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Original Article

Cellular Delivery of Nanostructured Poly(amido amine) Dendrimers and Establishment of a Simple Methodology upon Ninhydrin Reaction

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Abstract

Dendrimer based nanostructures have been increasingly used for delivery of drugs/genes. These nanosystems, as non-viral gene delivery systems, were shown to have relatively high transfection efficiency despite exerting somewhat cytotoxicity. In this current investigation, poly(amido amine) (PAMAM) dendrimers, generation (G) zero to five, PEGylated PAMAM G3 and a new quaternized PAMAM G4 were synthesized and further characterized using FT-IR and ^{1}H-NMR spectroscopies. The cellular uptakes and toxicity of these nanosystems were investigated using fluorescence microscopy and MTT assay, at which they revealed high internalization potential with low cytotoxicity in both T47D and MCF-7 cells. To establish a simple detection methodology, a ninhydrin reaction was performed on intact PAMAM full generations as well as PEGylated PAMAM G3 and quaternized PAMAM G4. Impacts of various factors such as reaction time, kinetic, reaction medium, and generation dependency of the reaction of dendrimers with ninhydrin were investigated. The best reaction conditions were determined and a simple and reproducible spectroscopic method was established. Upon these findings, we propose that this ninhydrin reaction based methodology may be considered as an easy approach for quantification of primary amines of nanostructured PAMAM dendrimer and its derivatives.

Keywords: Cellular delivery, L-carnitine; Ninhydrin reaction; Nanostructures; PAMAM dendrimers; PEGylated PAMAM.

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1. Introduction

Use of dendrimer based nanostructures, as drug/gene delivery systems, appears to improve the cell/tissue specific delivery and targeting paradigms. Thus, easy methodologies for synthesis and detection of these nanosystems appear to be an of note requirement for further advancement of the field. Of these nanostructures, the starburst poly(amido amine) (PAMAM) dendrimers are a group of half or full generation with unique properties and potential in medical applications such as drug and gene delivery [1, 2]. Surface groups of amine terminated (full) generations could be modified and conjugated with different ligands and functional moieties in many biological and non-biological applications [3, 4]. The presence of primary amino groups in full generations, moreover, means that during synthesis, conjugation and modification processes of each generation ninhydrin test [5-8] could be applicable for characterizing PAMAM and modified PAMAM dendrimers beside other methods, like SEC, HPLC, MALDI-TOF mass spectroscopy, FT-IR, NMR spectroscopy [9] both as their indicator in solutions and an indicator of amidation step during the synthesis process as well as an indirect estimator of the number of various attached groups and conjugated drugs and ligands at the surface of each PAMAM full generation. The reproducibility of the test, however, depends on a number of factors. For instance the color yield per mole of free amino groups in chitosan, as a linear polymer, could be affected by the percentage of free amino groups and the molecular weight (MW) of the polymer [10]. Moreover, for classical polymers, final conformation of each polymer chain in the dispersion medium is a crucial factor in determining the rate and stochiometry of the color produced and may result in a different calibration curves probably because of different accessibility of primary amino groups in the molecules.

In this study, Tomalia's starburst PAMAM dendrimer with ethylenediamine (EDA) core (G0 to G5), PEGylated form of generation three (mPEG-PAMAM G3) and L-carnitine attached generation four of PAMAM (QG4) were synthesized and characterized using FT-IR and ^{1}H-NMR. They were examined upon their potential for transfection of the human breast cancer cell lines, T47D and MCF-7 cells. A ninhydrin reaction was performed via a novel simplified UV-VIS spectroscopic method on various intact generations and modified G3 and G4. Factors such as reaction time and kinetics, medium and generation dependency of the reaction of synthesized dendrimers have been investigated.

2. Methods

2.1. Synthesis of poly(amido amine) dendrimers

The synthesis of poly(amido amine) dendrimers (PAMAM) with ethylenediamine (EDA) core up to the fifth generation (G5) was performed using the divergent procedure described by Esfand *et al*. [11]. The PEGylation of PAMAM G3 was carried out by activation of hydroxyl group of methoxy-terminated polyethylene-glycol 2000 Da (mPEG 2000) using p-nitrophenylchloroformate (PNC) as reported in the literature [12]. Carnitine molecules were conjugated to the surface of PAMAM G4 through activation of carboxylate groups of carnitine by EDC. Briefly, the pH of solution of 50 mg of PAMAM G4 in 5 ml of distilled water was adjusted to 7 by diluted HCl. Solution of 73 mg of L-carnitine (20 molar excess in comparison to the moles of primary amine of PAMAM) in 5 ml distilled water and 70 mg of EDC were added to the above solution. The mixture was stirred for 3 h at room temperature, dialyzed over 3 days against distilled and then deionized water by benzoilated 1200 Da dialysis tubing bag followed by lyophilization, results in a viscid

Table 1. Regression equation, correlation coefficient and range of linearity of PAMAM full generations reacted with ninhydrin in ethanol.

PAMAM generation	Regression equation	correlation coefficient (R^2)	Beer's low range (nmol amine/ml)
0	y=0.0156x+0.0186	0.9998	~ 20-154
1	y=0.0057x-0.0131	0.9997	~ 25-167
2	y=0.0058x-0.0059	0.9999	~ 25-150
3	y=0.0058x-0.0059	0.9999	~ 25-140
4	y=0.0058x-0.0189	0.9990	~ 25-135
5	y=0.0052x-0.0087	0.9938	~ 20-90

Note: "y" and "x" represent the absorbance unit (at 569 nm) and the concentration of amine (nmol/ml), respectively.

brownish yellow product (45 mg). ^{1}H-NMR (δH ppm): 4.5 (m, -CH*(OH)-CH2 of carnitine); 3.25-3.35 (s, N-CH2*-CHOH); 3.1-3.25 (m, PAMAM protons); 3.1 (s, -CH3 of carnitine); 2.87-3.02 (m, PAMAM protons); 2.6-2.85 (m, PAMAM protons); 2.45-2.58 (m, PAMAM protons); 2.2-2.42 (m, -CH2-CO of PAMAM and carnitine).

2.2. Size and zeta potential analysis

The size and zeta potentials of the dendrimers were determined using Malvern Nano ZS, Malvern Instruments Ltd., (Malvern, Worcestershire, UK). The dendrimers were prepared in distilled, degassed biologic-grade water and consecutively measured (×4). Both zeta potential and size determinations were performed at 25 °C. Prior to use, all glass and plastic wares were pre-washed with filtered water to minimize particulate contamination. For detection of the synthesized dendrimers, ninhydrin based reaction were conducted.

2.3. Cell culture

The human breast cancer cells (T47D and MCF cell lines) obtained from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran) were cultured at a seeding density of 4.0×104 cells per cm^2 onto the designated culture plates using RPMI 1640 (Gibco, UK) supplemented with 10% FBS (Gibco, UK), 100 μg/ml streptomycin (Invitrogen, Paisley, UK) and 100 units/ml penicillin G (Invitrogen, Paisley, UK). For cellular uptake and cytotoxicity assay, the 40-50% confluent cells were washed twice with serum free media (SFM), and then exposed to the prepared nanoparticles of polymers or polyplexes for 4 hr at 37 ºC incubation. After that, the cells were washed with SFM, replenished with normal culture medium and incubated at 37ºC for further 24 hr. The cultivated cells were then subjected to the designated analyses.

2.4. Fluorescent microscopy

Cellular internalization of the PAMAM (G3 and G5) nanoparticles was examined

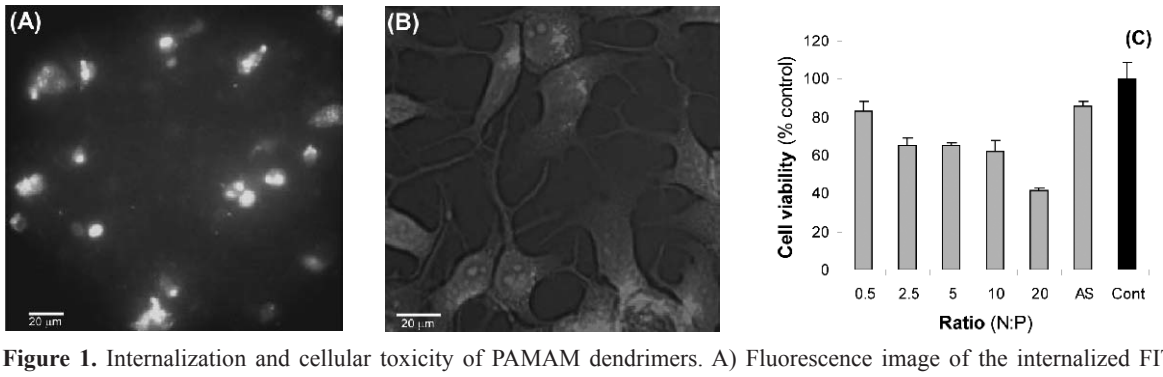

Figure 1. Internalization and cellular toxicity of PAMAM dendrimers. A) Fluorescence image of the internalized FITC-labeled anti epidermal growth factor receptor (EGFR) antisense (AS) complexed with PAMAM (G5) in T47D cells. B) Superimposed fluorescence and light microscopy image of the internalized Cy3-labeled anti EGFR antisense complexed with PAMAM (G3) in MCF-7 cells. C) Cytotoxicity of T47D cells treated with different ratios of PAMAM (G5):antisense polyplexes (N:P) compared to untreated control (cont.) cells.

Table 2. Regression equation, correlation coefficient and range of linearity of PAMAM full generations reacted with ninhydrin in DMSO.

PAMAM generation	Regression equation	correlation coefficient (R^2)	Beer's low range (nmol amine/ml)
0	y=0.0049x-0.038	0.9996	~ 35-370
1	y=0.005x-0.045	0.9984	~ 35-300
2	y=0.0048x-0.0417	0.9987	~ 35-300
3	y=0.0049x-0.0442	0.9978	~ 35-260
4	y=0.0053x-0.0424	0.9997	~ 35-200

Note: "y" and "x" represent the absorbance unit (at 604 nm) and the concentration of amine (nmol/ml), respectively.

using fluorescent microscope. At 40-50% confluency, the cells were transfected with the FITC or Cy3 labeled anti epidermal growth factor receptor (EGFR) antisense complexed with the dendrimers for 4 hr. The cells were then washed 3 times with sterile PBS prior to fixation. Fixation involved washing the cells 3 times with PBS, followed by 10 min. incubation with 2% formaldehyde in PBS at room temperature. Cells were then washed a further 3 times with PBS and mounted on slides using Vectashield Hardset mounting medium with or without DAPI nuclear stain (Vector Labs, Peterborough, UK).

The prepared samples were examined utilizing an Olympus BX51 compound fluorescence microscope equipped with a BX-RFA fluorescence illuminator and catadioptric UMPlanFL-BD objectives. Intermediate magnifications were obtained using a U-CA magnifying device (Olympus optical Co., Ltd. Tokyo, Japan) which was inserted between objective and camera. To optimize fluorescence excitation, U-MWU2 cube at 330-385 nm, U-MWB2 cube at 460-490 nm and U-MF2 cube at 546-563 nm were used for DAPI, FITC and Cy3, respectively. For some cases, the Z-stacks acquisitions were performed (i.e., 5 micrometer increment per focal step) using Hamamatsu C7780-10 cooled CCD (Hamamatsu photonic Co., Tokyo, Japan) as TIFF format in RGB mode (i.e., 12 bits per each channel). Acquired images (1344×1024 pixels resolution) were then imported to ImageJ 1.37 software (http://www.uhnres.utoronto.ca/facilities/wcif/imagej/) to produce the superimposed images. Depth of focus was improved automatically using the stack z-projection plugin at the expert mode (complex wavelet).

2.5. Cellular toxicity

Cytotoxicity of nanoparticles was evaluated using methylthiazoletetrazolium

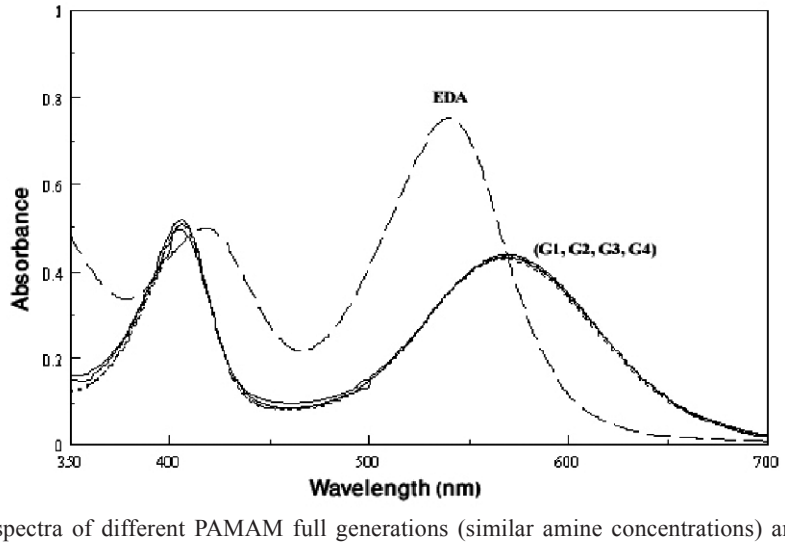

Figure 2. UV-VIS spectra of different PAMAM full generations (similar amine concentrations) and EDA reacted with ninhydrin in ethanol for 4 min. over boiling water bath. The reaction was performed in 1 ml ethanol, cooled and adjusted to 5 ml in a volumetric flask by ethanol-water mixture (50 v/v%). Abs: Absorbance.

Table 3. Evaluation of accuracy and precision of the developed method of intact PAMAM G2 and PEGylated G3 dendrimer reaction with ninhydrin in DMSO.

PAMAM generation	Added (nmol amine/ml)[a]	Detected (nmol amine/ml)[b]	RSD (%)	Accuracy[c]
G2	74.7	84.4	2.5652	12.99
	149.4	152.5	1.4982	2.07
	224.1	240.1	0.8266	7.14
mPEG-G3	35.4	34.6	0.6490	2.26
	44.6	42.9	1.5901	3.81
	55.2	53.0	0.3813	3.99

[a]theoretical calculations; [b]mean of three independent measurements; [c]absolute value of [(added-detected)/added]×100.

(MTT) assay (Sigma Aldrich Co. Germany) in T47D cells. Briefly, cultured cells in 96-well plates were exposed to various ratios of the PAMAM:Antisense (N:P) polyplexes for 4 hr. They were then washed once with phosphate buffered saline (PBS), replenished with normal culture medium and incubated at 37 ºC for 24 hr. The normal culture medium was replaced with 200 µl fresh media and then 50 µl MTT reagent (2.5 mg/ml in PBS) was added to each well. Following a 4 hr incubation period at 37 ºC, medium was removed and cells were exposed to 200 µl DMSO and 50 ml of Sorenson buffer (pH 7.4). Cultures were incubated for 30 min. at 37 ºC and then UV absorbance was measured at 550 nm using a spectrophotometric plate reader (SUNRISE TECAN, Austria).

3. Results and discussions

3.1. Size and zeta potential

The synthesized dendrimers yielded nano-scaled size (~50 nm) with zeta potential approximately+25 mV. These results are in consensus with previous reports on the size and surface charges of the commercially available starburst PAMAM dendrimers [13, 14].

3.2. Internalization and cellular toxicity of PAMAM dendrimers

As shown in Figure 1, internalization of PAMAM:antisense polyplexes in both cell lines revealed their transfection efficiency which was further examined using RT-PCR anlysis (data not shown). Panels A and B show successful delivery of FITC/Cy3 labeled anti EGFR antisense (AS) complexed with PAMAM (G5/G3) in T47D and MCF-7 cells, respectively.

Similar to previously reported results [13, 14], the cytotoxicity of T47D cells treated with different ratios of PAMAM (G5):antisense polyplexes (N:P) revealed

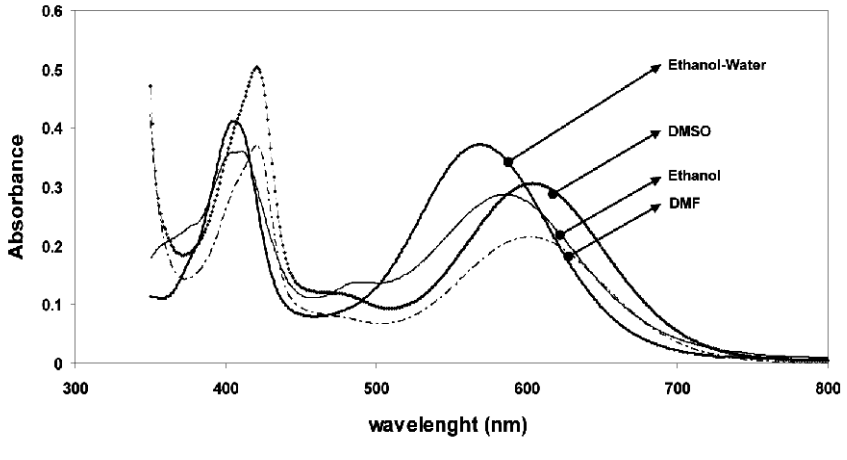

Figure 3. UV-VIS spectra of similar concentrations of PAMAM G2 reacted with ninhydrin for 4 min. in DMSO, DMF, ethanol and 50 v/v% ethanol-water mixture. Abs: Absorbance.

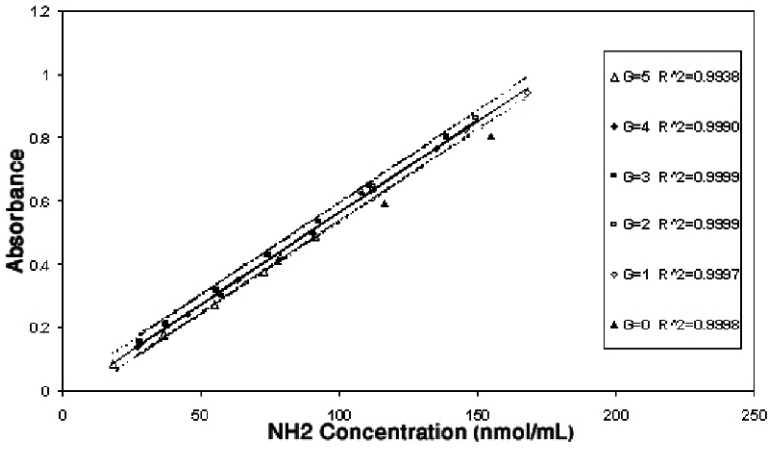

Figure 4. Calibration curve of different PAMAM full generations reacted with ninhydrin according to their primary amine contents in ethanol with 95% CI of the best-fit line of all data.

about 50-85% cytotoxicity compared to untreated control cells (Figure 1C). The greater the N:P ratio, the higher the toxicity.

3.3. Reaction of ninhydrin with PAMAM

The reaction of ninhydrin with EDA and various generation of PAMAM up to G5 was investigated first according to the method developed by Luo *et al*. [15]. EDA reacted with ninhydrin in absolute ethanol and produced a near purple color that possessed two maximum absorbances at 540 and 418 nm (after diluting with 50 v/v% ethanol-water mixture). However, full generations of PAMAM (G0 through G5) produced a violet to blue colored solution that had two λmax of 405 and 569 nm in their spectra in the mixture of ethanol-water (50 v/v%) and also two different λ_{max} of 411 and 586 nm in absolute ethanol (Figures 2 and 3, respectively). It has already been reported that the produced Ruheman Purple (RP) color is due to the ninhydrin reaction with primary amines [16].

Calibration curves of the reaction of different generations of PAMAM with ninhydrin were constructed separately (Figure 4 and Table 1). At least 5 points were selected for each generation. For all generations the curves were linear at amine concentrations of about 25 to 135 nmol/ml (except for G5). The best fit line of all points upon 95% confidence interval (CI) was plotted as an indicator of generation-dependency of PAMAM for such reaction. The 95% CI

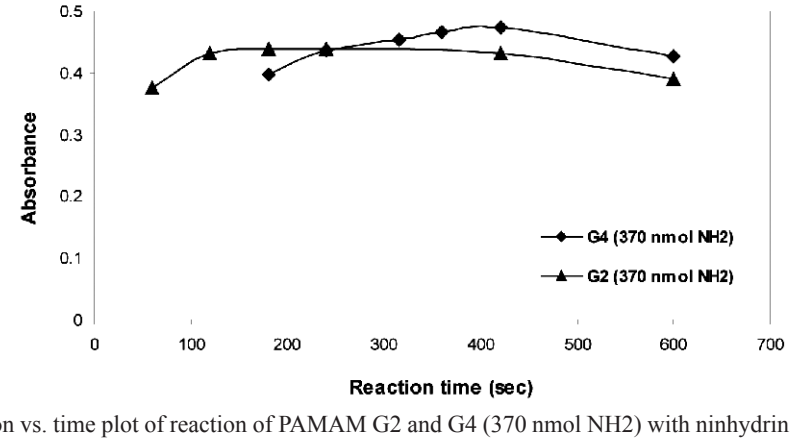

Figure 5. Absorption vs. time plot of reaction of PAMAM G2 and G4 (370 nmol NH2) with ninhydrin (750 µl of 4 mg/ml) in absolute ethanol, then diluted to 5 ml by 50 v/v% ethanol-water mixture (each point is mean of two independent measurements).

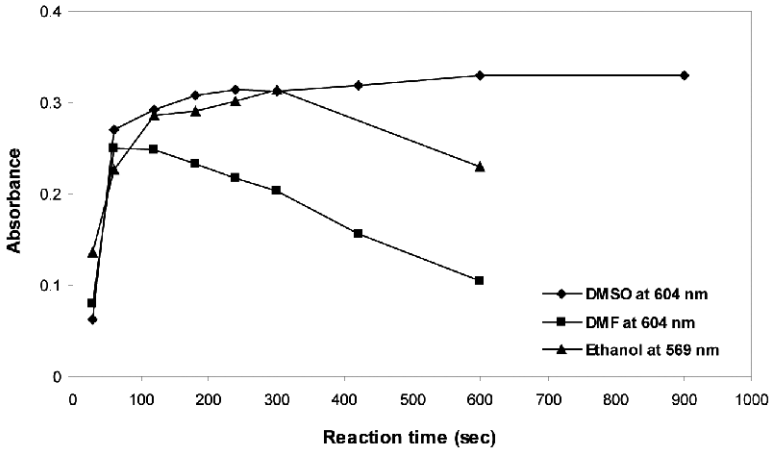

Figure 6. Stability of PAMAM G2 (fixed 371 nmol NH2) reacted with ninhydrin in different solvents over different reaction time periods (average of two independent measurements).

represents acceptable dispersion around the best fit line (RSD>10%) as shown in Figure 4. A better linearity ($R^{\wedge 2}$>0.9997) was observed for the lower generations (G0 to G3) compared to the higher generations (G4 and G5) ($R^{\wedge 2}\geq$0.9938). Upon our speculation, this is because of precipitation of produced RP color on the wall of the reaction flask during the reaction, a precipitate that could not be dissolved in ethanol or ethanol-water (50 v/v%) even by vigorously shaking.

3.4. The effect of the solvent on the ninhydrin reaction with PAMAM

The reaction rate of PAMAM with ninhydrin in all tested generations seems to be much higher than almost all amino acids tested. Such reaction can be completed within 30 sec to ~10 min. depending on their concentrations and generations, i.e. the higher generations need more time to reach the highest absorbance. Accordingly, PAMAM G4 reached its highest absorbance after about 6 min. (Figure 5), while the reaction time for G2 was less than 2 min. The reaction kinetics of G2 and G4 appeared to be a linear function of the amine concentration. After a defined period of reaction time (i.e., ~7 min. for G2 and G4), the resulting RP color started decomposing.

To find optimum conditions of the reaction and getting reproducible results with better linearity and stability, we investigated the potential of media other than ethanol (e.g., DMF and DMSO). The UV-VIS spectra of the

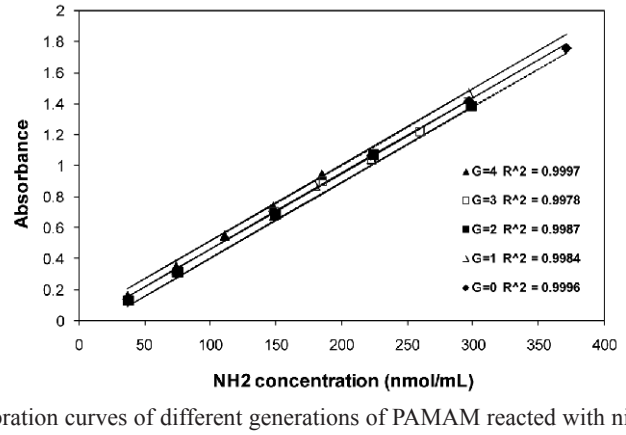

Figure 7. Constructed calibration curves of different generations of PAMAM reacted with ninhydrin in DMSO at optimized reaction conditions for all generations. 95% CI of the best-fit line was plotted as an indicator of generation dependency of PAMAM in this reaction.

resulting product in four different media are shown in Figure 3. The maximum absorption wavelength in aprotic solvents shifted to the higher wavelengths (419 and 604 nm in DMSO and DMF, instead of 405 and 569 nm in ethanol-water solvent). The effect of solvent on the absorption spectra of RP color have been well documented by Friedman [16]. In aprotic and polar solvents (e.g., DMF and DMSO), the absorbance and accordingly the apparent color of the resulting solution was different (green), while the intensity of the resulting color was weaker than that of the ethanol-water.

3.5. The effect of the reaction time

Completion of the reaction required 7 min. for either of the generations of PAMAM from G0 through G5 in ethanol. There is no significant difference (student t-test, $p>0.05$) between absorbance intensity at 3 min. and 7 min reaction time for G≤3 using the method described above.

Figure 6 shows the stability of RP color in different media over different reaction time periods. The DMSO was found to be a suitable solvent since the product represented no degradation over a period of 15 min. compared to other media, in which the degradation occurred in direct relation to time. For example in ethanol, after 7-8 min., the resulting color started to fade and after 10 min. about 10-30% degradations occurred.

We found that DMF was not a suitable medium for this reaction because the product yielded the lowest stability and color intensity (Figure 6). It showed the lowest absorbance even after applying the best reaction time (2 min). After 10 min., the reaction mixture showed about 58% reduction in absorbance. The reaction kinetics in DMSO and DMF were similar to that of ethanol. However, based upon the mentioned results in DMF, the color produced was degraded rapidly over a designated period of time (Figure 6).

Figure 7 and Table 2 show linearity and 95% CI boundary of the best fitted line of PAMAM full generations reacted with ninhydrin in DMSO for 4 min.

over boiling water bath. Having compared data of Figure 7 and Table 2 with Figure 4 and Table 1, it can be deduced that there are little deviations from linearity. Besides, the developed color fits best with the Beer's law when DMSO is used as the reaction medium. The higher generations displayed little deviations even at high concentrations in comparison to the other solvents tested. Further, PAMAM revealed a lower generation dependency in DMSO in comparison to ethanol.

As shown in Figures 6, 7 and Table 2, the best reaction medium loomed to be DMSO regarding the stability of the product and

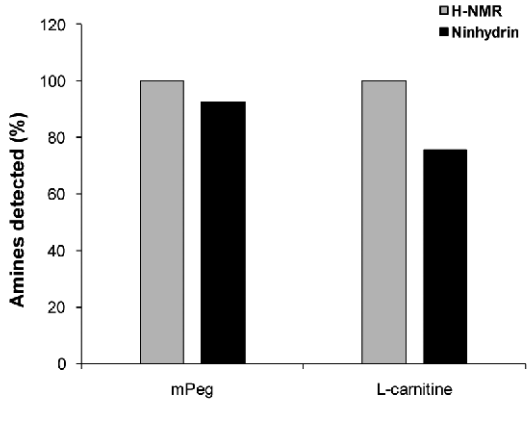

Figure 8. Comparison of ninhydrin and ^{1}H-NMR method for detection of unreacted amine groups of PAMAM G4 and G3 after conjugation with L-carnitine and mPEG respectively.

linearity of the curve. The reaction condition seemed to be simple and rapid; moreover, the calibration curves resulted in better linearity and repeatability over a wider range than ethanol.

3.6. Validation of the developed method

Table 3 shows the accuracy and precision of ninhydrin reacted with G2 and mPEG PAMAM G3. Represented data show that the developed method possesses a good repeatability beside the best linearity in DMSO as shown in Figure 7 and Table 2.

3.7. Reaction of mPEG-PAMAM G3 and QG4 with ninhydrin

After optimization of the reaction in DMSO for intact dendrimers, the method was used to quantify the number of attached mPEG chains and L-carnitine to the surface of the PAMAM G3 and G4, for which approach first the direct estimation was performed by ^{1}H-NMR spectroscopy. For estimating number of conjugated mPEG chains to the surface of PAMAM G3, the ^{1}H-NMR methodology was exploited through comparison of the peak integral at 2.5 ppm of methyl protons adjacent to the tertiary nitrogen group of PAMAM with the sharp singlet (3.23 ppm) of methoxy end group belonging to the mPEG. As a result, approximately 16.02 mPEG chains were found to be attached to the surface of each molecule of PAMAM G3 and each mole of the resulting molecule displayed a molecular weight (MW) of about 38950 Da. The ninhydrin test was carried out with a solution of 2 mg/ml of mPEG PAMAM under optimum conditions in DMSO, resulting ~15 moles of unreacted primary amines at the surface of each molecule (Figure 8).

In the case of QG4, comparison of the integral of the multiplet peak at 4.5 ppm (belonging to –CH*OH- of attached carnitine) with the peak at 2.5 ppm of methyl protons of PAMAM also provided 7.4 molecules of attached L-carnitine per molecule of PAMAM G4, corresponding to an average MW of 15406 Da for the final conjugate (Figure 9). Further, ninhydrin detected 75.5% of 56.6 moles of unreacted amine groups that were shown by the direct estimation method using ^{1}H-NMR (Figure 8).

At all concentrations of mPEG-PAMAM G3, the estimated number of primary amines in ninhydrin method was similar to that obtained by ^{1}H-NMR. The small difference observed (i.e., 0.1-1.9 lower primary amino groups per mole of PAMAM) is deemed to be due to the spatial hindrance of reactive

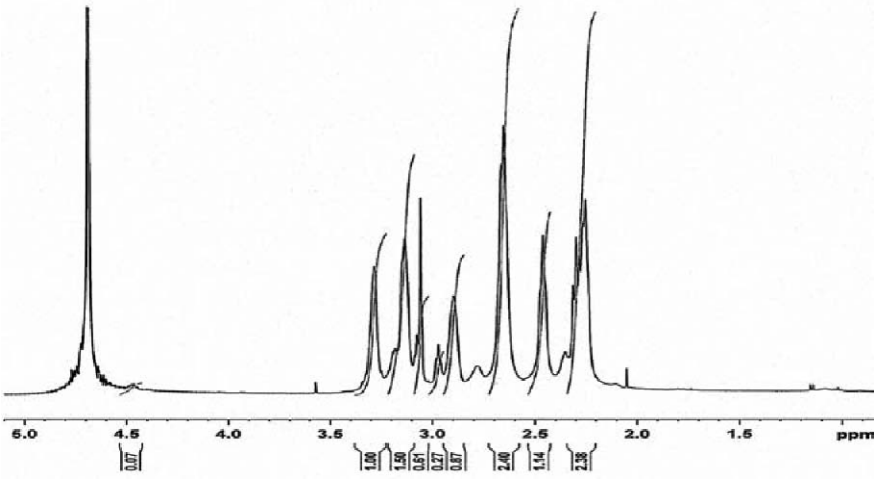

Figure 9. ^{1}H-NMR spectrum of QG4 in D_2O.

primary amines as consequence of the presence of mPEG chains, resulting in some amine groups not reacting with ninhydrin molecules. However, the estimated numbers using the ninhydrin method (1-10% lower than the estimated primary amine of each mole of PAMAM by NMR) were satisfactorily reproducible.

The amount of free amine for QG4 resulted from ninhydrin test was not similar to the ^{1}H-NMR data, that is the difference between the two methods (i.e., ninhydrin and ^{1}H-NMR methods) in the case of QG4 could be due to the presence of side reaction between the free amine group and the EDC in the previous step.

Reaction of ninhydrin with ordinary linear polymers such as chitosan were shown to have different behaviors depending upon their structures, MW, degree of deacetylation and branching [10]. We speculate that this could be due to the fact that their chain conformations in solution depend on the degree of flexibility of polymer-backbone, polarity, ionic strength and availability of proton ions in the medium as well as the shielding and hindering of some of the reactive amino groups. It should be also evoked that only part of the reactive primary amines of a polymer chain loom to be available that can lead to some non-stochiometry and different rates, kinetics and color yields. Contrarily, PAMAM dendrimers have a unique homogenous globular symmetrical nano-scaled shape in solvents and almost all the surface primary amines of PAMAM full generation molecule in the polar protic and aprotic media examined appeared to be accessible. Consequently, it is expected that all generations have identical kinetics and ideal stochiometry (Figures 4, 7 and Tables 1, 2). Further, because of a wide MW dispersity of classical polymers and many ligands that have been attached to the surface of PAMAM dendrimers, in some cases estimation of number of attached ligands per mole of dendrimer can not be satisfactorily accomplished using methods such as NMR, SEC, and etc. However, simple methods like ninhydrin may benefit such need.

4. Conclusion

Based upon our findings, we witnessed that the synthesized PAMAM dendrimers condensed the desired antisense and the resultant polyplexes were successfully delivered to the target cells. The cellular toxicity were largely dependent on the generation of PAMAM dendrimers and the ratio of dendrimers:antisense. The optimized method of ninhydrin titration of NH2 groups on intact PAMAM and modified dendrimers in DMSO appeared to be a simple yet reliable method that resulted in markedly high precision and wide rage of linearity. Thus, we propose utilization of this method along with other more sophisticated approaches may favor the quantification of intact full generations of cationic polymers such as star burst PAMAM dendrimers and its derivatives.

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