine the biological activities of the molecules in this study as well.

Experiment

Materials

Nanoclay 1–135 (C10A) was provided from Esan-Eczacıbaşı. The organic modifier of Nanoclay 1–135 is dimethyl, benzyl, hydrogenated tallow, quaternary ammonium

cation with the particle size range of < 15 μ, where tallow is ~ 65% C18, ~ 30% C16, ~ 5% C14 [15]. For the synthesis of *N*-cyclohexylacrylamide (NCA) monomer; cyclohexylamine (99.9%), acryloyl chloride (97%) and triethylamine (99%), (Aldrich) were used. For nanocomposite synthesis; NCA was used as monomer and benzoyl peroxide (recrystallized before use) (BPO) (Aldrich) was used as initiator and pure tetrahydrofuran and pure hexane were used as solvents.

Instrumental measurements

The FTIR spectra of all samples were performed with a PerkinElmer Spectrum Two (UATR) IR spectrometer in the range of 4000–450 cm^{-1}. XRD patterns were obtained using a Bruker Axs D8 Advance diffractometer with a back monochromator and a Cu target and Kα ($\lambda = 1.5418$ nm) in $2\theta = 10°–45°$ (step of 0.01º, at room temperature). Scanning electron microscope observation was recorded with a Zeiss Evo LS 10 at 25 kV. Thermal analyzes were obtained with a Hitachi 7000 TGA/DTA/DTG simultaneous system at a heating rate of 10 °C/min in nitrogen atmosphere.

Preparation of poly(NCA)/organoclay nanocomposites

NCA monomer and homopolymer were re-synthesized according to the literature (Fig. 1) [16–19]. Poly(NCA)/organoclay nanocomposites were prepared with in situ method. 3% and 5% amount of C10A organonanoclay was dispersed in tetrahydrofuran and stirred by magnetic stirrer at 65 °C for 24 h. 1 M NCA monomer was dissolved in tetrahydrofuran at room temperature in another flask. BPO was added as a free radical initiator to the 3% and 5% organoclay added monomer mixture in separate bottles. In magnetic stirrer, polymer/organoclay composites were synthesized being stirred continuously for 48 h with the help of back cooler. The composites were precipitated in excess hexane, removed from impurities, dried in the oven and sifted through a 20micron sieve.

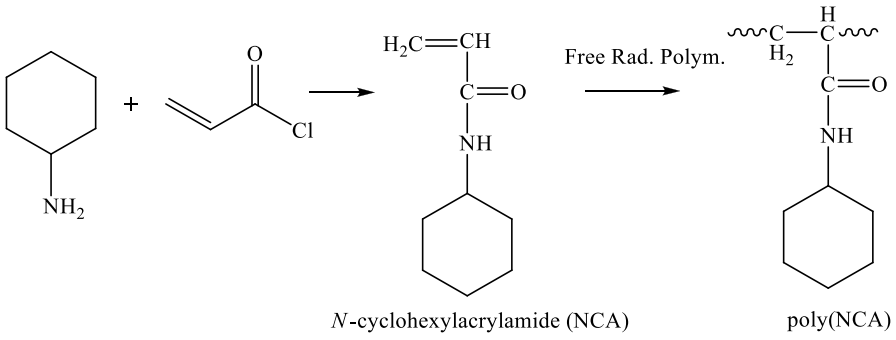

Fig. 1 Synthesis of NCA monomer and homopolymer [16–19]

Antiproliferative effect of organoclay, poly(NCA) and their nanocomposites on HeLa cell line

In this present work, cytotoxicity was evaluated by the XTT assay (Biological Industries, USA). The Hela cells were seeded into 96 well plates at a concentration of 5×10^4 cells/well and they were exposed to different concentrations for 48 h together with C10A organoclay, poly(NCA) and their nanocomposites. Then, the culture solution in each well was aspirated and XTT solution was added. The results of the absorbance measured at 450 nm were directly proportional to the number of viable cells in each treatment by a micro culture plate reader (BioTEK). The 50% inhibitory concentration (IC50) was calculated from obtained values.

Results and discussions

FTIR spectroscopy

Clay contains molecules such as SiO_2, Al_2O_3, MgO. When the FTIR spectra of montmorillonite clay are examined, it was observed that the O–H stretch vibration peaked at 3624 and O–H bending vibration at 1450 cm^{-1}, Si–O stretch at 1010 and bending vibration at 514 cm^{-1}, Al–OH vibration at 913 cm^{-1} and Mg–O vibration at 475 cm^{-1} [15, 20–26]. The C10A organoclay peaks are observed in the clay as mentioned above. Also, C10A organoclay includes dimethyl benzyl alkyl chain quaternary ammonium chloride structures used in its own modification. The following are the peaks from the chemical modifier; aliphatic N–CH_3 vibration at 2840 cm^{-1}, aliphatic CH_2 vibration at 1465 cm^{-1}, symmetric and asymmetric C–H stretching vibration at 2920 cm^{-1} and aromatic C=C stretching vibration at 1644 cm^{-1} [15, 20]. The most common characteristic bands observed for poly(NCA) units in nanocomposites are seen in (cm^{-1}); 3288 and 1536 (stretch and bending N–H vibration) and 1648 (C=O amide stretch) [16–18]. In nanocomposites, all these peaks from the homopolymer are observed. On the other hand, some of the characteristic peaks of organoclay are also seen in composites. Cyclo CH_2 stretch vibration in the ring from NCA homopolymer, aliphatic N–CH_3 vibration from the chemical modifiers of the organoclay and symmetric-asymmetric C-H stretch vibrations are overlapped and it was observed that it gave two separate peaks at 2927 and 2855 cm^{-1}. It is also observed in nanocomposites where Mg–O vibration from organoclay peaks at 463 cm^{-1} and Si–O stretch peaks at 1033 cm^{-1}. From these results, it can be said that the organomodified clay presents in polymer matrix as is reported by other articles [15, 20–26]. Figure 2 shows FTIR spectra of poly(NCA)/3%C10A and poly(NCA)/5%C10A nanocomposites.

XRD measurements

X-ray diffraction is the preliminary technique to verify whether the layered structure has altered or not. The characteristic crystalline peaks of the diffraction angle of

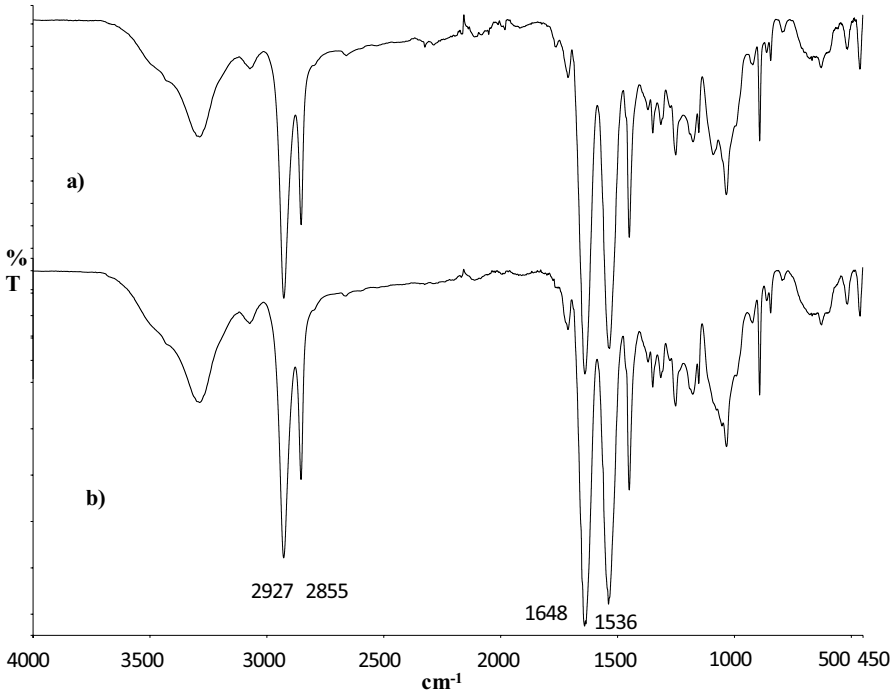

Fig. 2 FTIR spectra of (**a**) poly(NCA)/3%C10A (**b**) poly(NCA)/5%C10A nanocomposites

C10A organoclay was $2\theta = 5.4°$, 20°, 22° ($d = 1.64$, 0.44, 0.40 nm) [15, 20, 21], The distribution of the polymer between the layers of the clay causes a clear XRD peak in the nanocomposites to be unreadable. The absence of component-specific peaks in nanocomposite materials can be explained by the fact that the polymer is intercalated between clay layers and clay layers become so irregular that they cannot give an XRD signal [15, 20–27]. Therefore, it can be considered as an exfoliated structure. Also, all peaks present in the XRD curve of the clays are not observed in the nanocomposites. The XRD patterns of the poly(NCA)/organoclay nanocomposites are shown in Fig. 3a, b.

Morphological study using SEM

SEM micrographs were used for further characterization of nanocomposites. The homogeneous distribution of nanoparticles from SEM photographs is presented in Fig. 4a, b. As seen in the SEM micrographs, the clay was dispersed in the polymer matrix. The increase in the amount of clay resulted in particle size. It can be observed that nanocomposites have a porous-perforated structure. SEM micrographs show that the organoclay is compatible with the NCA homopolymer and they are interacting between the polymer–clay surfaces. Exfoliated structure was observed in the XRD results and confirmed with the help of SEM effects [15, 20–27].

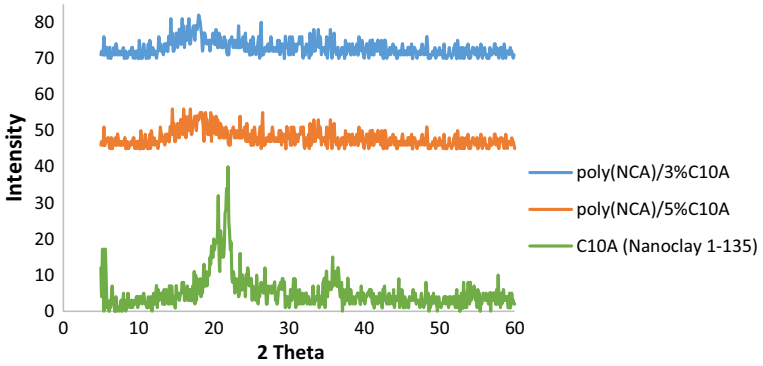

Fig. 3 XRD patterns of (**a**) poly(NCA)/3%C10A (**b**) poly(NCA)/5%C10A

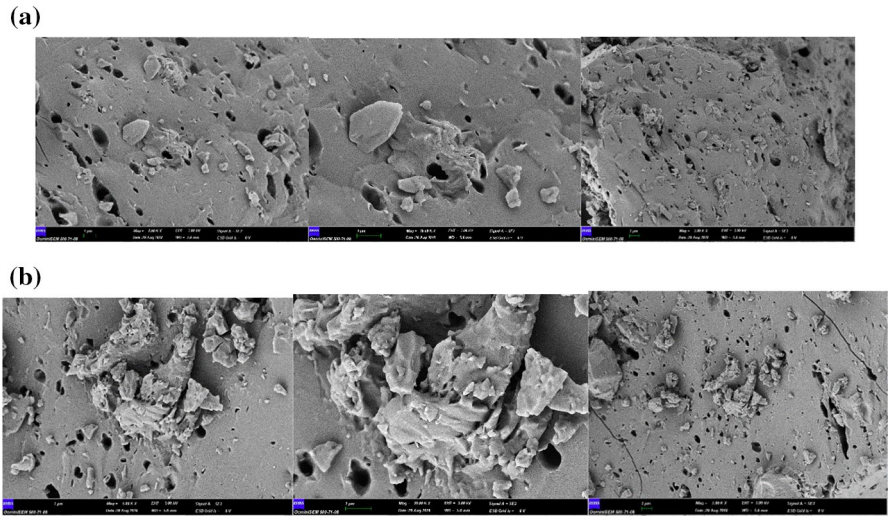

Fig. 4 SEM micrographs of (**a**) poly(NCA)/3%C10A (**b**) poly(NCA)/5%C10A

Thermal characterization

Thermogravimetric analysis method helps determining the thermal stabilities of polymer/clay nanocomposites and provides information about their thermal behavior. The decomposition temperature and the temperature at weight loss are taken as a measure of thermal stability [28]. When nanocomposites' thermal behaviors are compared, it is observed that composites containing more clay have better thermal stability. These thermal changes prove that polymer chains break into the clay galleries and form nanocomposites. The nanodispersion of polymer molecules in silicate layers limits thermal movement, which increases thermal stability. It was observed that thermal stability of nanocomposites formed with

clay increased with the increasing amount of clay in the composite, resulting in a clay thermal barrier [15, 20–27]. It is also known that the thermal data of composites synthesized with a small amount of clay filler give close results [20–27]. The thermal properties of nanocomposites were determined by TGA/DTA/DTG simultaneous system. The degradation of the composites from the thermogram was observed at three levels. Important thermal results for nanocomposites are given in Table 1, the thermal curves of nanocomposites are shown in Fig. 5.

Antiproliferative activity on HeLa cell line for organoclay, poly(NCA) and their nanocomposites

The results of cytotoxicity analyses of different concentrations revealed that C10A organoclay was cytotoxic against cancerous cell line (Fig. 6a). In our previous study, IC50 value of NCA monomer was found 0.07 M for HeLa cells [29]. Compared to the C10A organoclay, nanocomposites poly(NCA)/3%C10A (IC50:230 µg/ml) and poly(NCA)/5%C10A (IC50:181 µg/ml) have lower toxicity on cancer cells (Fig. 6b, c). The cytotoxic effect of NCA homopolymer without clay was not observed at highest dose (2000 µg/ml) on cells. According to the obtained results, it was observed that the toxicity of the synthesized nanocomposite structure on HeLa cell decreased.

Till now, for the delivery of the drugs in cancer treatment, several studies have been performed on nanocomposite which was synthesized from polymer and clay molecules. The use of polymers in the synthesis of these nanocomposites has a more effective role in the binding and transportation of anticancer drugs due to their superficial properties [30] and also polymeric structure enhances immune escape in live systems [31]. Nanoclay has an important role in cancer cells as reported in in vitro/in vivo toxicity studies. Zhang et al. reported that DOX-kaolin MeOH nanoclay showed dose-dependent therapeutic effects in vitro [32]. In another research, Bunyatova et al. demonstrate that intercalated hybrid complexes containing a combination of various anticancer sites, such as free and complexed carboxyl, trithiocarbonate, amine and ammonium cations significantly induced cell death in breast cancer [33]. Hosseini et al. (2018) analyzed cytotoxic effect of doxorubicin-bentonite nanoclay complex on melanoma cancer cells [34].

Table 1 Some thermal data for nanocomposites

Sample	Max. decomp. temp. (°C)	Temp. of 50% weight loss at (°C)	%Weight loss at 400 °C	%Weight loss at 450 °C	%Weight loss at 500 °C	%Residue at 550 °C	%Residue at 600 °C
poly(NCA)/3%C10A	412	394	56	94	95	5	4
poly(NCA)/5%C10A	413	395	55	94	95	5	5

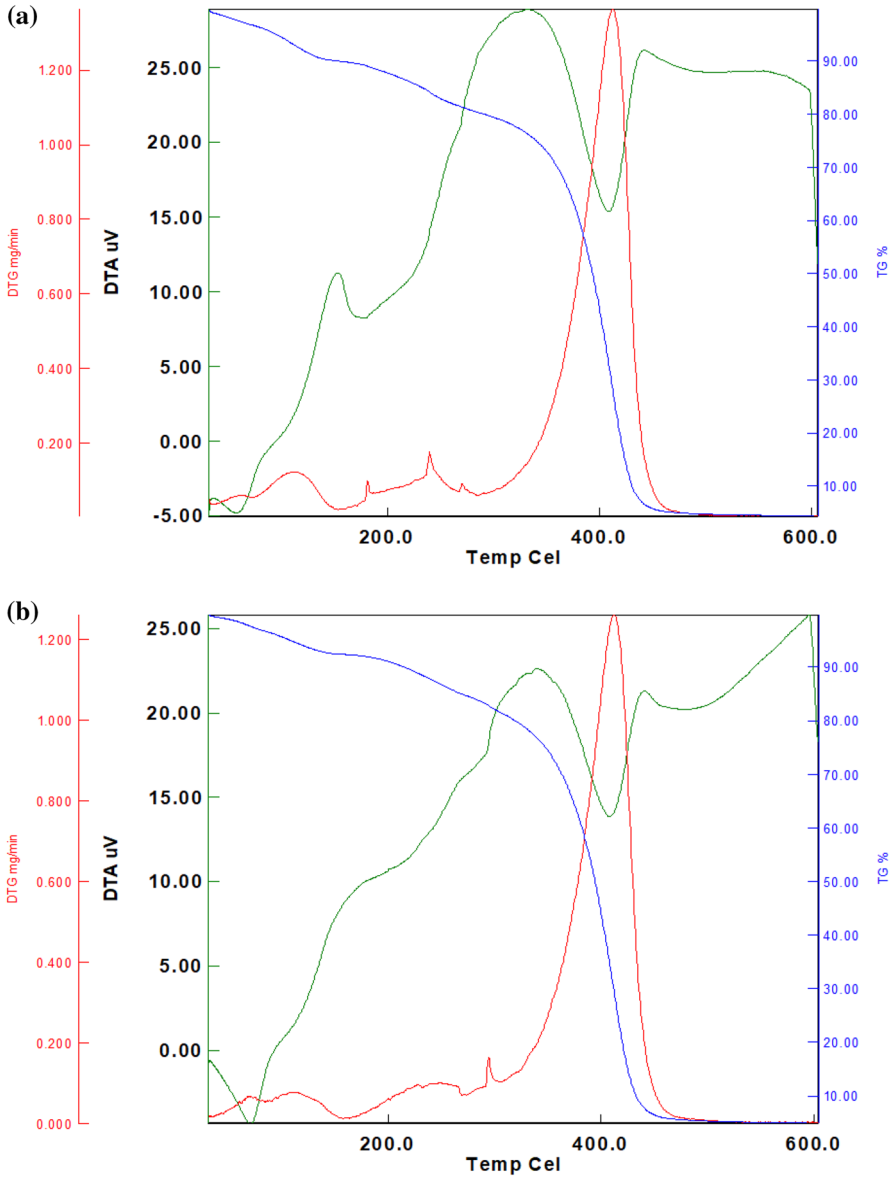

Fig. 5 The TGA/DTA/DTG curves of the (**a**) poly(NCA)/3%C10A (**b**) poly(NCA)/5%C10A nanocomposites

Conclusion

In this research, polymer/organoclay-based nanocomposites synthesis, characterization and thermal properties of poly(*N*-cyclohexylacrylamide) (poly(NCA)) were investigated by in situ polymerization. FTIR, XRD, SEM and TGA techniques were

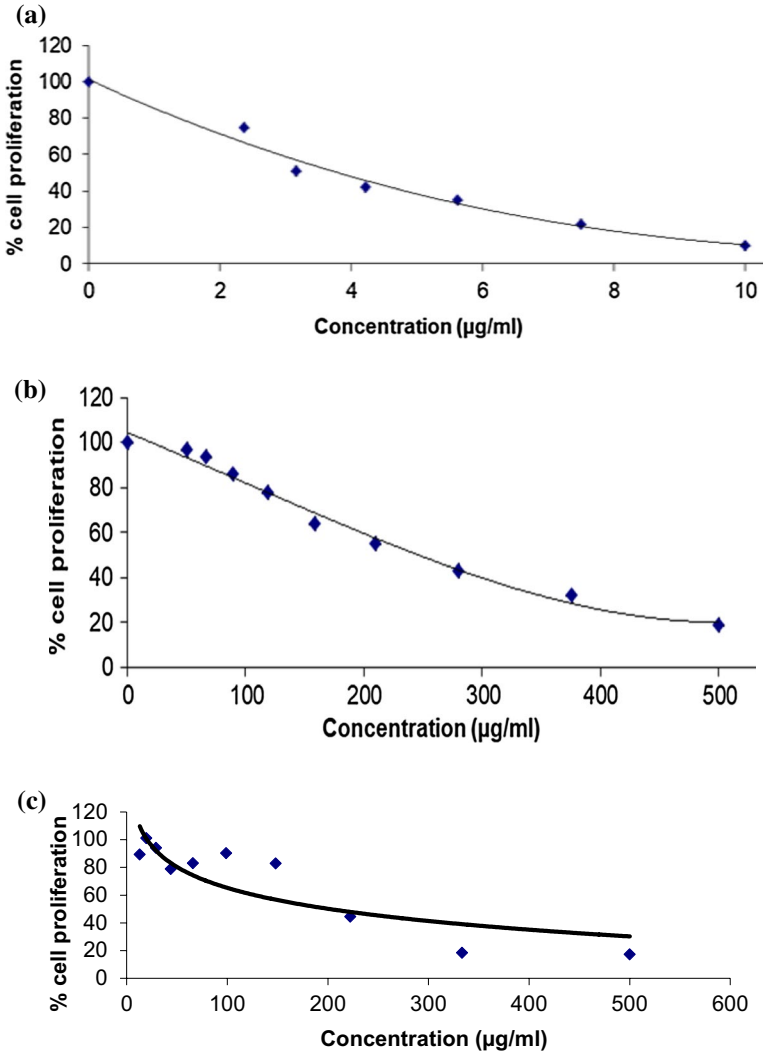

Fig. 6 Cytotoxicity analyses of (**a**) C10A organoclay (IC50:3.5 µg/ml), (**b**) poly(NCA)/3%C10A (IC50:230 µg/ml), (**c**) poly(NCA)/5%C10A (IC50:181 µg/ml) on HeLa cells, respectively

used at characterizations of nanomaterials. From XRD, SEM and thermal measurements, it was observed that the morphology of nanocomposites was exfoliated when the clay content in the polymer matrix was kept at 3% and 5%. TGA/DTA/DTG simultaneous system was used for thermal analysis and it was observed that the thermal stability of nanomaterials increased as the clay rate increased. In our study, cytotoxicity of C10A organoclay, NCA homopolymer and their nanocomposites against HeLa cancer cells was analyzed by cytotoxicity assay and the results showed that nanocomposites were promising for cancer treatment. Further research

on anticancer activity of nanocomposites to develop practical applications might be warranted in different types of cancer cells and in vivo.

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