

Novel delivery based anionic linear globular dendrimer²-zidovudine nano-conjugate significantly decreased retroviral activity

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Abstract: Human diseases like viral organisms for example, hepatitis, HIV and etc., attack the health and caused large mortality in populations by many years. So finding novel delivery vehicles based antiviral drugs employing nano-materials is of high universal interest. In current approach a very biocompatible biodegradable nano-biopolymer anionic linear globular dendrimer second generation G2 was elaborately conjugated to a well-known anti-HIV drug Azidovudine and thereafter was characterized by different analytical techniques like AFM, Zeta sizer, ¹HNMR, FTIR and LC-Mass spectroscopy. Then, Anionic Linear Globular DendrimerG2-Zidovudine Nano-Conjugate was assessed on human normal cells (toxicity assay by XTT test) and also HIV cell model and the results showed that Anionic Linear Globular DendrimerG2-Zidovudine Nano-Conjugate Significantly Decreased Retroviral Activity without any human cell toxicity respectively. Based on current experimental data such nano-compositions is proposed for further *in vivo* anti-HIV assays as well.

Keywords: Viral organisms, HIV, nano-biopolymer anionic linear globular dendrimer second, Generation G2.

INTRODUCTION

The human immunodeficiency infection (HIV) is a lentivirus based sickness that prompt to very much recognized viral illness known as Acquired Immunodeficiency Syndrome (AIDS) (Douek *et al.*, 2009). AIDS is portrayed by some dynamic disappointment of the human immunological circumstances permits deadly pathogens to vanquish the wellbeing. Generally, such illness brought on to a 9-11 years' survival with no helpful methodologies, in light of the infection subtype. Retroviral pathogens contaminate human immunological typical cells, for example, T partner cells for the most part called as CD4⁺ checked cells or macrophages (Cunningham *et al.*, 2010). Retroviral disease because of numerous more systems incorporating pyroptosis or apoptosis in cells, brought on to diminish in CD4⁺ T assistants' levels too (Cunningham *et al.*, 2010, Doitsh *et al.*, 2014, Grag *et al.*, 2012). At the point when CD4⁺ T cell sums diminished to a range beneath a basic stage, this circumstance leads cell intervened insusceptibility irregularity and the essential tainted organs is being readied vulnerable to speak to the ailment's signs. Retroviral infection is portrayed as a generally round considering 120 nm as breadth, in short such infection is littler than a typical red platelet (RBC) ~ 60 times (McGovern *et al.*, 2002). Its structure comprises of two comparatives single-stranded positive RNAs which encode the nine vital qualities including 2000 retroviral

protein-P24 duplicates of cone shaped capsid composition. Its RNA is thoroughly influenced to P7 nucleocapsid proteins as well as related catalysts vital for virion creation like switch transcriptase or proteases and ribonuclease likewise integrase. As a subsequent, P17 protein encompassed the said capsid to make the fundamental obligation out of virion nanoparticle. Furthermore, such nanoparticle has been influenced by viral envelope which it is the lipid bilayer driven from human cell layer while the resultant made the infection molecule. The viral envelope comprising host cell proteins and in addition moderately few arrangement of the retroviral Envelope, in which including a structure making three glycoproteins 120 furthermore 41. Resultant protein, allows the HIV infection to tie to target have and in addition next retroviral activities, for example, breaker to cell film improving intracellular viral operators likewise beginning the outstanding irresistible turn (Chan *et al.*, 1997, Klein *et al.*, 2010). As expressed over, the said surface retroviral protein is a main marker for HIV immunization looks into and developments. The most substance of the trimetric acclaimed envelope spike is comprising of extraordinary sort of N-connected glycans with high cell thickness which portrayed by antibodies in which including in avoiding ordinary cell maturation (Behrens *et al.*, 2016), Cupo *et al.*, 2016, Pritchard *et al.*, 2015). Zidovudine (ZDV) is all around perceived as recently assigned hostile to HIV pharmaceutical measurements shape which named as azidothymidine (AZT), used to deal with every one of the means of HIV/AIDS malignancy (Douek *et al.*, 2009). Such

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pharmaceutical dose frame diminished in retroviral replication and additionally enhancing all the clinical manifestations. There is sufficient proof to bolster such medication as forestalling HIV transmission, on the off chance that counteractive action of mother to youngster transmission during childbirth or after any infrequent needle stick mischance. Extra writing data demonstrated a remarkable patient reaction to zidovudine (Mitsuya *et al.*, 1985, Richman., 1995, Wainberg *et al.*, 2005). Yet a few reports showed AZT-safe troubles so such medication is ordinarily utilized in mix with other retroviral sedates in centers in an exceptional antiretroviral regimen known as profoundly dynamic antiretroviral treatment (HAART). AZT has been broadly utilized as post-presentation bar together with another antiretroviral, lamivudine, significantly lessening the risk of HIV disease taking after the essential single introduction to the infection, (for example, a needle-stick harm including blood or body liquids from an individual distinguished or associated with being tainted with HIV)⁹⁻²⁴ (however its utilization in life has for the most part been supplanted by tenofovir as a part of a 3 tranquilize combination)(Levy., 1993). As a result, it is a need for producing novel AZT delivery systems to overcome such problems using nanoparticle based systems. Dendrimers were first developed as a special kind of nano-sized polymers or bio-polymers which enabled to inter to the human cells specifically cancerous or viral affected hosts as well (Okeye and Picker., 2013). Dendrimers divided into different groups based on its surface charge and generation as well. Two well-known dendrimers based on positive charges (PAMAM) and negative charge of its surface (anionic linear globular dendrimer-ALGD) were recently well cited in the literature for different drug delivery or molecular imaging purposes (Hu and Hughes, 2013). PAMAM due to its positive surface charge has shown some cellular toxicity. In contra verse, other type due to negatively surface charged has shown no cellular toxicity as well as easy synthesis pathway and cost beneficence comparing to that of PAMAM. Anionic linear globular dendrimer G2 is a novel negatively charged generation of nano-polymers recognized in the family of dendrimers. Its synthesis is easy and cost effective. Its application as a novel safe intracellular vector to deliver a well-known anti-HIV drug zidovudine to provide a smart anti-retroviral bomb. In such situations large amounts of drug is uniquely delivered into the target cells (infected cells) and due to biodegradability of nano-biopolymer in a time course kinetic drugs is being presented to intracellular malignancies as well (Assadi *et al.*, 2016, Ensoli *et al.*, 2014, Memo *et al.*, 2010).

MATERIALS AND METHODS

Materials

PEG 600 was purchased (from Merck, Germany). All synthesis catalysis and mediators for preparation of synthesized nano-drug including Dicyclohexylcarbo-

diimide, Sulfo NHS as well as dimethylsulfoxide, citric acid and silver were also provided from Merck, Germany. Additionally, pSPAX.2, pmzNL4-3 or pMD2.G plasmids were prepared for transfection respectively. In molecular and cellular experiments, HEK293 cells and DMEM primary culture medium were employed. HEK was considered as normal human cell line and is an abbreviation from human embryonic kidney origin and elected for such experiments due to higher level endurance and or multiplication.

Synthesis of anionic linear globular dendrimer second generation G2

According to previous publication of the current project with slightly modifications, the second generation G2 dendrimer synthesis was started by a reaction between dicarboxylated PEG 600 (as the core of dendrimer) and dicyclohexylcarbodiimide (DCC) in anhydrous DMSO supported by calcium chloride as well as employing the condition of darkness and vacuum. Dicyclohexylcarbodiimide activated poly ethylene glycol by esterification protocol. Thereafter, citric acid moiety was replaced with dicyclohexylcarbodiimide and as a result the color of solution was slowly tended to orange, after 1 hours from the study the reaction molarity of materials was then repeated and the reaction mixture was allowed to be mixed for 3-4 days. After a period of 3-4 days stirring by addition of 5-10 ml water the reaction was stopped and the solid precipitate filtered off and got out from the study and the resultant solution was purified by dialysis buffer using spectrum dialysis bag (USA) with cut off 500Da, or sephadex G-25 column, for a period of one day of time respectively. Final dialyzed solution was lyophilized and characterized by HNMR and FTIR as second generation G2 dendrimer (Scheme 1).

Preparation of drug (zidovudine)-dendrimerG2 conjugates

Due to significant increased tendency between nano-azidovudinemolecules to the oxygen atoms on surface of G2 dendrimer, the reaction and conjugation between azidovudine and dendrimer is possible by using EDC a carbodiimide family and sulfo-NHS/CaCl₂ as catalysts and water resistance agents as wells. In special condition by using isolation and light protection, the reactant molecules mixture was stirred for at least one week. As stated above, Nano-drug conjugated from other free un-reactant molecules such as azidovudine or EDC and sulfo-NHS/CaCl₂ was separated by dialysis using (500-1000Da cut off-spectrum Company. During the analyses the Final product (liquid or lyophilized) was lastingly protected from light and air. Structure of product was assessed by FT-IR, Mass spectroscopy and ¹HNMR spectrums and also compared with G2 dendrimers data from previous publications. Size and charge of the nano-conjugate was assessed as well. AFM Imaging data of azidovudine-dendrimerG2 conjugated was also obtained for more detailed comparisons (Scheme 1).

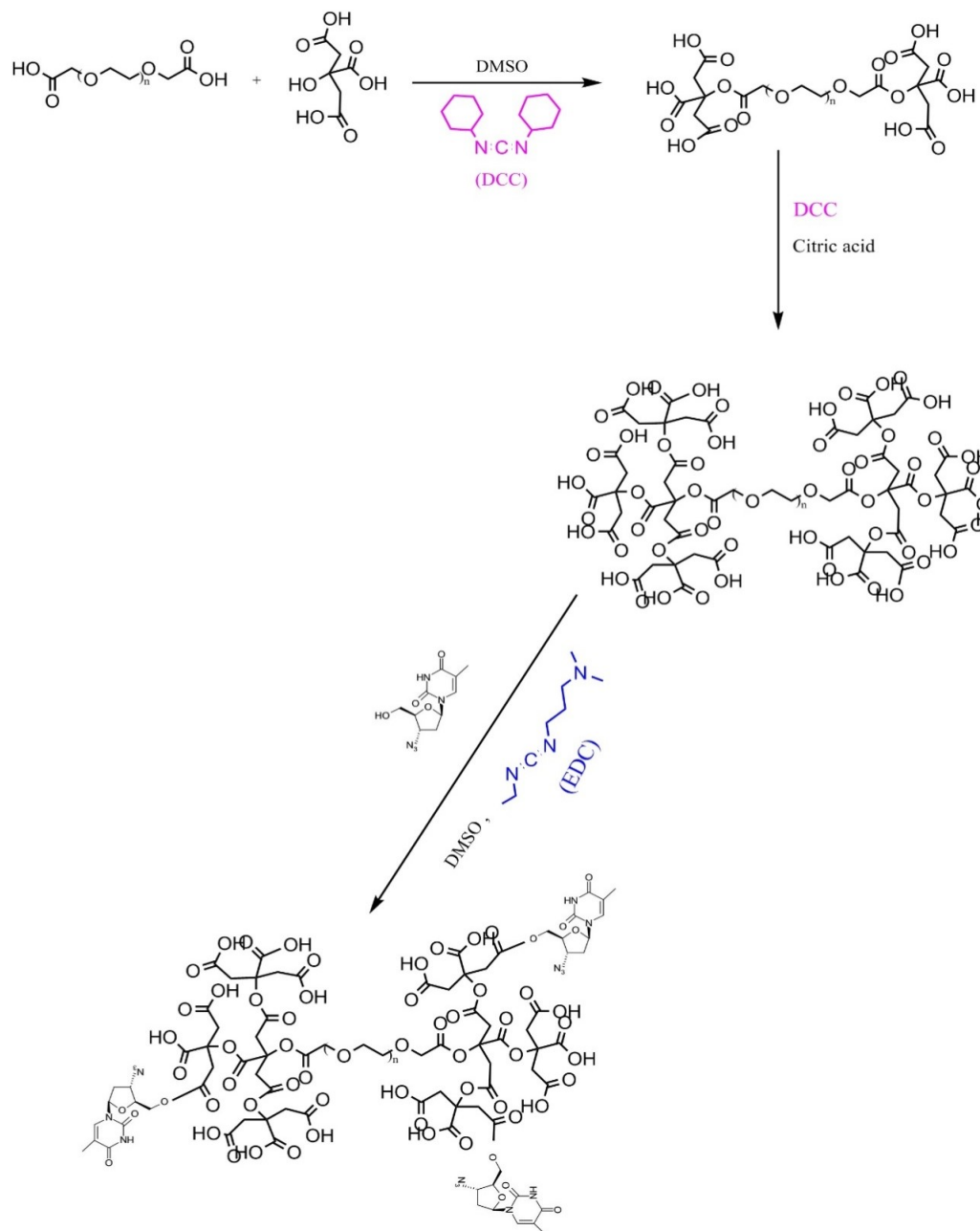
Stability assessment

Such recommended conveyance based adversely charged second era PEGylated dendrimer conjugated to AZT (Azidovudine) security at various pH (4, 7.3 and 9) and mediums (DMSO, Water and PBS) and times (6, 12, 24 hours) at room temperature, was surveyed by Thin Layer Chromatography (TLC) and UV spectroscopy ingestion at 266 nm (in light of estimation of adjustment bend)

separately. It ought to be noticed that U.V ingestion for anionic straight globular dendrimer is contrasted from that of AZT also.

Cell culture and antiviral assessments*Cells, transfection and virus production*

As indicated by our past productions, to deliver HIV-1 SCR virions pseudo wrote by VSVG, Qiagen test was



Scheme 1: Demonstration of Chemical synthesis of AZT-conjugated negatively charged PEGylated dendrimer.

additionally utilized. Briefly, HEK293T cells were precisely refined in DMEM (Dulbecco's modified Eagle's way of life medium, Sigma, USA) and from there on enhanced by a mind boggling plan including with FBS (15 %), penicillin (100 U/ml), streptomycin (100 lg/ml), and glutamine (2 mmol/l); as likewise hatched in 5% CO₂ at 37°C whole a six cell culture well-plate since plates achieve an aggregate sums of $\sim 1.2 \times 10^6$. Transfection

process was planned and did by utilizing a standard Lipofectamine 2000 in view of Qiagen convention. pSPAX. 2 plasmids, pmzNL4-3 as well as pMD2.G were simultaneously transfected into HEK293T cells to give proviruses. A short time later, said cell containing plates were hatched for no less than 24 h in 37 °C and 5 % CO₂. A synthetic operator called HEPES (4-(2-hydroxyethyl)- 1 piperazineethanesulfonic (25 mM) was utilized to control

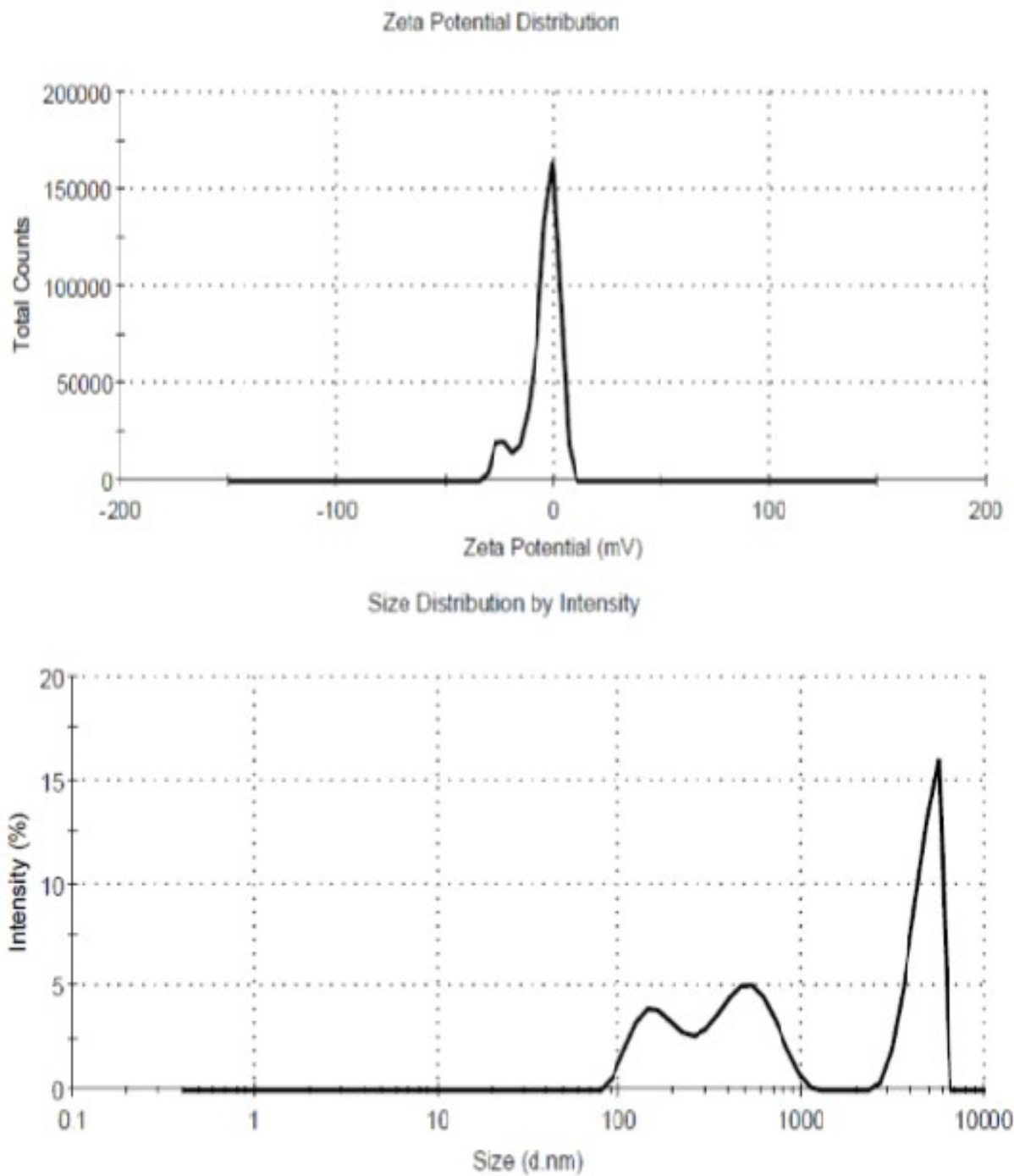


Fig. 1: size distribution and zeta potential of Azt-G2 dendrimer .

the phone culture pH at transfection step. To deliver a huge infection stock, transfected cells supernatants were extravagantly reaped at 24, 48, and 72 h post transfection, and afterward after filtration were performed by ultracentrifugation for 2h in 60,000g at 4 OC [29]. The resultant pelleted virions were broken up by appropriated measures of RPMI 1640 by delicate blending overnight at 40C to stop any inadvertently infection total, then

evaluated by P24 ELISA test pack (Biorad) and put away at - 700C to be utilized (Mitsuya *et al.*, 1985). To compute SCR HIV-1 virions pseudo-typed P24 titer by stomatitis infection surface VSVG vesicular glycoprotein HEK cells were individually utilized. A 60* 104 HEK cells include were vaccinated every 24 well containing 250 μ l of culture arrangement and afterward after 600ng of P24 was drop wised to every well. A while later, the specified cells

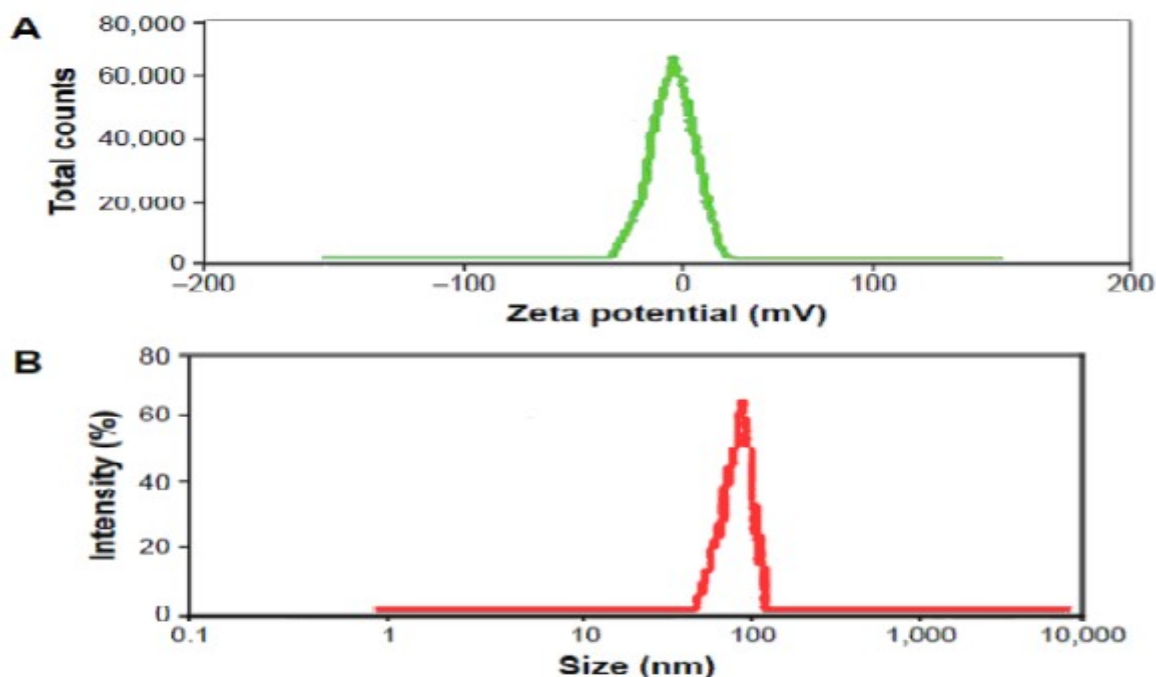


Fig. 2: (A), zeta potential distribution; (B) size distribution of G2.

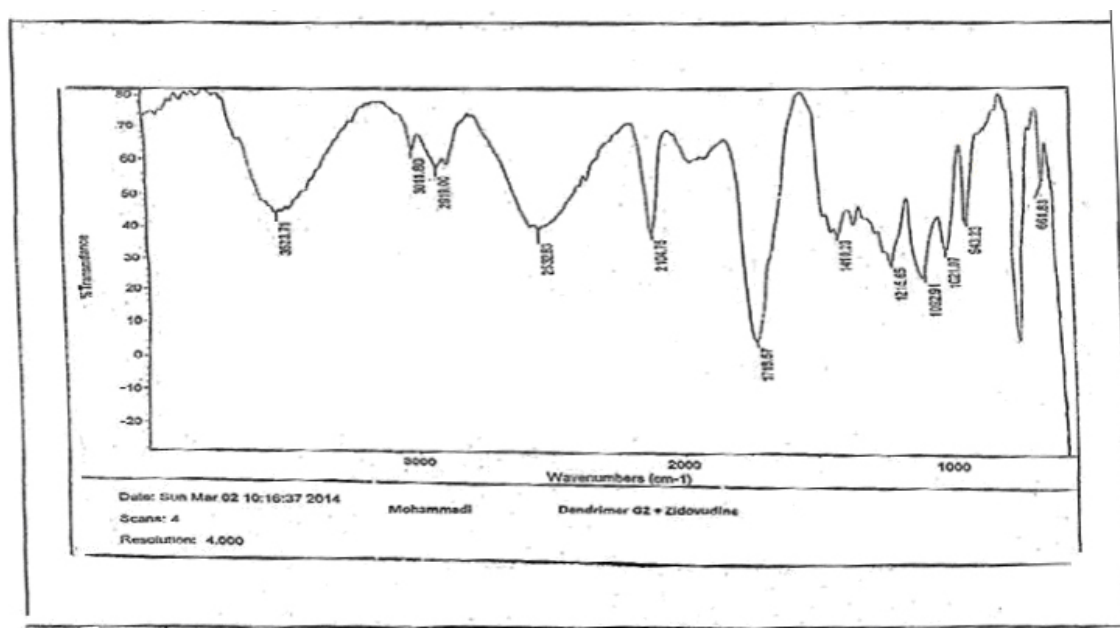


Fig. 3: FTIR spectrum related to AZT-G2 dendrimer conjugation.

were as often as possible washed each 5 h by an answer of 10% FBS. At that point, the supernatants from coming about arrangements were expelled and supplanted with new culture arrangement. Ultimately, P24 focus was approved through the ELISA catch convention (Biomerieux) completed by the assembling organization. All the medication, nano-sedate/controls were broken up in dimethylsulfoxide (DMSO) with various measurements extending from 10 μ M up to 10mM. The investigations were completed in triplicate. Tranquilize; nano-sedate/controls Toxicity were viably and in addition independently evaluated on the HEK cells. CC50 (cytotoxicity limit 50 %) was evaluated. By separating CC50 by IC50 gave TI (remedial file) and additionally SI

(selectivity file) (TI or SI = CC50/IC50). Antiviral impacts of various specialists were likewise separated by TI or SI markers.

Evaluation of the new nano-drug effectiveness on HIV replication

A human embryonic kidney cells measure of 60×10^3 in each well of a 24 well plate containing 250 μ l of cell culture solution was provided. SCR HIV virions and 600 ng P24 were then also added to each well. The nano-drug and other controls were elaborately added in four concentrations (20, 40, 80 and 120 μ M) to experimental wells as repeated in triplicate. After period of 5h, the

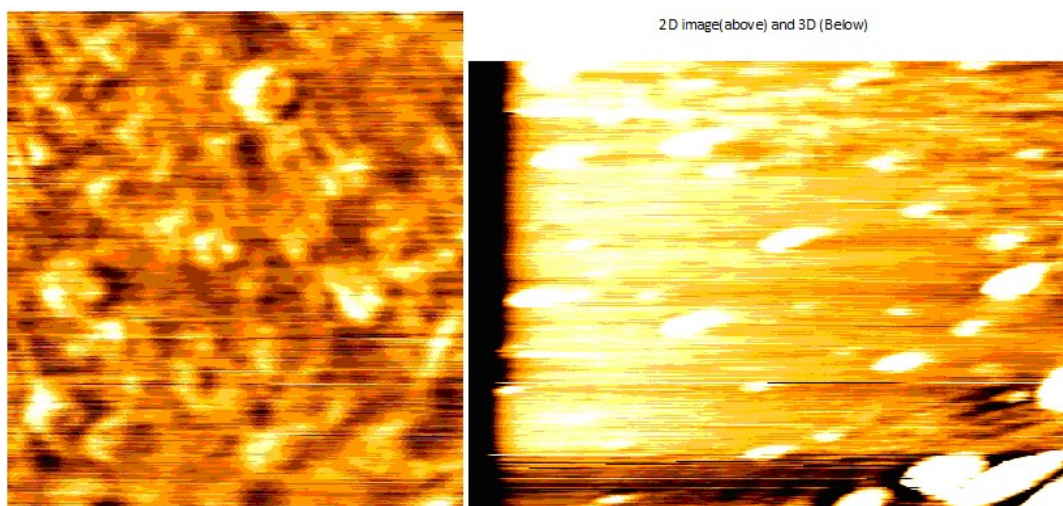
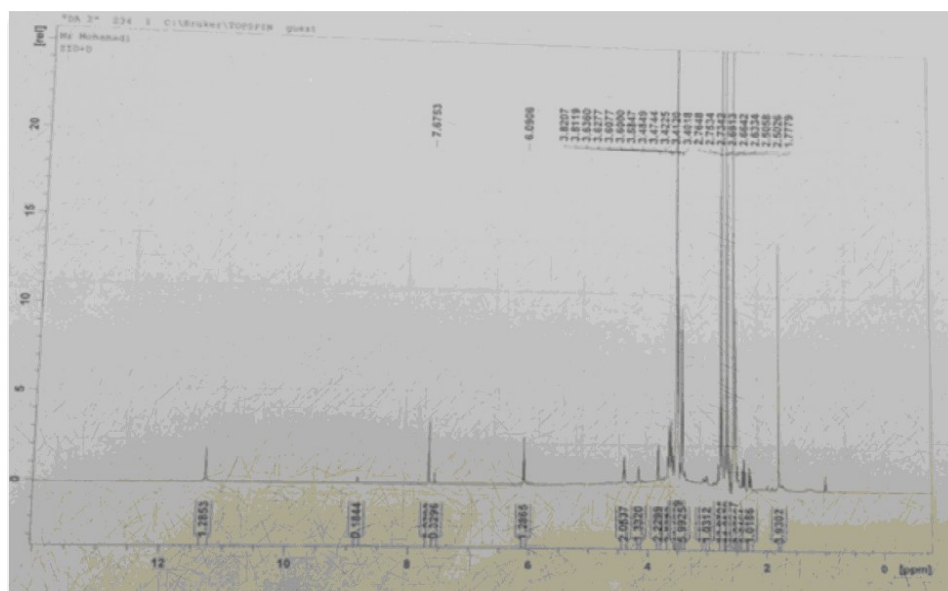


Fig. 4: AFM image of AZT-G2 dendrimer conjugation.



treated wells were washed twice with DMEM/FBS 10% solution (Mitsuya *et al.*, 1985). Thereafter, nano-drug and other controls as well as additional control named as fresh medium with the same amounts were added. The resultants treatments were inoculated at 72h, the supernatant was then gathered. Finally, level of P24 of the separated supernatant was determined with ELISA apparatus (Biorad). Related ELISA test was carried out and finally the concentration was evaluated. To evaluate cellular proliferation, XTT method (Roche) was applied as well. To reduce test errors phenol red solution was employed.

STATISTICAL ANALYSIS

Statistical analysis was done by SPSS software version 17 (IBM SPSS Statistics, IBM Corporation,

Chicago, IL). Excel software was used for expressing of results as Means $\pm$ SD.

RESULTS

Results of Dendrimer G2-Zidovudine AZT conjugation

The molecular size, charge, and morphology have significant change following the AZT-G2 dendrimer conjugation. An expansion in the size/poly disparity was seen and the zeta potential remains constant negative in the conjugates as compared with the dendrimer. The constant negative charges might come from the carboxyl groups of G2 and the Azido group of AZT moieties being conjugated (fig. 1 and 2). The same approving evidence was achieved by atomic force microscopy that observed slightly spherical particles (fig. 3). Fourier-

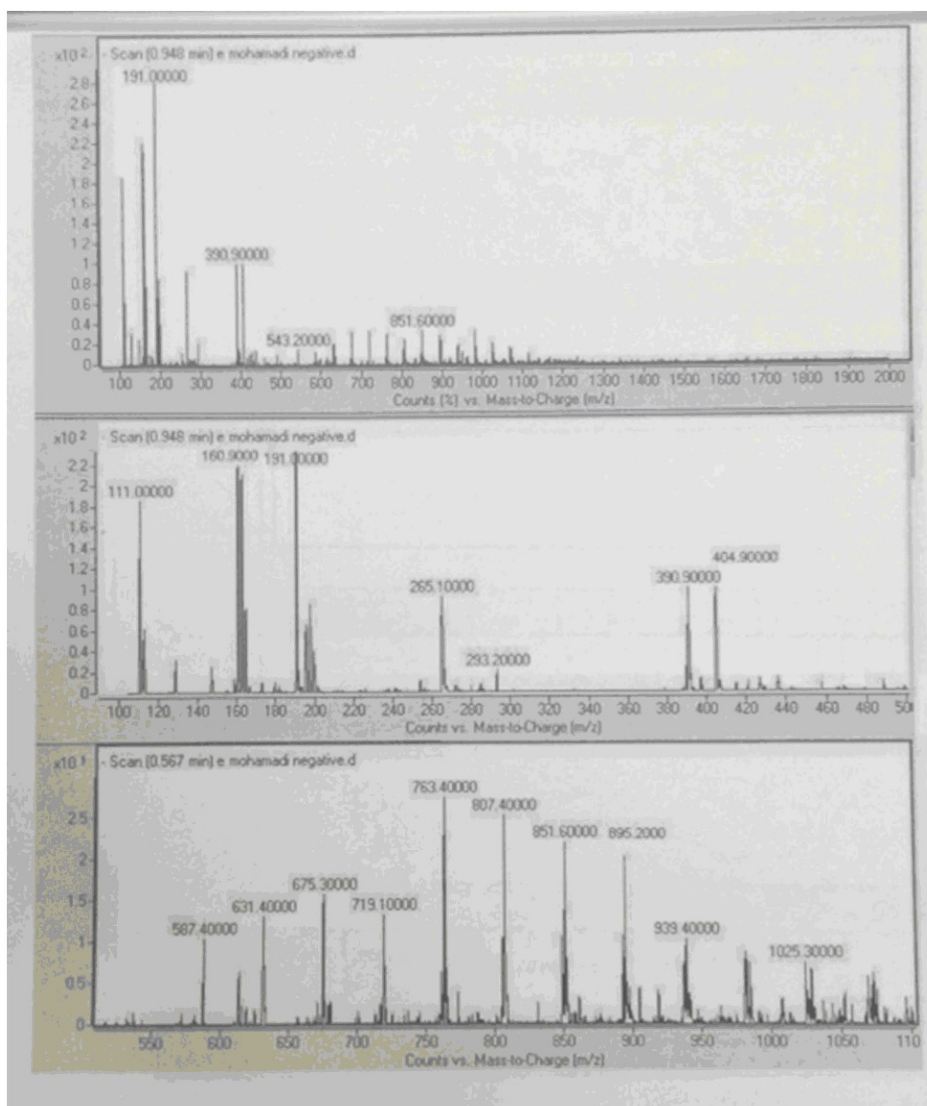


Fig. 6: AZT conjugated dendrimerG2 LC-Mass spectrum data which confirmed the conjugation of AZT molecules to citric acid of dendrimerG2 surface.

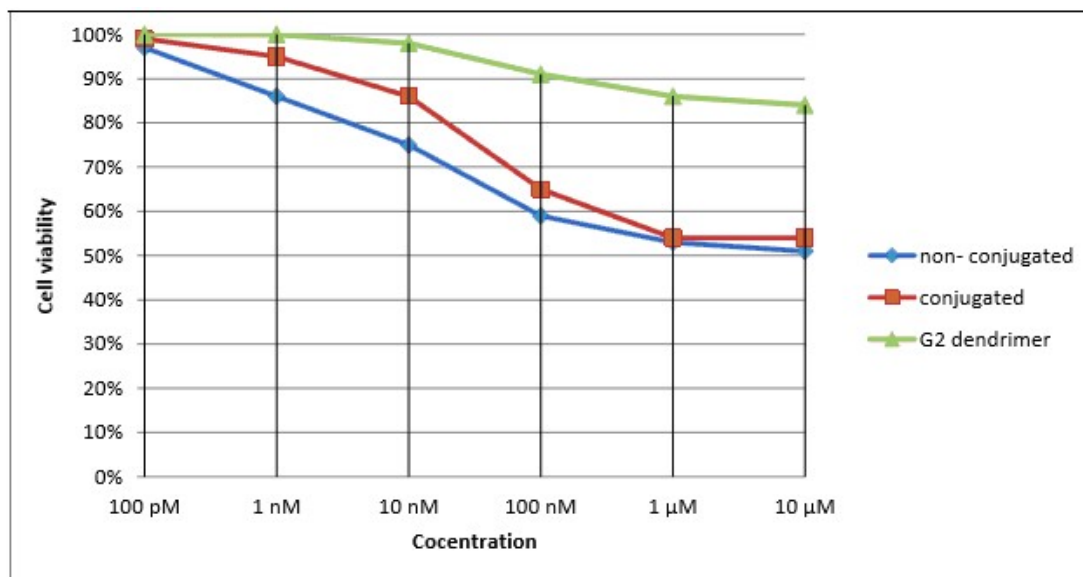


Fig. 7: Result of XTT assay on HEK cells, as percentage cell viability at different concentration, compared using AZT. AZT-G2 dendrimer. And dendrimer.

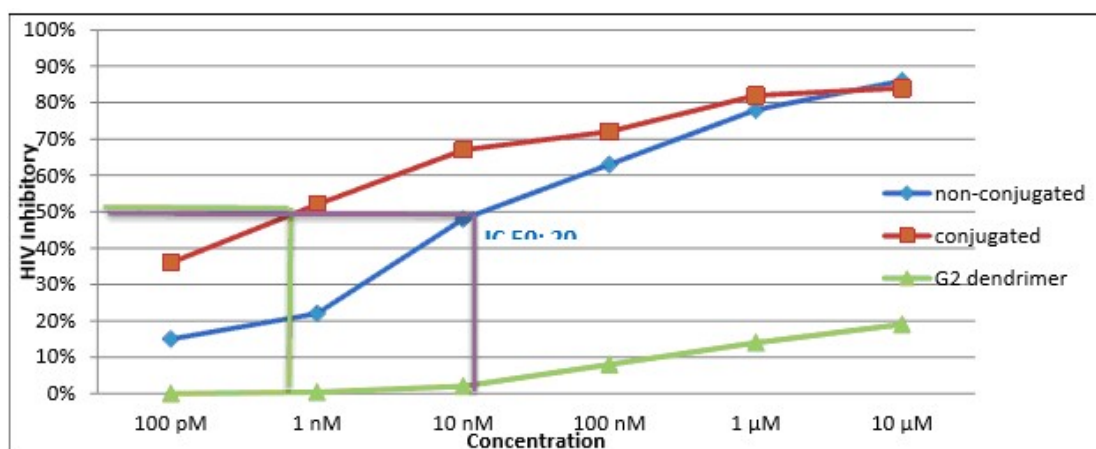


Fig. 8: Effects of increasing concentration of AZT. AZT-G2 dendrimer and G2 dendrimer. on P24 Ag production.

Transform infrared (FTIR) spectroscopy was applied to approve the existence of the G2-AZT conjugate in the region of 400-4000 cm^{-1} . The peak 1410 cm^{-1} is related to the C-C asymmetric stretching band existing in the Zidovudine drug. Peaks ranging from 1021 cm^{-1} to 1092 cm^{-1} in C=O groups shows an ester linkage, indicating the conjugation of Zidovudine to G2 through the ester linkage. The broadened peaks ranging from 1600 cm^{-1} to 1718 cm^{-1} relate to carbonyl amide groups from dendrimer which is bystander from reaction. Both The peak 2104 cm^{-1} which shows Azide band and the peak 3011 cm^{-1} that relates to N-H stretches of Amide group contribute to Zidovudine. Broadening peak at 2523 cm^{-1} is related to O-H of dendrimer. The peaks at 661 cm^{-1} and 943 cm^{-1} at relates to aromatic group. fig. 4 and 5 shows the HNMR spectrum and AFM related to CBL-G2 dendrimer conjugation that confirmed results came from FTIR. For a meticulous approval of dendrimer, the AZT conjugation to

G2, LC/MS was used (fig. 6). Compared with the intact different fragmentation patterns in the AZT-G2 dendrimer confirmed the conjugation was formed. In fig 6 results of mass spectroscopy was depicted as for example 293 regarding to conjugation of AZT to citric acid dendrimer G2 from OH- side and losing H_2O molecule. Or 675 fragments are regarding to conjugation of two AZT moiety to one side of one citric acid surface of second generation. In addition of carboxyl acid from first generation citric acid 719 is reached and etc.

Stability assessment

No noteworthy arrival of zidovudine was seen amid 6 and 12 hours of assessment at any pH at any medium at all from nano-conjugate. These discoveries showed a decent soundness for such nanoparticle based pharmaceutical conveyance approach. At time 24 hours, a marginally discharge from the conjugate was discovered 11% which

to delineate some precariousness for dendrimer at pH=4 in DMSO lattice as it were. Other pH and mediums did not trouble nano-conjugated based lamivudine discharge at 24 hours separately.

Cytotoxic effects of G2-AZT conjugation on HEK293T Cells

The results of XTT assay for AZT, AZT-G2 and G2 in concentrations of 100 pm and 1, 10,100 nm and 1, 10 μ M are shown on fig. 7. As it is obvious, increasing the concentration in all three groups leads to decreasing cell viability. Significant differences can be seen between G2 and the two other groups. As concentration of AZT and AZT-G2 got higher the strength against cancerous cell between those groups is got closer, so that in high concentration, 1 and 10 μ M AZT and AZT-G2 had overlap.

Inhibition of HIV p24 production (replication assay) by G2-AZT conjugation

The HIV replication assay was performed using VSVG pseudo-typed SCR HIV-1 virions, which are only able to replicate for 1 cycle. This study confirmed the ability of the test G2-AZTconjugation to inhibit the replication of HIV virions from the time of entry to that of release from the host cells. According to the results, it can be concluded that the AZT-G2 dendrimer inhibited the production of HIV-1 p24 Ag by more than 50% in the strikingly lower concentration compared to AZT alone was calculated to be 20nm/mL for AZT alone and 900 pm/mL for AZT-G2 dendrimer) (fig. 8).

DISCUSSION

With infection of target cell by a create HIV-1 virion, a break is made on the surface of the telephone on account of an association happening between the layers of the host cell and the virion. The substance of the viron enter the cytoplasm of the cell through that break and a short time later the switch interpretation will be set up to be done (Hu and Hughes., 2013). Memory T cells are fundamental concentrations for HIV (Ok eye and Picker, 2013). HIV torment changes into one of the fundamental issues of the critical prosperity general which has its social likewise, budgetary weight for general prosperity. Roughly 34 million people have HIV among which 24 million considered AIDS-related passing, and 2.6 million new defilements (Ensoli *et al.*, 2014). As a result of loss of counter acting agent, antiviral drugs have been the best moving toward point (Kim *et al.*, 2010). Anionic Linear Globular Dendrimer G2 applies anticancer activity through concentrations that no striking extreme *in vivo* risk would create (Assadi *et al.*, 2016). The major tried stood up to by treatment of HIV is Antiviral drug resistance where advancing viral replication and persevering prescription presentation cause the decision

of safe strains. In order to confront with this test a couple attempts have been driven joins streamlining of host factors and medicine transport and the change of new drugs (Strasfeld and Chou, 2010). Closure of disease replication through joint effort with cell pathways is the present system (Galdiero *et al.*, 2011). Blend antiretroviral treatment has continuously propelled cure, however the truth is the lifetime and its responses besides the insufficient in patients in whom the contamination makes resistance must to be viewed as (Momo *et al.*, 2010). The purpose of this study is the ability of nanotechnology in change the present cure and giving new supportive system against HIV/AIDS. The biochemical characteristics of dendrimers, including their monodies persity, water dissolvability, steady stacking limit and significant number of functionalizable periphery packs make these macromolecules strong match for appraisal as both explanatory and accommodating gadget. Using of safe nanocarriers, for instance, anionic straight globular dendrimers is charming in this matter (Alavidjeh *et al.*, 2010). In this study we explored a couple of centralizations of G2-dendrimer AZT-conjugated compound against replication Of HIV and surveyed cytotoxicity of the new Compound in different obsessions in connection with both AZT and G2 dendrimer alone. Investigate showed the viral p24 levels diminished as an outcome of conjugating inside Dendrimer on the surface of monocytes and lymphocytes. HIV-1 replication was checked by p24 antigen production. As a result; it shows up AZT G2-Dendrimer conjugation could add to the diminishing of HIV-1 replication in polluted patients by cell increase limitation rather than by a quick antiviral action. Whereas, AZT particularly stifled disease replication. HIV replication rate and survival was measured after utilization of a couple of merging's of new compound in relationship with pharmaceutical alone. An investigation was driven by Perise-Barrios, *et al*, Notably, a half decreasing in p24 release was watched when CD4 T lymphocytes were agonized with second time carbosilane dendrimers (2G-NNsss16 and 2G-03NN24) Furthermore, cell augmentation measures reflected the non-proliferative effect 2G-NN16 and 2G-03NN24 dendrimers had on CD4 T lymphocytes after 48 h of treatment (Perise-Barrios *et al.*, 2014). It displayed that the anionic direct globular dendrimer up to the concentration level of 0.5mg/mL had not lethality in relationship with the negative control (a cell advancement of 108% was seen at this obsession (Alavidjeh *et al.*, 2010). Eventual outcomes of this study demonstrated that all groupings of G2-dendrimer AZT-conjugated could limit HIV capably and were estimations subordinate. Considering the loss of intense against retroviral drugs, other than the alleged exceptionally dynamic hostile to retroviral treatment regimens prompting to numerous potential unfriendly impacts, assessing potential nano medicine-based hostile to HIV medications is of awesome significance.

CONCLUSION

In this study, we found that G2-Dendrimer AZT has detectable hostile to retroviral action.

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