Composite of AuNPs@SiO₂NPs@[(NIPAM)-*b*-(Ala)] and its activity on leukemia cells

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MS received 10 October 2017; accepted 12 January 2018; published online 3 December 2018

Abstract. Synthesis and characterization of thermo-sensitive block copolymer of N-isopropyl acryl amide-*b*-Alanine [(NIPAM)-*b*-(Alanine)] thin film and its doping with (AuNPs)-(SiO₂NPs), (gold and silica nanoparticles) were reported. Further, composite effect on K562 (leukemia) cells was examined based on *in vitro* cell-based studies. The synthesis of SiO₂NPs was followed through facile Stober's sol-gel synthesis methods. The individual morphology of [(NIPAM)-*b*-(Alanine)] thin film, AuNPs and SiO₂NPs including [(NIPAM)-*b*-(Alanine)]@(Au)-(SiO₂NPs) composite was confirmed by using TEM instrumentation. [(NIPAM)-*b*-(Alanine)] thin film was embedded with gold and silica nanoparticles followed by the sonication. The average size of AuNPs is 16 nm and for SiO₂NPs, it is ~368 nm (in diameter). Synthesized composite [(NIPAM)-*b*-(Alanine)]@(Au)@(SiO₂NPs) is biocompatible for mankind use. However, composite used to examine the inhibitory activity on K562 cells and it shows ~78% inhibition, which is significant value for 24 h treatment under humidified atmospheric conditions.

Keywords. Composite; gold nanoparticles; silica nanoparticles; cell inhibition; [(NIPAM)-*b*-(Alanine)].

1. Introduction

Synthesis and development of benign/harmless composites in nanoscale were of a great interest and also the approach of scientists and researchers across the globe [1]. Now-a-days nanotechnology was extended to cure various diseases and cancer is one of them. Detailed study and observations are needed to cure diseases like cancer without any cytotoxicity and side effects [2]. Even though there are vast number of surgery techniques and methods including variety of anticancer drugs, they all have their own limitations, while using in mankind applications. Potential tools such as N-isopropyl acryl amide-*b*-Alanine [(NIPAM)-*b*-(Alanine)]@(Au)-(SiO₂NPs) composite can fulfil those limitations based on their biocompatible, biodegradable and easy removal from body after its functions [1,2]. As per literature, [(NIPAM)-*b*-(Alanine)]@(Au)-(SiO₂NPs) type composite shows significant effect to cure all types of diseases. For example, FeO₃NPs, AgNPs, ZnONPs, etc. show potential applications in pharmaceutical and biomedical fields. Similarly, [(NIPAM)-*b*-(Alanine)] also has medicinal importance to cure several diseases including various cancers [3]. However, amino acid-based di-BCP [(NIPAM)-*b*-(Alanine)]@(Au)@(SiO₂NPs) show potential health

benefits, which have active functional groups to fight against cancer-causing factors. [(NIPAM)-*b*-(Alanine)]@(Au)-(SiO₂NPs) also has antimicrobial (antibacterial and antifungal) activities and anticancer activities too [4]. It has high parasitic, insecticidal and anti-malarial activities. It has anti-cancerous activity through prevention of their reproduction and reduces and suppresses the abnormal growth in cells and tumours [2–5]. The usage [(NIPAM)-*b*-(Alanine)]@(Au)-(SiO₂NPs) can be extended to several cancers, such as lung, colon, breast, skin, ovary, liver, etc. [6]. The [(NIPAM)-*b*-(Alanine)] thin film embedded with gold (Au) and silica (SiO₂) nanoparticles shows enhanced effect on cancerous cells [7]. Our main target is to develop nontoxic composite with multifunctional activities towards the pharmaceutical and biomedical fields especially for cancer treatment [8–10]. As per the literature, [(NIPAM)-*b*-(Alanine)]@(Au)-(SiO₂NPs) were demonstrated as excellent drug store for therapeutic applications [9–12]. [(NIPAM)-*b*-(Alanine)] soft thin films are remarkable and effective host materials for doping of gold, silica nanoparticles to increase their activity on cancer cells [13]. However, [(NIPAM)-*b*-(Alanine)] alone shows minimal effect on cancer cells and it can be enhanced with the combination with Au (gold) and silica NPs, which can easily enter inside the cells through the penetration

Figure 1. Steps involved in the synthesis of [(NIPAM)-*b*-(Alanine)] compound. Photos (a, b, c) show the colour change while stirring (d) for gold nanoparticles and (e) TEM image of composite and (f) schematic for composite.

and permeable capacity [11–16]. Gold nanoparticles (GNPs) are stable and crystalline. Particle size should be in the range of 50–200 nm. The particle size <100 nm are ideal for pharmaceutical and biomedical applications. [(NIPAM)-*b*-(Alanine)] thin film was doped with silica nanoparticles followed by the incorporation of gold nanoparticles [16–18]. The mixture or composite of [(NIPAM)-*b*-(Alanine)]@(Au)-(SiO₂NPs) was termed as composite for cancer treatment and it shows enhanced activity on cancer cells through the increased inhibition on leukemia cells [17–23].

2. Materials and methods

2.1 Materials

Chemicals required for the synthesis of composite of [(NIPAM)-*b*-(Alanine)]@(Au)@(SiO₂NPs) were purchased with high purity and used without further purification. Ammonium hydroxide (NH₄OH) (>98%, Sigma Aldrich), ethanol (CH₃CH₂OH) (>98.5%), TEOS (tetraethyl orthosilicate) (>98%, SDFCL), carbon-coated copper grids (Gilder grids, 200 mesh, 3.05 mm OD, centre and rim-mark, Copper, Ted Pella Inc.), Whatmann filter paper, pH paper, phosphotungstic acid (PTA) (>93.8%, Sigma Aldrich),

methylthiazoltetrazolium (MTT; >99.9%, Sigma Aldrich), magnetic bead, magnetic stirrer cum hot plate, sample viols, micropipettes, glass slides, centrifuge tubes, K562 and HEK293 cells, RPMI media for cell culture.

2.2 Synthesis of [(NIPAM)-*b*-(Alanine)]

The thermo-sensitive NIPAM of ~2.6 g was dissolved in ~50 ml of dry tetrahydrofuran (THF) solvent and it was used to mix-up with the solution of alanine (Ala). Further, ~2.6 g of amino acid-based chiral polymer alanine was separately dissolved in ~40 ml of dd-H₂O (double-distilled water). Both NIPAM and Gly solutions were mixed with each other under continuous stirring for 12 h with the feeding rate of 1.5 ml min⁻¹. After 12 h stirring, the precipitate colour was white (figure 1a). The mixture of solutions was further stirred for 24 h at ~25°C. After 24 h stirring, its colour was changed to pink colour syrupy liquid (figure 1b). The remaining solvent from the reaction chamber was removed through the rotary evaporation under reduced pressure and allowed to dry at room temperature (RT) by using desiccators (figure 1c). After ~48 h, thin film formation was observed at the bottom of the beaker. The yield of the final synthesized [(NIPAM)-*b*-(Alanine)] thin film is 4.1/5.2 g, which is equal to ~78.8% (figure 1) [22].

Figure 2. TEM images (a–c) for AuNPs acquired from lower to higher magnifications and (d) SAED pattern for AuNPs.

2.3 Synthesis of SiO_2 NPs

Synthesis of silica nanoparticles (SiO_2 NPs) was carried out through the facile modified Stober's sol–gel method. In a 200 ml beaker, ~ 50 ml of ethanol and ~ 15 ml of double-distilled water were stirred at 600 rpm followed by the addition of ~ 5 ml of TEOS solution with feeding rate of ~ 1 ml per min. The stirring was further continued for 2 h at RT [23]. To the above mixture, ~ 6 ml of ammonium hydroxide (base with pH of ~ 12.8) solution was added with feeding rate of $\sim 400 \mu\text{l min}^{-1}$. The milky white gel formation was observed. The stirring was continued for 24 h at RT [24,25]. Hence, the mixture was separated by using high speed centrifugation at 10,000 rpm for 10 min. The supernatant was decanted and settled residue was washed with ethanol until the base smell goes out. The compound of SiO_2 was separated from centrifuge tube and dried at $\sim 600^\circ\text{C}$ for 12 h. The yield of the

synthesized SiO_2 is ~ 2.6 g. Then, well-dried silica powder was dispersed in $\sim 500 \mu\text{l}$ of acetone and used for the sample preparation for transmission electron microscopy (TEM) imaging.

2.4 Synthesis of AuNPs

The hydrogen tetrachloroaurate/auric chloride (HAuCl_4) of 1.4 g was taken in a 150 ml round bottom flask (RB) and it was added with 50 ml of methanol (MeOH) and allowed to stir for 8 h at 45°C . After 20 min stirring, the mixture was added with 0.56 g of sodium borohydride (NaBH_4) reducing agent and stirring continued for 8 h. The sodium metal from NaBH_4 was degraded with the MeOH solvent. The final yellowish solution was used to separate the gold (Au) nanoparticles through the centrifugation at 2000 rpm for 6 min and obtained AuNPs

Figure 3. HRTEM images of AuNPs. Images (a, b) are acquired at lower to higher magnification. Images (c, d) show lattice parameters (d-spacing with $2.3 \text{ \AA}/0.23 \text{ nm}$).

were used for TEM characterization. Figure 1d, sample vial, shows the physical appearance of synthesized AuNPs.

2.5 Composite [(NIPAM)-b-(Alanine)]@(Au)@(SiO₂NPs)

An *in situ* synthesis method was allowed for the preparation of composite between [(NIPAM)-b-(Alanine)], AuNPs and SiO₂NPs. However, $\sim 500 \mu\text{g}$ thin film of [(NIPAM)-b-(Alanine)] was dissolved in 1 ml of EtOH solvent and it was added with $\sim 200 \mu\text{g}$ of synthesized SiO₂NPs as well $\sim 100 \mu\text{g}$ of AuNPs in a 3 ml cryo vial. Further, sample was allowed to disperse through the sonication at RT for 5 min. The well-dispersed sample was used for the sample preparation for TEM characterization and cell inhibition assays.

2.6 Cell culture and maintenance

For cell culture and maintenances, we have taken 6 mm Petri (Labteck) plates to seed the cells with suitable media

and maintained in CO₂ incubator with the set temperature at $\sim 37^\circ\text{C}$ and 5 vol% of CO₂ under humidified conditions. Both the cells (K562 and HEK293) were cultured and allowed to grow in Dulbecco's modified Eagle (DMEM) media, which is supplemented with penicillin (streptomycin) (2%), 10% of FBS (foetal bovine serum), amphotericin (0.15%) in an atmosphere of 5% CO₂ at $\sim 37^\circ\text{C}$. The fresh media was added to the plates for every 24 h till the cell viability and inhibition assays were completed.

2.7 Cellular inhibition assay

An MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] assay was performed to examine and evaluate the activity of prepared composite of amino acid-based di-BCP [(NIPAM)-b-(Alanine)]@(Au)@(SiO₂NPs) by using two different cell lines. *In vitro* cell-based studies were performed for the cell inhibition studies. The following

Figure 4. TEM images (a, b) correspond gold and silica nanoparticles without [(NIPAM)-*b*-(Alanine)]. Images (c, d) show composite of [(NIPAM)-*b*-(Alanine)] doped with SiO₂ and AuNPs.

different cell lines were used for the inhibition assay. Such as cancer (K562) and healthy (HEK293) cell lines were added with the above mentioned (prepared composite) and allowed for the treatment for 24 h. As mentioned in figure 5, five different concentrations (100, 200, 300, 400 and 500 $\mu\text{g ml}^{-1}$) of the prepared composite was added to the wells of 96-well plate with the density of 10×10^5 cells well⁻¹ and allowed to interact each other for inhibition on cancer cells. A blank well corresponds to the cells (as cultured) without any prepared composite of [(NIPAM)-*b*-(Alanine)]@(Au)@(SiO₂NPs) show zero inhibition.

2.8 Cellular imaging

The treated K562 (blood cancer) cells were once again added with 200 μl (1 mg, 0.01 mmol) of the green fluorescent fluorescence isothiocyanate (FITC), which dissolved in DMSO and it mainly stains the cell membrane

for cell morphology studies. Further, blue fluorescence ((2-(4-amidinophenyl)-1H-indole-6-carboxamide), DAPI, 1 mg, 0.1 mmol) dissolved in distilled water and 200 μl was added to the treated cells to stain the genetic material of DNA/RNA specifically A–T (adenine–thymine) rich regions in the nucleus. However, FITC shows the excitation and emission at 490 and 525 nm (λ_{max}), whereas DAPI shows excitation and emission wavelengths at 350 and 470 nm, respectively.

3. Results and discussion

3.1 Morphology of composite

The morphology of synthesized composite of [(NIPAM)-*b*-(Alanine)]@(Au)@(SiO₂NPs) and individual silica and gold nanoparticles were characterized through the TEM

imaging. From the TEM images, it was concluded that [(NIPAM)-*b*-(Alanine)] was doped with SiO₂NPs and SiO₂NPs were doped with AuNPs. Here, [(NIPAM)-*b*-(Alanine)] was acting as host for SiO₂NPs and SiO₂NPs are the host for AuNPs. However, [(NIPAM)-*b*-(Alanine)]@Au@SiO₂NPs is a composite of multiple components. The thin sheet of BCP [(NIPAM)-*b*-(Alanine)] is a main host for SiO₂ and AuNPs [1–3]. It can be referred as hybrid of different components. However; synthesized SiO₂NPs are spherical shaped with an average size of 368 nm (in diameter). Interestingly, these SiO₂NPs have shell with the size of ~18 nm, hence, SiO₂NPs can be referred as core shell nanoparticles as well (figure S2). The silica nanoparticles were distributed evenly with good consistency. The block co-polymeric [(NIPAM)-*b*-(Alanine)] thin film was seen between silica nanoparticles, which is holding or binding them with the help of electrostatic interactions. The binding nature/interactions between SiO₂NPs and [(NIPAM)-*b*-(Alanine)] is mainly due to the presence of organic functional groups such as keto (C=O), amide (–CH₂–NH), hydroxyl (–OH) and charge from SiO₂NPs [24–26]. As per the literature, silica nanoparticles are amorphous in nature. The morphology of AuNPs was confirmed through the TEM imaging and they are very much smaller in size, which is a novel characteristic property to consider them for biological applications. It is essential to perform the TEM imaging to evaluate the properties such as size, shape, etc. Figure 2 shows the distribution of huge number of GNPs with inconsistent size and shape. These irregularities depend on synthesis methods. Based on synthesis methods, their size and shape can be tuned with rough and smooth surfaces on GNPs. In figure 3, HRTEM corroborates the lattice parameters, such as d-spacing properties of GNPs/AuNPs, which is in the range of ~2.3 Å (~0.23 nm). However, figure S1 illustrates the distribution of particles through the sonication effect. Aggregation between GNPs was avoided through the sonication and dispersion solvent (ethanol). The average size of GNPs is 16 nm (in diameter, figure S3). Interestingly, these GNPs size was regulated from ~50 to ~10 nm. The GNPs are stable and crystalline in nature with fcc (face centered cubic) structure. From figures 2 and 4, it is clearly evident that [(NIPAM)-*b*-(Alanine)] was embedded or doped with nanoscale silica nanoparticles followed by the doping with GNPs. Doping of SiO₂NPs and AuNPs depends on sonication methods. The nanoparticles of SiO₂ and gold were incorporated inside the soft thin film of [(NIPAM)-*b*-(Alanine)] upon sonication. However, red circles in figure 4c, d show the association of AuNPs with polymer thin film and yellow circles show the interaction between silica nanoparticles and polymer thin film. The images in figures 2 and 4 are acquired from lower to higher magnifications and show the morphology of composite of [(NIPAM)-*b*-(Alanine)]@SiO₂@AuNPs. Since [(NIPAM)-*b*-(Alanine)] has low melting point (~55°C), it can burn or create charge effect during TEM imaging. To avoid such problems at higher accelerating voltages (200 kV), sample was added with phosphotungstic acid (PTA) solution.

Figure 5. Cell inhibition assay on cell lines. Synthesized composite of [(NIPAM)-*b*-(Alanine)]@Au@SiO₂NPs shows more inhibition on cancer (K562) cells and inhibition is negligible on non-cancer (HEK293) cells.

Furthermore, PTA forms a thin protective layer on the surface of the samples and it resists the charge effect, while imaging. However, positive surface charge of silica nanoparticles helps in surrounding the polymer thin film to form composite. Notably, gold nanoparticles are also positively charged (Au⁺) and it enhances the interactions between polymer film and SiO₂NPs. After structural and morphological confirmations, this composite [(NIPAM)-*b*-(Alanine)]@SiO₂@AuNPs was used to examine the inhibitory activity by using two different types of cell lines. The very small-sized gold nanoparticles can easily enter inside the cells through the penetration and permeation processes and show inhibitory activity on cancer cell metabolism [22–25].

3.2 Cellular inhibition assay

Further, composite of [(NIPAM)-*b*-(Alanine)]@SiO₂@AuNPs used to evaluate the cell inhibition capacity on two different cell lines, such as HEK293 (human embryonic kidney) are normal (healthy or non-cancerous) cells and K562 (chronic myeloid leukemia blood cancer) cells [27,29]. However, well-cultured cells with the density of ~6 × 10⁴ cells per well was poured in the wells of 96-well plate. Cells were added with five different concentrations (i.e., 100, 200, 300, 400 and 500 µg ml^{−1}) of [(NIPAM)-*b*-(Alanine)]@SiO₂NPs@AuNPs composite and allowed to incubate for 24 h in CO₂ incubator at humidified atmospheric conditions (~37°C). There is a blank well which consists cells as cultured, i.e., without any composite materials. After 24 h incubation, 96-well plates were taken out from CO₂ incubator and used to analyse cell inhibition assay by using multiplate reader. The obtained data used to plot bar

Figure 6. Laser scanning confocal microscopic images of K562 cells acquired after 24 h treatment with synthesized [(NIPAM)-*b*-(Alanine)]@(AuNPs)@(SiO₂NPs) composite.

graphs and it shows increased inhibition trend as the increase in concentration of composite from 200 to 500 $\mu\text{g ml}^{-1}$. Figure 5 shows increase in inhibition with the increase in concentration of composite [28,29]. The concentration $>200 \mu\text{g}$ shows $>50\%$ cell inhibition with IC₅₀ of 0.1 mmol concentration of composite [27,29]. The composite concentration with $\sim 500 \mu\text{g}$ shows enhanced and significant cell inhibition on K562 cancer cells, which is $>78\%$. Inhibition value is significant and ever reported with such composites. Blank well is the control for zero inhibition without composite for both the cell lines. Interestingly, inhibition is observed on cancerous (K562) cells only. The inhibition on healthy HEK293 cells is negligible, which is $<3\%$. The inset in figure 5 illustrates the inhibition on non-cancer (HEK293) cells with prepared composite of [(NIPAM)-*b*-(Alanine)]@(SiO₂NPs)@(AuNPs) for 24 h treatment. From

the MTT assay results, it is evidenced and confirmed that these synthesized [(NIPAM)-*b*-(Alanine)]@(SiO₂NPs)@(AuNPs) composite is compatible for normal cell lines and shows significant inhibition on cancer cells. The most increased inhibition on K562 cells is because of active functional groups in [(NIPAM)-*b*-(Alanine)] and easy insertion of smaller gold nanoparticles inside the cancer cells.

3.3 Cellular imaging

The [(NIPAM)-*b*-(Alanine)]@(SiO₂NPs)@(AuNPs) composite-treated K562 cells were used for cellular imaging by using laser scanning confocal microscopy (LSCM) for morphological observations. Figures 6 and S4 illustrate the morphology of K562 cells, which is stained with green fluorescent dye, FITC and blue fluorescent dye, DAPI.

However, FITC stains the cell morphology (which binds to cell membrane) and DAPI mainly stains genetic material, which is present in DNA and RNA. DAPI specifically binds to adenine–thymine (A–T)-rich regions of nucleus of the cell. Figures 6 and S4 show the distorted, and ruptured cell morphology for cells, which are treated with developed composite for 24 h. Basically, K562 cells are spherical in shape, but from figures 6 and S4, we can see the distorted morphology for treated cells [27,29].

4. Conclusion

The block copolymer [(NIPAM)-*b*-(Alanine)] thin film was successfully doped with silica and gold nanoparticles. The synthesized [(NIPAM)-*b*-(Alanine)]@(SiO₂NPs)@(AuNPs) composite was characterized by using TEM imaging. It was synthesized through the facile and modified addition polymerization approaches. Composite does not show any inhibition on healthy HEK293 cells, that means it is bio-safe for mankind applications. The activity of composite was measured based on MTT assay methods and resultant data. From this, the composite shows enhanced inhibition activity on leukemia (K562) cells which is >78% after 24 h treatment. Furthermore, such biocompatible block copolymer with gold and silica nanoparticles can be used to treat various cancers.

Acknowledgements

We would like to acknowledge University Grants Commission for financial assistance under the Rajiv Gandhi National Fellowship Scheme.

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