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Comparing the transfection efficiency of cationic monomer ratios in vinylimidazole and aminoethyl methacrylate copolymers

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ABSTRACT

Employing polycations as non-viral gene delivery vectors has been extensively studied owing to their safety, efficiency and possibility of modifying them in an intended way compared with viral vectors. However, the main challenge is finding a biocompatible and transfection-efficient polymer. In this study, 2-aminoethyl methacrylate (A) and 1-vinyl imidazole (V) were copolymerized at three different molar ratios by a free radical polymerization method and novel biocompatible polycations with narrow molecular weight distribution were obtained. The resulting copolymers were used for condensation of plasmid DNA (pDNA) at different N/P ratios followed by physicochemical characterizations of resulting polyplexes. At N/P ratio of 2, the nanoplexes were smaller than 120 nm. The optimum formulations were stable in presence of polyanions and capable of protecting the condensed pDNA against nucleases. The polyplexes having V to A molar ratio of 1:1 were the most efficient carrier in transfecting HeLa cells and were introduced as a promising non-viral vector.

1. Introduction

Gene therapy is an approach that relies on delivering functional gene (nucleic acid or its analogues) into the target cells by a vector to manipulate and alter gene expression so as to cure or prevent diseases like congenital abnormalities and cancer (Patil et al., 2019; Wahane et al., 2020; Zu and Gao, 2021). The main impediment in this process is finding a proper carrier to load the genetic substance and deliver it to the target site safely and efficiently. An efficient vector not only carries the gene but also protects it from digesting enzymes in the blood stream and in the cell endosome (Liu et al., 2010). Generally, gene delivery carriers are classified into viral and non-viral vectors. Although viral vectors like adenovirus, cytomegalovirus, lentivirus, vesicular stomatitis virus, and retrovirus have gained attention due to high and efficient gene transfection, they have serious disadvantages like toxicity, immunogenic problems, carcinogenicity, and high cost of large scale production (Butt et al., 2022; Taghdiri and Mussolino, 2024; Yin et al., 2014). Hence, non-viral vectors with advantages consisting biocompatibility, low immunogenicity, flexibility to modification, low cost, and no restriction in size of the loaded genetic material has received scientists' attention (Jeong and Nah, 2017; Li et al., 2015; Ma et al., 2021; Vijayanathan et al., 2014; Zinchenko, 2016). Among non-viral carriers, cationic polymers have been extensively studied and employed. They can form complexes with DNA by neutralizing the negative charge of its phosphate groups and the electrostatic interaction between them leads to formation of a complex spontaneously called polyplex. Consequently, the presence of positive charge moieties like amino groups in polymer structure is essential (de Ilarduya et al., 2010; Freitag and Wagner, 2021; Lai and Wong, 2018; Zeng et al., 2019; Zeng et al., 2018). Moreover, an efficient polyplex is the one that is stable and capable of overcoming biological barriers, *i.e.* sheltering the genetic material from serum and extracellular medium nucleases, escaping from endosomes to deliver its cargo to cytoplasm

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and its site of action (Chrysostomou et al., 2022; Varkouhi et al., 2012). Polycations including chitosan (Rahmani et al., 2019), poly-L-lysine (PLL) (Veleva-Kostadinova et al., 2018; Yang and Luo, 2023), poly (ethylenimine) (PEI) (Bono et al., 2020), poly(2-dimethylaminoethyl methacrylate) (PDMAEMA) (Panchal and Vasava, 2024) and poly-amidoamine (PAMAM) dendrimers (Mekuria et al., 2021; Tarach and Janaszewska, 2021) are the frequently used polymers for this purpose. PEI is the prominent polymer in this category because it can function as a pH sensitive polymer and is able to bind to protons entering the endosome, which leads to an influx of protons and consequently water to this vesicle that induces its swelling and bursting before lysosomal degradation of the genetic material (Martens et al., 2014). Furthermore, different studies demonstrated that modifying polymers with histidine or imidazole-containing moieties confers the endosomal escape feature to the polymer and increases its gene delivery capability (Mehta et al., 2024; Mishra et al., 2006). For instance, polyplexes of poly(imidazole/2-dimethylaminoethylamino) phosphazene demonstrated meaningfully greater transfection efficacy in comparison with poly (2-dimethylaminoethylamino) phosphazene and PEI 25 K discretely (Yang et al., 2008). In addition, it was shown that imidazole ring in urocanic acid-modified chitosan (Kim et al., 2003) and histidylated polylysine (Bello Roufaï and Midoux, 2001) increased transfection efficacy due to the fact that imidazole heterocycle pKa is almost 6 and it is protonated at endosomal pH, inducing endosomal bilayer disruption and the release of polyplex to the cytosol (Asayama et al., 2010; Dhanya et al., 2018). Other polymers that exhibit endosomal escape are polyvinyl imidazole derivatives. The homopolymer, polyvinyl imidazole, exhibits considerable buffering capacity at endosomal pH, although it is not able to interact and make complex with genetic materials like DNA since imidazole ring groups are not significantly charged at pH 7.4 (Asayama et al., 2007). To enhance interaction of polyvinyl imidazole with the genetic material, different approaches including alkylation of polymer and adding amino ethyl group to the imidazole ring were employed to quaternerize the amine moiety in the imidazole ring and aid the polymer in developing a stable complex with DNA (Asayama et al., 2010; Asayama et al., 2007). In addition, it has been demonstrated that even 0.5 to 2 % modification of imidazole moieties with 1-bromopropane raised polymer- DNA complex formation significant (Danilovtseva et al., 2019). Furthermore, copolymerization of vinyl imidazole and amine containing monomers or block polymers is another approach to obtain stable polyplexes. Polymerization of N-ethyl pyrrolidine methacrylamide with 1-vinylimidazole has been effective in producing stable DNA-polymer complex together with efficient endosomal escape and gene transfection (Velasco et al., 2012). Moreover, reducible poly(2-dimethylaminoethyl) methacrylate-*block*-polyvinyl imidazole has been synthesized and manifested superior transfection activity to reducible poly(2-dimethylaminoethyl) methacrylate. This copolymer was synthesized with single disulfide bond in the backbone by oxidizing their dithioester-terminated polymers using ethyl xanthogenate as chain transfer reagent (Yu, 2012). Additionally, in another study, 1-vinylimidazole copolymerized with methyl acrylate and a mixture of synthetic oligopropylamines was grafted to the methyl acrylate blocks. The grafted copolymer exhibited superior interaction and binding capacity with DNA oligonucleotide compared with unmodified copolymer due to presence of amine groups in grafted moieties (Strelova et al., 2021).

Positive charge of 2-aminoethyl methacrylate homopolymer at physiologic pH (pKa approximately 7.6) makes it an appropriate choice for gene delivery, owing to the capability of primary amine in interacting with plasmid DNA and producing complex effectively (Ji et al., 2011; Thompson et al., 2008; Trützschler et al., 2018). Furthermore, the amine moiety aid endosomal escape and elevate the chance of gene expression. Besides, it was demonstrated that poly(2-aminoethyl methacrylate) (PAEM) accelerated endosomal escape of plasmid DNA, compared to poly(2-dimethylaminoethyl methacrylate) bearing tertiary amine, which stems from the ability of charged groups of this polymer to exhibit notably membrane interaction instead of proton sponge effect (Trützschler et al., 2018). Therefore, to overcome the drawback of poly 1-vinylimidazole, copolymerization of 2-aminoethyl methacrylate and 1-vinyl imidazole is beneficial to improve DNA condensing property and gene transfection.

Furthermore, the copolymer of 1-vinylimidazole and 2-aminoethyl methacrylate not only takes advantage of forming hydrogen bond and π-π interaction with nucleotides of the genetic material by means of the imidazole ring of 1-vinylimidazole, but also it is capable of interacting with the genetic material by electrostatic bonds (Li et al., 2023; Zakharova et al., 2024). The amine group of 2-aminoethyl methacrylate in copolymer structure gets protonated at neutral and physiologic pHs and impart the positive charge to the polymer, which is essential to have an efficient interaction with negative charged genetic material and condense it to form a polyplex. Moreover, one advantage of the copolymer polyplexes in comparison with polyplexes of poly 1-vinyl imidazole might be forming more stable complexes and not releasing their cargo in blood circulation by means of the positive charge of the amine groups at physiologic pH, which helps to provide electrostatic interaction with phosphate groups of the genetic material and to keep the formed stable polyplex. Additionally, the existence of imidazole rings along with primary amino groups in 1-vinylimidazole and 2-aminoethyl methacrylate copolymer structure might improve endosomal escape and gene transfection compared with other polymers like PEI and chitosan. These polymers have only primary amino groups in their structure. It was indicated in different studies that histidine modified PEI and chitosan had superior transfection efficiency compared to unmodified polymers, which stems from the imidazole ring of histidine and its role in buffering endosome (Chang et al., 2010; Salmasi et al., 2015). Furthermore, the presence of imidazole and 2-aminoethyl methacrylate groups together in the present copolymer can compensate the drawbacks of the studied homo or copolymers shown in Table 1 and provide the advantageous features mentioned above.

The aim of the present study was developing a suitable polycation material as a non-viral carrier for gene delivery by a simple free radical copolymerization of 1-vinylimidazole and 2-aminoethyl methacrylate and studying the effect of the obtained copolymer on particle size, zeta potential, stability and transfection efficacy of resultant polyplexes.

2. Materials and methods

2.1. Materials

2-Aminoethyl methacrylate hydrochloride (90 %, stabilized) was from Acros Organics (New jersey, USA), 1-vinylimidazole, 2,2 azobisisobutyronitrile (AIBN), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA), dimethyl sulfoxide (DMSO) was from Merck Chemical Company (Darmstadt, Germany), fetal bovine serum (FBS), trypsin and Dulbecco's modified Eagle's medium (DMEM) were purchased from BioIDEA (Tehran, Iran), L929 and HeLa cell lines were obtained from the Pasteur Institute (Tehran, Iran), DNA size marker (100–10,000 bp), 6× DNA loading dye, DNA Safe Stain and Agarose gel were supplied from SinaClon BioScience (Iran), pLOX-CWgfp plasmid was from Addgene (USA) and DNA-fectamine was purchased from Bio Basic (Toronto, Canada).

2.2. Synthesis of copolymer

2-Aminoethyl methacrylate hydrochloride (A) and 1-vinylimidazole (V) with molar ratios of 1:1 (AV), 2:1 (A2V), 3:1 (A3V) were dissolved in the mixture of isopropanol and water (1:1 *v*/v) in a round-bottom bottle, equipped with a magnetic stirrer bar, under nitrogen atmosphere. Then, the initiator AIBN (1 % $w_{initiator}/w_{monomer}$) was added to the mixture, while stirred at 750 rpm and kept at 60 °C for 29 h. Afterward, the reaction mixture was dialyzed by a dialyzing tube (cut-off 2000 Da) against deionized water for 24 h and was freeze-dried to obtain the

Table 1
Advantage and disadvantages of studied homo or copolymers containing imidazole or 2-aminoethyl methacrylate monomers in their structure.

Polymer	Structure modification	Drawbacks	Advantages	Reference
Poly 1-vinylimidazole	None	Forming unstable polyplex at physiologic pH	Buffering endosome and successful endosomal escape	(Danilovtseva et al., 2019)
Aminated poly vinylimidazole	Attachment of aminoethyl group to imidazole ring	Low transfection efficiency	Forming stable polyplex at physiologic pH	(Asayama et al., 2007)
Butylated poly vinylimidazole	Attachment of Butyl group to imidazole ring	–	Improved endosomal escape, Superior transfection efficiency than aminated poly vinylimidazole and PEI in HepG2 cells	(Asayama et al., 2010)
Poly 2-aminoethyl methacrylate	None	Low transfection efficiency in presence of serum	Higher stability of high molecular weight polymer polyplexes against heparin sulfate, acceptable transfection efficiency in serum free medium	(Ji et al., 2011)
Pegylated poly 2-aminoethyl methacrylate	Covalent attachment of poly etylene glygol to poly 2-aminoethyl methacrylate	–	Improved cell viability and transfection efficiency in presence of serum	(Santo et al., 2019)
poly (2-dimethylaminoethylamino) phosphazene	Attachment of 2-dimethylaminoethy-lamino to polyphosphazene	Cell cytotoxicity, Low transfection efficiency	Forming complex with genetic material	(Yang et al., 2008)
poly(imidazole/ DMAEA) phosphazene	Attachment of imidazole and 2-dimethylaminoethylamino to polyphosphazene	–	Stable polyplex, higher transfection efficiency and lower cell cytotoxicity than poly (2-dimethylaminoethylamino)phosphazene	(Yang et al., 2008)
Poly (N-ethyl pyrrolidine methacrylamide-*co*-1-vinylimidazole)	Copolymerization of N-ethyl pyrrolidine methacrylamide and 1-vinylimidazole	–	Higher transfection level in presence of serum compared with poly (dimethylaminoethyl methacrylate)	(Velasco et al., 2012)
poly(1-vinylimidazole-*co*-2-aminoethyl methacrylate)	Copolymerization of 1-vinylimidazole and 2-aminoehtyl methacrylate	–	Stable polyplex complex at neutral and physiologic pHs, lower cell cytotoxicity and increased transfection efficiency in copolymers with higher 1-vinylimidazole content	Present study

polymer.

2.3. Characterization of polymers

2.3.1. Fourier transform infrared spectroscopy (FTIR)

FTIR spectrophotometric assessment of copolymers were carried out (FTIR 6300, Jasco, Japan) in the 400 to 4000 cm^{-1} range by preparing potassium bromide discs and compared with FTIR spectrum of physical mixture of monomers to validate the production of copolymers (Fattahi et al., 2013).

2.3.2. ^{1}H NMR spectroscopy

^{1}H NMR measurement was done to confirm the formation of copolymers and to calculate the ratio of monomers in the structure of copolymers. All spectra were obtained by 400 MHz AV-400 Bruker spectrometer (Germany) by employing D2O as a solvent.

2.3.3. Gel permeation chromatography (GPC)

Gel permeation chromatography in aqueous medium (Waters 1515 pump, Shodex SB-805 HQ column) was carried out to measure molecular weight of polymers.

2.4. Cell viability assay

The MTT assay was conducted to assess the toxicity of poylcations on L929 and HeLa cell lines. For this purpose, the cells were cultured in DMEM medium containing 10 % FBS, 100 U/mL penicillin at 37 °C and under 5 % CO_2. L929 and HeLa cells were seeded in separate 96-well plates at the concentration of 7×10^4 and 7.5×10^4 cells/ mL, respectively. After 24 h, the polycations (AV, A2V, A3V) in the range of 6.25 µg/mL to 100 µg/mL were added to wells and the cells were incubated for 24 h. After the incubation period, medium was replaced with fresh medium containing MTT (5 mg/mL) and plates were incubated for 4 h in CO2 incubator. Then, all media was removed and formazan crystals were dissolved by adding DMSO to each well. Finally, a microplate reader (Biotek, ELx808, USA) was employed for determination of the absorbance at 570 nm. The relative cell viability at different concentrations was calculated by the following equation:

$$\text{Cell viability}\ (\%) = \frac{\text{absorbance of sample} - \text{aborbance of blank}}{\text{absorbance of control} - \text{absorbance of blank}} \times 100 \quad (1)$$

The control group contained cells with no treatment and the blank was culture medium with no cells and no treatment.

2.5. Preparation of polyplex

Polyplexes of polymers and plasmid DNA (pDNA) were prepared using N/P ratios of 0.25 to 16 by incubation of 500 µL of copolymer solution in deionized water at different concentrations with 500 µL pDNA at a fix concentration of 20 µg/mL. Afterwards, the mixture was stirred at 750 rpm for one hour at room temperature. The resultant polyplexes (AV/pDNA, A2V/pDNA and A3V/pDNA) were used for further experiments.

2.6. Particle size and zeta potential of polyplexes

The hydrodynamic diameter, polydispersity index (PDI), and zeta potential measurements of the polyplexes were conducted by dynamic laser light scattering technique (Zetasizer 3600, Malvern Ltd., Worcestershire, UK) at room temperature at a scattering angle of 90°, in triplicate.

2.7. Gel retardation assay

To assess the binding stability of polyplexes prepared at different N/P ratios, 10 µL of the prepared polyplexes of each polymer (equal to 1 µg pDNA) were loaded onto a 0.8 % agarose gel containing 0.5 % Tris/ Borate/EDTA (TBE) buffer and 0.1 % DNA Safe Stain. The electrophoresis was performed for 25 min at 95 V and the resulting gel was analyzed using the UV Gel doc (CAMAG Reprostar 3, Switzerland).

2.8. Heparin displacement assay

To determine the stability of polyplexes, the optimum N/P ratio of AV/pDNA, A2V/pDNA and A3V/pDNA containing 1 μg pDNA were incubated with different concentrations of heparin sulphate (heparin to DNA weight ratios of 0.5 to 16) for one hour at 37 °C and electrophoresed on a 0.8 % agarose gel containing 0.5 % TBE buffer and 0.1 % Safe Stain for 25 min at 95 V. The gel was analyzed by the UV Gel doc (Gwak et al., 2017).

2.9. Serum stability test

The stability of polyplexes was investigated against serum nucleases by incubating equal volume of optimum polyplexes of AV/pDNA, A2V/pDNA and A3V/pDNA with equal volume of FBS (final concentration in medium was 50 %) for 72 h at 37 °C and samples were collected at specific time intervals to be analyzed by gel electrophoresis. The same procedure was done for naked plasmid as control.

2.10. DNase I protection assay

To determine the efficacy of the polymer in preserving condensed plasmid against *in vivo* nucleases, the DNase I protection assay was conducted. In this procedure, the optimum polyplex of each copolymer (2 μg pDNA) were incubated with 0.5 μL DNase I (1 u/μL) and 1 μL of 10× reaction buffer containing MgCl2 for 15, 30, and 60 min at 37 °C. To cease the enzyme digestion, 1 μL EDTA containing buffer was added to the specimen and was incubated for 10 min at 65 °C. Then, the mixture was run on a 0.8 % agarose gel containing 0.5 % TBE buffer and 0.1 % Safe Stain for 25 min at 100 V along with 2 μg plasmid DNA and the optimum polyplex containing 2 μg plasmid DNA without enzyme as controls.

2.11. Scanning electron microscopy (SEM)

Size and morphology of optimum polyplexes of each copolymer type were studied by SEM (Leo 1430 VP scanning electron microscope, Germany). A droplet of optimum polyplex of each copolymer was dried on the sample holder and samples were observed after being coated with a fine layer of gold.

2.12. Cytotoxicity of polyplexes

Cytotoxicity of the optimum polyplex of each copolymer on HeLa cells was evaluated by MTT assay. The cells (7.5×10^4 cells/ mL) were added to a 96-well plate and incubated for 24 h. Afterward, the optimum polyplex of each copolymer (containing 1 μg plasmid DNA) was added to wells and the plate was incubated for 48 h. Then, the rest of the procedure was conducted according to Section 2.4 and the cell viability was calculated (Eq. (1)).

2.13. Transfection efficiency

To assess the gene transfection efficacy of polycations, the number of transfected cells producing green fluorescent protein (GFP) were quantified by flow cytometry (BD FACSCalibur, USA). For this purpose, HeLa cells at a concentration of 25×10^4 cells/well were seeded in a 6-well plate. After 24 h, the medium was discarded and 2 mL fresh serum-free medium containing the optimum polyplex of each polymer, naked plasmid and DNAfectamin-plasmid complex as control (10 μg plasmid DNA) were added to wells and incubated for 4 h. Then, the medium was aspirated and 2 mL serum-containing fresh medium was added to each well. After 48 h incubation of cells under optimum culture conditions, the medium was discarded and cells were washed by cold PBS, trypsinized and collected in tubes for further evaluation by flow cytometry.

2.14. Statistical analysis

The analysis of variances (ANOVA) and LSD *post hoc* test was carried out for statistical evaluation of obtained data. The results were shown as mean ± standard deviation and the differences between groups with $p < 0.05$ were considered as statistically significant.

3. Results and discussion

3.1. Fourier transform infrared spectroscopy (FTIR)

The purified polymers were characterized by FTIR to confirm their synthesis. Fig. 1A depicts the FTIR spectrum of a physical mixture of 2-aminoethyl methacrylate and 1-vinylimidazole. Absorption bands at 1714.41 and 3413.39 cm^{-1} were associated to C═O stretching (ester group) and the N—H bending (primary amine) of the 2-aminoethyl methacrylate, respectively (Deng et al., 2009; Esselin et al., 2016). Bands at 657.607 cm^{-1} and 738.602 cm^{-1} represented the C—N stretching of imidazole ring and the C—N bond connecting the ring to the vinyl group, respectively (Pekel et al., 2004; Pekel et al., 2001; Talu et al., 2015). The strong peak observed at 1647.88 cm^{-1} belonged to stretching vibration of C═C of both monomers. As illustrated in spectra B—D, the broad peaks about 3600–3250 cm^{-1} were related to the N—H bending of primary amine (Deng et al., 2009). The bands at 1728.87, 1727.91 and 1729.83 cm^{-1} belonged to C═O stretching in 2-aminoethyl methacrylate. Furthermore, the peaks around 660–663 cm^{-1} and 751–754 cm^{-1} indicated the C—N stretching of imidazole ring and C—N bond attaching this ring to the polymer backbone, respectively (Pekel et al., 2001; Talu et al., 2015). The short peaks at 1608.34, 1612.2 and 1609.31 cm^{-1} belonged to C═C stretching. The observed bands demonstrated that both monomers took a part in the synthesis of copolymer. In addition, the peak at 1647.88 cm^{-1} became shorter and broader in the spectra of copolymers that demonstrated the decrease in portion of C═C bond and formation of C—C bond in backbone of the copolymers in favour of formation of copolymer (Talu et al., 2015).

3.2. ^1H NMR spectroscopy

To confirm the successful production of the copolymers and determine the actual molar ratio of 1-vinylimidazole to 2-aminoethyl methacrylate hydrochloride in the final product, ^1H NMR spectroscopy was carried out. Fig. 2 shows 1H NMR spectra of copolymers, the peaks at 4.13 and 3.17 ppm indicated the two methylene groups in aminoethyl moiety of 2-aminoethyl methacrylate monomer (Chen et al., 2019). Furthermore, the signals between 6.4 and 7.73 ppm belonged to CH groups of imidazole ring in 1-vinylimidazole (Deng et al., 2011; Wang et al., 2014). The molar ratio of the two monomers in the copolymers was calculated by the following equation:

$$[1-\text{vinylimidazole}] \Big/ [2-\text{aminoethyl methacrylate}] = \frac{\left[\frac{I_{(a+b+c)}}{3}\right]}{\left[\frac{I_{(d)}}{2}\right]} \quad (2)$$

Where $I_{(a+b+c)}$ is the integral of a, b, c peaks related to imidazole ring hydrogens and $I_{(d)}$ is the integral of CH2 group of 2-aminoethyl methacrylate unit at 4.21 ppm, which are equivalent to the three and two protons, respectively. The molar feed ratio and the final molar composition of each copolymer are indicated in Table 2. The calculated molar ratio of 1-vinylimidazole to 2-aminoethyl methacrylate in AV, A2V and A3V copolymers were 1.02, 1.83 and 2.8, respectively. The resulting molar ratio for AV was close to the molar feed ratios, although the actual molar ratios of A2V an d A3V were lower than the molar feed ratios. The NMR spectra confirmed the formation of copolymers and contribution of both monomers in their structures at different ratios. The obtained results demonstrated that the contribution of 1-vinylimidazole was

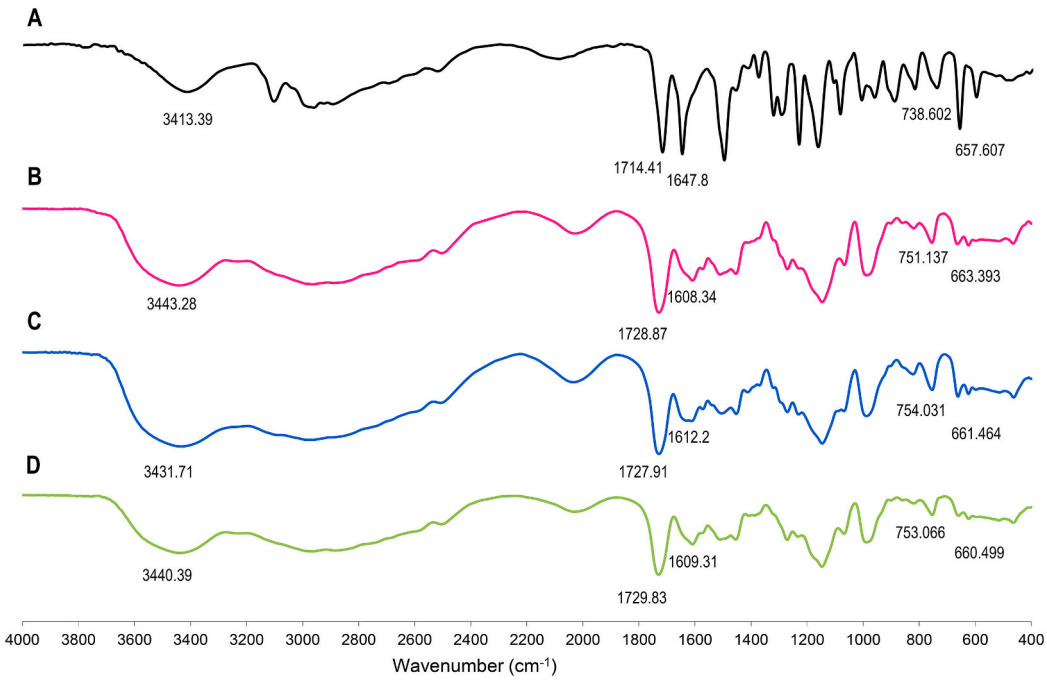

Fig. 1. FTIR spectra of (A) physical mixture of 2-aminoethyl methacrylate hydrochloride and 1-vinylimidazole monomers, (B) AV, (C) A2V and (D) A3V copolymers.

slightly greater than 2-aminoethyl methacrylate in the structure.

3.3. Gel permeation chromatography (GPC)

GPC technique was employed to determine the molecular weight and polydispersity of synthesized copolymers. Table 2 summarizes the data obtained for each copolymer. Molecular weight and polydispersity index of resultant copolymers were between 8000 and 16,000 Da and lower than 1.2, respectively. Low polydispersity index values were in line with another study regarding copolymerization of vinyl imidazole and N-ethyl pyrrolidine methacrylamide (Velasco et al., 2012). A narrow molecular weight distribution was reported by employing complicated methods like RAFT polymerization to polymerized N-isopropylacrylamide and 1-vinylimidazole although, in the present study we obtained it by controlling the reaction parameters precisely. Moreover, the PDI value, which is below 1.2 confirmed that the reaction condition was suitable (Nguyen et al., 2021; Whitfield et al., 2019).

3.4. Cell viability assay

To assess the impact of each copolymer on cell viability, an MTT assay was performed after 24 h of incubation with L929 and HeLa cell lines. (Fig. 3A). The relative cell viability of L929 and HeLa cells increased at 24 h incubation period with decreasing copolymer concentration from 100 to 6.25 μg/mL. At concentration of 12.5 μg/mL or below, the viability of L929 was above 75 %. According to ISO 10993-5:2009, the cell viability percentage above 75 % indicated that the copolymers were not toxic (Iso, 2009). Also, AV and A2V copolymers were found to be nontoxic even at concentration of 25 μg/mL in contrast to A3V polymer. Furthermore, the copolymers demonstrated cell viability greater than 75 % at concentration of 12.5 μg/mL or below for HeLa cells, and as the copolymers did not reduce cell viability more than 30 % based on ISO10993-5:2009 guideline, they were considered non-cytotoxic at this concentration range. In contrast to A2V and A3V copolymers, which were toxic at concentration above 12.5 μg/mL, cell viability of 74.48 % was observed for AV copolymer indicating its less toxicity compared to other copolymers. Additionally, the AV copolymer presented a considerably higher viability of 94.7 % at concentration of 6.25 μg/mL compared to other copolymers ($p < 0.05$). It has been demonstrated that amine groups serve as active sites inducing cell toxicity owing to the interaction of positive charged amine groups with negative charged cell membrane and subsequently affecting its integrity (Kravicz et al., 2019; Mapfumo et al., 2024), which might be the reason for higher cytotoxicity of A2V and A3V copolymers possessing more amine groups compared to AV polymer. Similarly, a study demonstrated that cytotoxicity of urocanic acid (containing imidazole ring) modified poly(2-(2-aminoethoxy) ethoxy) phosphazene polyplexes decreased by increasing the urocanic acid conjugation degree (Yang et al., 2010).

3.5. Particle size and zeta potential

The pDNA at fixed concentration of 20 μg/mL was mixed with copolymers at different concentrations to prepare polyplexes. The hydrodynamic size of polyplexes was evaluated by dynamic light scattering method. Fig. 3B shows the zeta potential and the hydrodynamic diameter of polyplexes of each copolymer at various N/P ratios. The data demonstrated that the net charge of the polyplexes was negative at $N/P = 0.25$. Furthermore, the zeta potential reached to around zero at $N/P = 0.5$ and the polyplexes were considerably large probably due to the fact that the amount of the copolymer was not enough to condense pDNA efficiently and produced a compact complex. Additionally, the charge of the polyplexes was not sufficient to stabilize them and prevent aggregation. Also, the PDI above 0.5 at this N/P ratio for all polyplexes clearly demonstrated the broad particle size distribution and formation of heterogeneous particles (Rahat et al., 2021). However, by adding more copolymer and reaching N/P of 1, the copolymer constructed a compact structure with DNA, slightly positive charge and the mean particle size decreased. By the increment of polymer concentration at N/P ratios above 1, there was a positive correlation between zeta potential and particle size with N/P ratio in all polymer types (Fig. 3B). The positive surface charge of polyplexes conferred upon them the ability of attaching to negative charged cell membrane having a crucial role in cellular uptake of polyplexes (Hamelmann et al., 2022; Liu et al., 2017). Moreover, the surface charge of A3V/pDNA polyplexes at N/P ratios of 2–12 and 2–16 was greater than AV/pDNA and A2V/pDNA polyplexes, respectively ($p < 0.05$). It might be due to the higher imidazole content

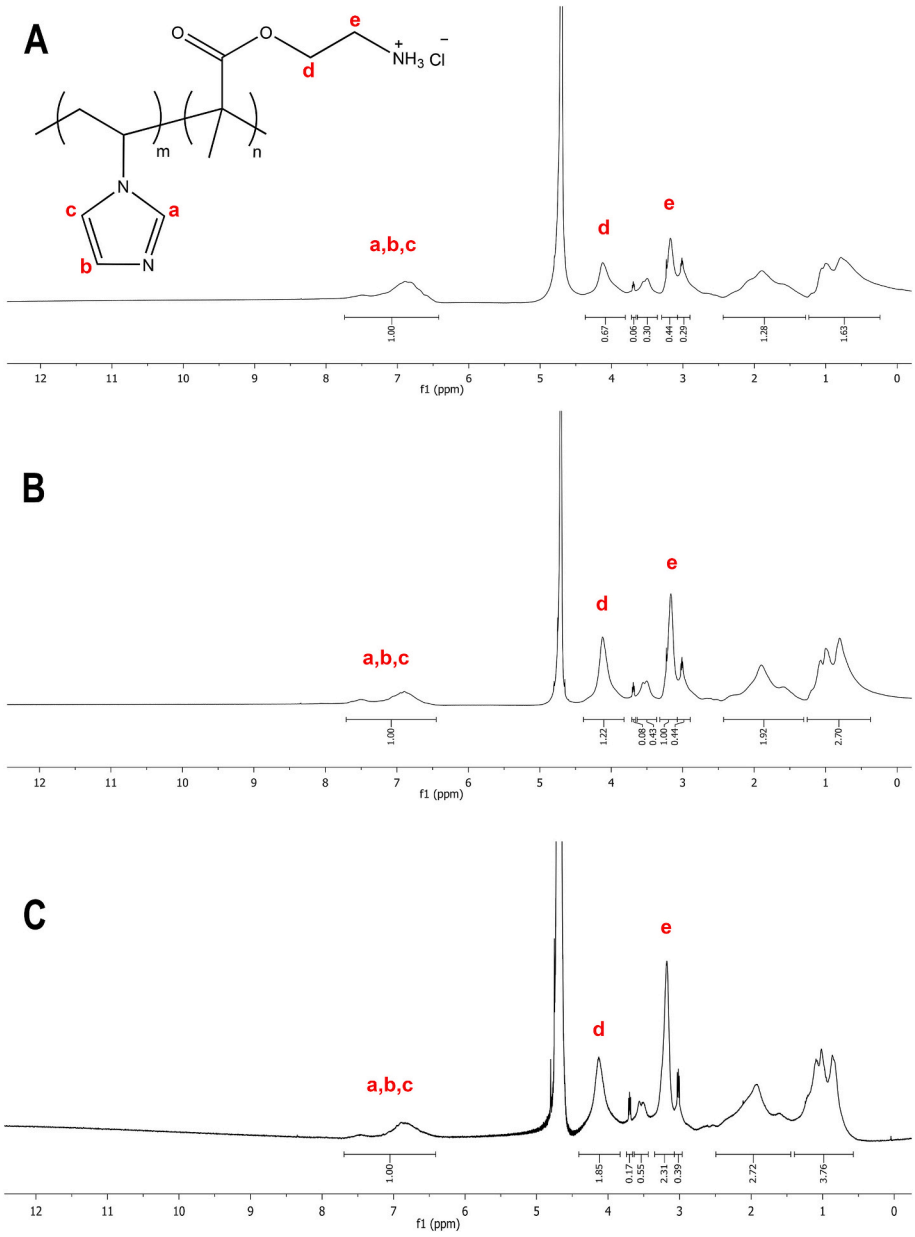

Fig. 2. NMR spectra of (A) AV, (B) A2V and (C) A3V copolymers.

Table 2
Characteristics of synthesized copolymers (A: 2-aminoethyl methacrylate and V: 1-vinylimidazole).

Copolymer	Feed molar ratio of 2-aminoethyl methacrylate to 1-vinylimidazole	Actual molar ratio	M_w (Da)	M_n (Da)	Polydispersity index (PDI)
AV	1	1.02	16,338	13,764	1.187
A2V	2	1.83	8863	7410	1.196
A3V	3	2.8	11,783	9844	1.197

of AV/pDNA and A2V/pDNA compared to A3V/pDNA. The imidazole ring of 1-vinylimidazole is capable of creating intramolecular hydrogen bonds with amine groups of 2-aminoethyl methacrylate, which probably resulted in conformational changes and subsequently less amine groups were exposed at the surface of the nanoparticles to provide the polyplexes with the positive charge (Zakharova et al., 2024).

3.6. Gel retardation assay

Gel electrophoresis was done to assess the capacity of the synthesized copolymers to condense pDNA efficiently. Fig. 4A depicts the effect of N/P ratio on DNA binding ability of each copolymer. AV, A2V and A3V polyplexes showed resembling condensation patterns and they were capable of forming complexes with DNA and immobilizing it from N/P ratio of 1 to 16, while free DNA bands were observed at N/P ratio of 0.25. The results indicated that the capability of all tested copolymers in retarding DNA mobilization increased by increasing N/P ratio and amount of copolymer (Kim et al., 2003; Lu et al., 2015; Wang et al., 2016). Moreover, this pattern was in line with polyplexes zeta potential results where the surface charge of nanoparticles shifted to positive charge and increased by increasing copolymer content and N/P ratio. As the amount of copolymer increased, more amine groups were available to interact with fixed amounts of phosphate groups of DNA plasmid and the resulting nanoparticles were capable of impeding electrophoretic

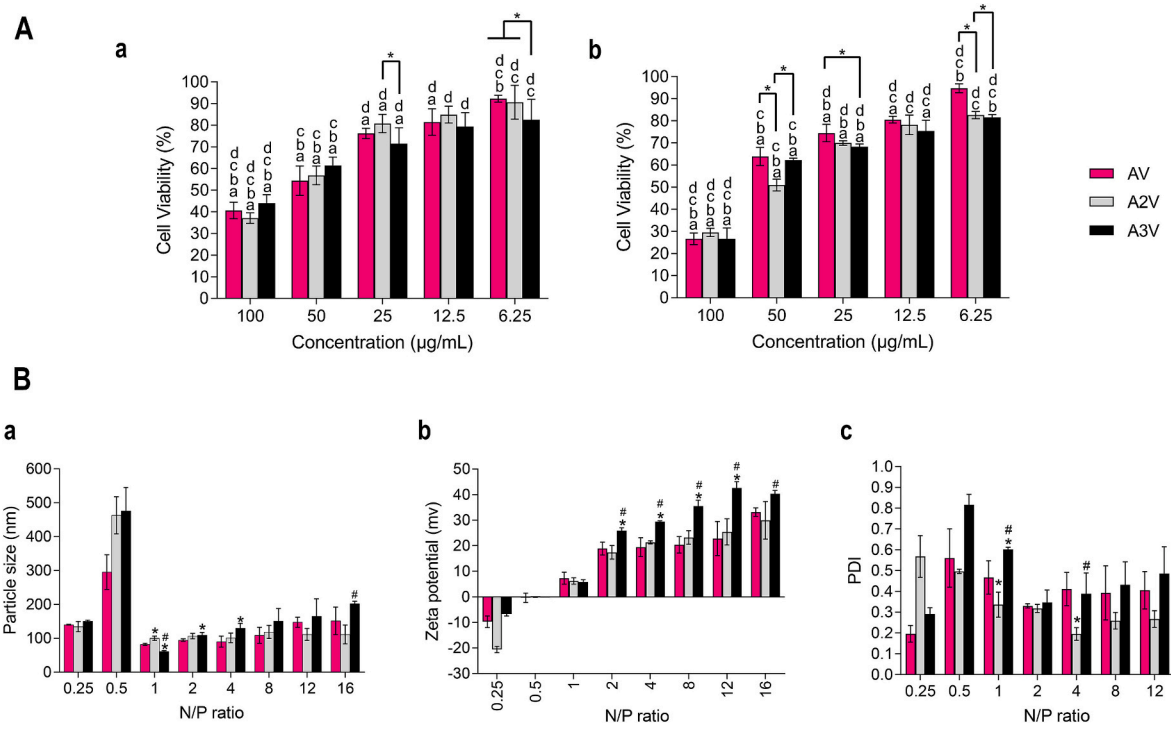

Fig. 3. (A) Cell viability of a) L929 and b) HeLa cells after 24 h of incubation with different copolymers at concentrations of 6.25, 12.5, 25, 50 and 100 μg/mL. For each copolymer: [a] $p < 0.05$ *vs* the cell viability at copolymer concentration of 6.25 μg/mL, [b] $p < 0.05$ *vs* the cell viability at copolymer concentration of 12.5 μg/mL, [c] $p < 0.05$ *vs* the cell viability at copolymer concentration of 25 μg/mL, [d] $p < 0.05$ *vs* the cell viability at copolymer concentration of 50 μg/mL and * shows $p < 0.05$, and (B) physical properties including a) particle size, b) zeta potential and c) PDI of AV/pDNA, A2V/pDNA and A3V/pDNA polyplexes prepared at different N/P ratios. *$p < 0.05$ *vs* AV/pDNA polyplex at each N/P ratio and [#] $p < 0.05$ *vs* A2V/pDNA polyplex at each N/P ratio.

mobility of plasmid on gel.

3.7. Optimum N/P ratios

The results of particle size and zeta potential measurement and gel retardation assessments were evaluated to determine the optimized N/P ratio for preparation of AV/pDNA, A2V/pDNA and A3V/pDNA polyplexes to be used in further experiments. Particle size and surface charge of the nanoparticles are critical factors that determine their biodistribution, half-life in blood circulation, cell membrane interaction and cellular uptake. Nanoparticles smaller than 10 nm will be cleared off rapidly by kidney filtration and those larger than 200 nm will be uptaken by RES. Furthermore, it was shown that nanoparticles smaller than 150 nm had efficient cellular uptake and transfection ability (Diana and Rekha, 2017; Shang et al., 2014). Regarding zeta potential, polyplexes possessing positive charge can affect the cell membrane function by interacting with its proteoglycans and stimulate cellular uptake (Augustine et al., 2020; Saadat et al., 2019). Additionally, nanoparticles with surface charges of ±15–30 mV are considered colloidally stable (Sohail et al., 2022).

Based on above mentioned criteria, polyplexes with positive charge in the range of 15–30 mV, appropriate size distribution and particle size capable of immobilizing DNA were opted as optimized polyplexes. For all copolymers, the obtained polyplexes at $N/P > 2$ might be positive enough to adhere to the negative charge cell membrane and facilitate the uptake process of polyplexes. Furthermore, they had zeta potentials greater than +15 mV, which guaranteed their stability. Statistical analysis demonstrated that for all polyplexes the particle size, PDI and zeta potential did not change significantly when N/P ratios increased from 2 to 8 ($p > 0.05$). The polyplexes of N/P 2–16 retarded DNA mobilization efficiently. Moreover, the increase in the copolymer content in polyplexes can affect the release of genetic material and makes it difficult for the plasmid to be released from polyplexes. Additionally, at N/P ratio of 2, the nanocarriers were smaller than 120 nm and they were compact enough to be uptaken by cells through endocytosis (Chen et al., 2019). Hence, the N/P ratio of 2 was selected as the optimum for preparation of AV/pDNA, A2V/pDNA and A3V/pDNA polyplexes.

3.8. Heparin displacement assay

To investigate the stability of polyplexes and their efficacy in retaining their integrity against extracellular polyanions, optimal N/P ratio of each copolymer was incubated with varying amounts of heparin (0.5–16 *w*/w) for 1 h at 37 °C and the mixture was run on agarose gel. Fig. 4B indicates that polyplexes prepared from all copolymer types could maintain the DNA in competition with heparin at all weight ratios and no free DNA band was observed. The results demonstrated that all polyplexes had biological resistance over polyanions like heparin sulfate, which was in line with the results of stability of poly(1-vinylimidazole) polyplexes that were stable up to the heparin/siRNA ratio of 3 (*w*/w), while our present polyplexes were more stable and they kept their integrity when the heparin/DNA ratio reached 16 (w/w). This might be due to the presence of amine groups of 2-aminoethyl methacrylate units in copolymers that led to more efficient condensation of the plasmid by establishing more stable electrostatic interactions at neutral pH (Kandasamy et al., 2020).

3.9. Serum stability test

Prepared polyplexes were incubated with FBS at a final concentration of 50 % up to 48 h to evaluate the efficacy of designed vectors in protecting DNA against serum components and nucleases. All the polyplexes maintained their integrity and stability for two days (Fig. 5A), while the naked plasmid was degraded after 1 h and the free DNA smear band became faded up to 24 h and disappeared after 48 h (Fig. 5A). The results indicated that all the polyplexes were efficient in preserving the

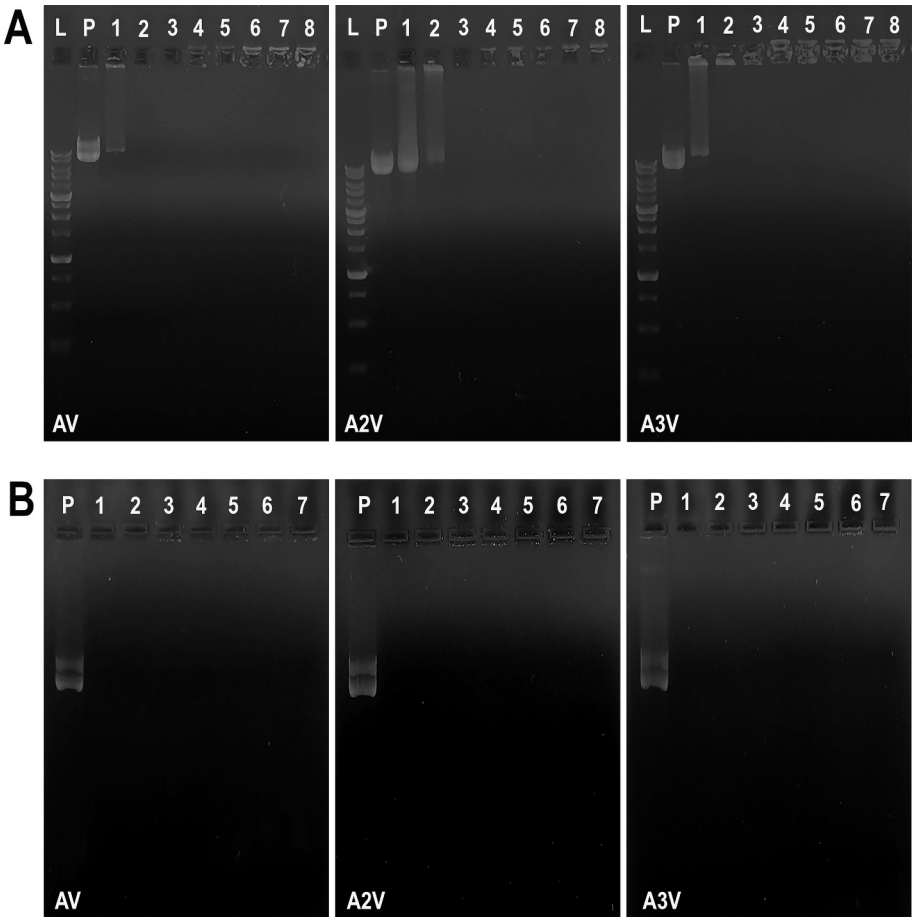

Fig. 4. (A) Gel retardation assay of different polyplexes. Lane 1: DNA ladder, Lane 2: naked plasmid, Lanes 3–10: polyplexes prepared at N/P ratios of 0.25, 0.5, 1, 2, 4, 8, 12 and 16, respectively, (B) Heparin displacement assay of different polyplexes. Lane 1: naked plasmid, Lane 2: optimum polyplexes without addition of heparin, Lanes 3–8: heparin: plasmid weight ratios of 0.5, 1, 2, 4, 8 and 16, respectively.

pDNA in presence of 50 % serum for 48 h and they may have stability in biological fluid against serum proteins and enzymes. The obtained results agree with Chou et al. (Chou et al., 2014) findings who demonstrated that histidine-lysine peptide polyplexes showed considerable robustness to 50 % serum incubation up to 24 h owing to non-ionic interaction of imidazole moieties of histidines with siRNA.

3.10. DNase I protection assay

In order to assess the protection efficiency of polyplexes against nuclease enzymes like DNase I, they were incubated with DNase I (0.5 IU) for 15, 30, and 60 min at 37 °C and then were run on agarose gel. Fig. 5B represents the digestion results of the polyplexes and naked plasmid as control. The naked plasmid was digested thoroughly after 15 min and there was no band of free DNA. On the contrary, AV/pDNA, A2V/pDNA and A3V/pDNA polyplexes sheltered the loaded plasmid against DNase I and were retained at the baseline well like undigested polyplexes. These results were in consistency with gel retardation and serum protection assay results (Fig. 4A and 5A) indicating that all three types of polyplexes at N/P ratio of 2 condensed DNA completely and protected the cargo against DNaseI, the serum nuclease, efficiently, which stemmed from the perfect entanglement of DNA with the synthesized copolymers that hindered its accessibility to enzyme (Dehshahri et al., 2022; Jiang et al., 2007).

3.11. Scanning electron microscopy (SEM)

SEM was performed to evaluate the morphology and particle size of the optimum polyplexes obtained from each copolymer (Fig. 6A, B, C). The images indicate that the copolymers formed complexes with pDNA in spherical shape that demonstrated efficient pDNA condensation. Most of the particles had a particle size in the range of 90–120, 80–120 and 100–135 nm for A2V/pDNA, AV/pDNA and A3V/pDNA, respectively (Fig. 6D, E, F). However, the aggregates observed in A3V/pDNA (Fig. 6C) are due to the aggregation that occurred during the drying process in sample preparation before imaging. The resultant particle sizes were in consistency with DLS measurements, which confirms suitability of the produced polyplexes sizes (less than 150 nm) and can assist better cellular uptake of the carriers (Modra et al., 2015).

3.12. Cytotoxicity of polyplexes

To assess to cytotoxicity effect of the optimum polyplex of each copolymer on HeLa cells, MTT assay was conducted. As depicted in Fig. 7A, the viability of treated cells was not declined under 70 % and the polyplexes are considered nontoxic to HeLa cells according to ISO 10993-5:2009 guideline (Iso, 2009). The cell viability of AV/pDNA polyplexes treated cells was higher than A2V/pDNA and A3V/pDNA polyplexes treated ones ($p < 0.05$) and the obtained results are in consistency with Mapfumo et al. (Mapfumo et al., 2024) study. They reported that cytotoxicity of polyplexes composed of histidine containing copolymers decreased by increasing the histidine content owing to the fact that imidazole group of histidine was not protonated at physiological pH and subsequently it did not affect cell membrane integrity and cell viability.

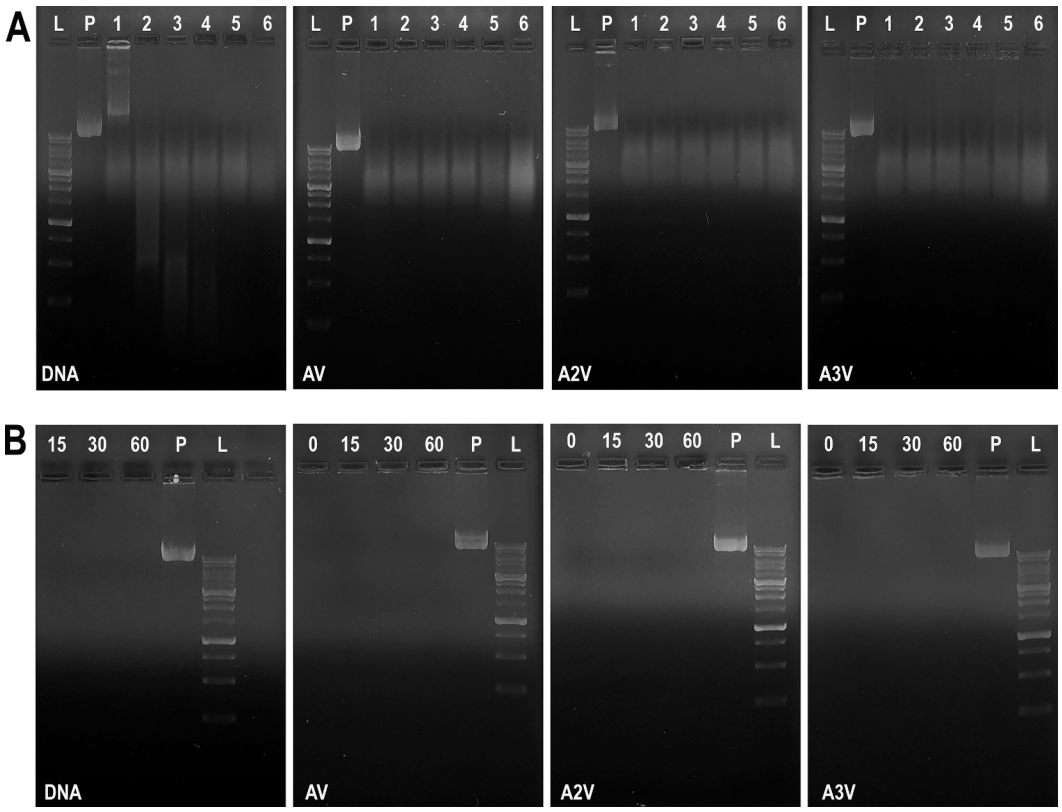

Fig. 5. (A) Serum stability of naked plasmid and optimum polyplexes. Lane 1: DNA ladder, Lane 2: naked plasmid, Lanes 3–8: after incubation with 50 % FBS at 37 °C for 0, 1, 3, 6, 24 and 48 h and (B) DNase I protection assay of naked plasmid and optimum polyplexes after 15 (Lane 1), 30 (Lane 2) and 60 min (Lane 3) of incubation with DNase I (0.5 IU) at 37 °C. P (Lane 4): plasmid without DNase I addition, L (Lane 5): DNA ladder.

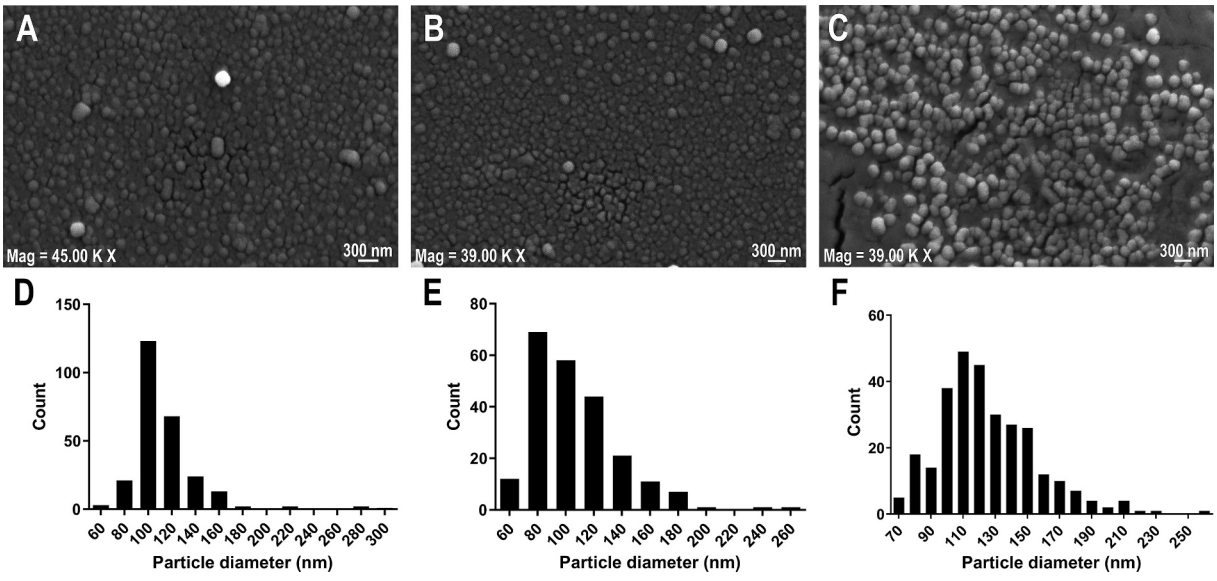

Fig. 6. SEM images and particle size distribution histograms of optimum (A, D) A2V/pDNA, (B, E) AV/pDNA and (C, F) A3V/pDNA polyplexes.

3.13. Transfection efficiency

Investigating transfection efficacy of copolymers compared to DNAfectamin was carried out by flow cytometry. The data indicated that DNAfectamin, AV/pDNA, A2V/pDNA and A3V/pDNA polyplexes transfected HeLa cells 45.2 %, 21.8 %, 10 % and 11 %, respectively (Fig. 7B, C and D). The naked plasmid treated group transfected 5.02 % of cells. The transfection efficiency of polyplexes were higher than naked plasmid indicating that non-condensed DNA did not have the ability to overcome barriers like nucleases and cell membrane without aid of gene delivery vectors (Bai et al., 2017; Yang et al., 2008). In addition, by increasing the vinyl imidazole/ aminoethyl methacrylate ratio from A3V/pDNA to AV/pDNA, the transfection efficiency increased possibly due to the fact that the higher amount of primary amine moieties in A2V and A3V copolymers led to its stronger binding to the genetic material, thereby hindering its dissociation from the polyplex, which resulted in

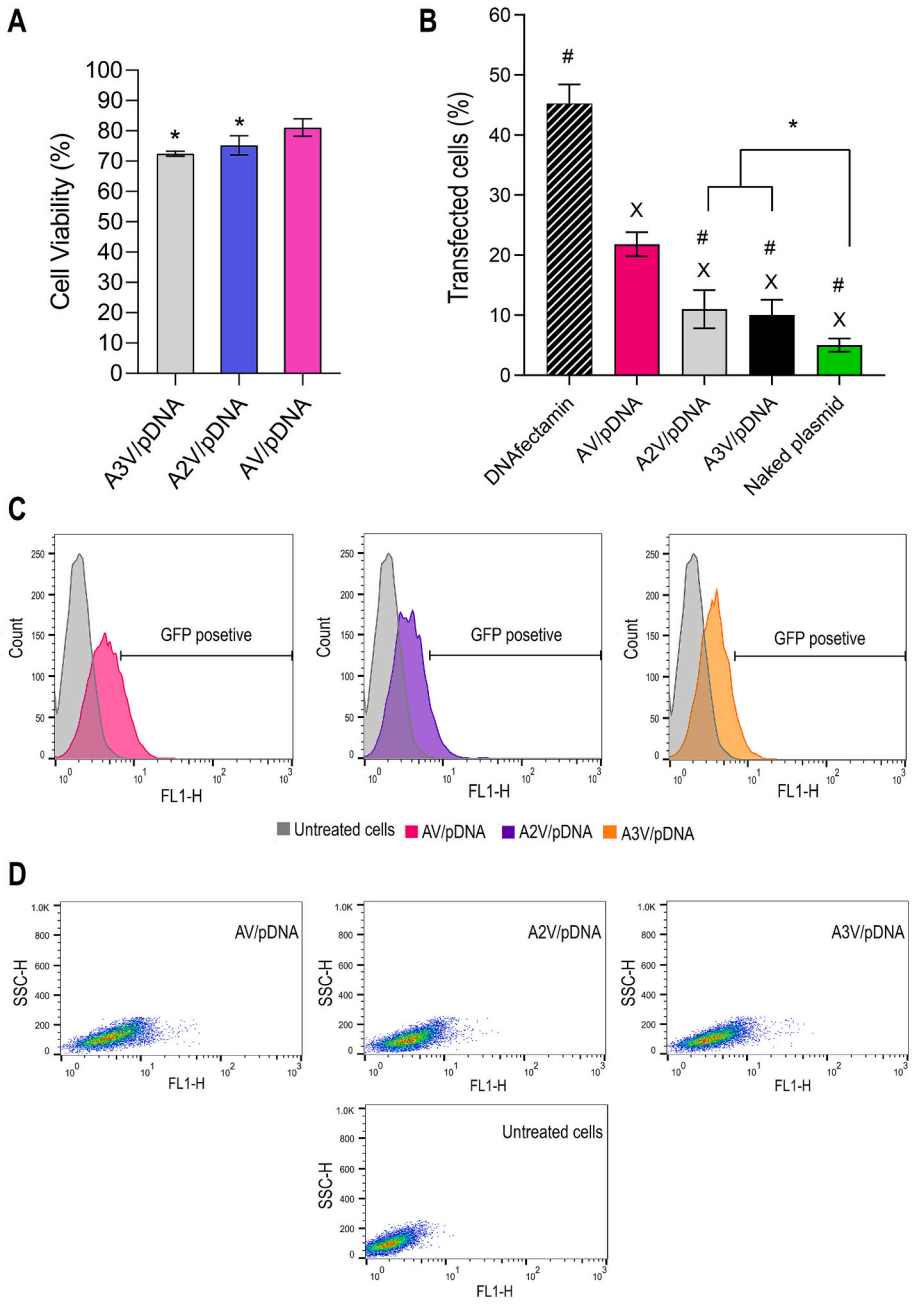

Fig. 7. (A) Cell viability of HeLa cells after 48 h of incubation with optimum polyplex of each copolymer (containing 1 μg plasmid DNA). * shows significant difference ($p < 0.05$) with AV/pDNA group. (B) Percentage of transfected HeLa cells, determined by measurement of GFP expression using flow cytometry. X shows $p < 0.05$ compared to DNAfectamin group, # shows $p < 0.05$ compared to AV/pDNA group and * symbol shows significant difference ($p < 0.05$) between the compared groups on the fig. (C, D) Flow cytometry histograms and scatter plots of transfected cells by optimum polyplex of each copolymer, respectively.

lower transfection efficiency of A2V/pDNA and A3V/pDNA (Smith et al., 2011). Furthermore, the lower buffering capacity of A2V and A3V because of their lower vinyl imidazole content might be another reason for the less transfection efficiency observed for A2V/pDNA and A3V/pDNA compared to AV/pDNA polyplexes (Barua et al., 2011). The obtained results are in line with Kim et al. (Kim et al., 2003) study who enhanced transfection efficiency of urocanic acid-modified chitosan polyplexes. It was reported that by increment in urocanic acid (bearing imidazole ring) substitution the buffering capacity of polyplexes enhanced and subsequently more efficient endosomal escape happened. Furthermore, Q. Xia et al. (Xia et al., 2024) demonstrated that imidazole content had a vital role in transfecting cells and when the grafting ratio of imidazole containing group / ionizable amine containing group in Poly(succinimide) polymer was 45 %:28 % or 60 %:13 %, the highest transfection efficiency was achieved in HEK293 cells. Moreover, as another reason, the superior transfection performance of AV/pDNA polyplexes compared to A2V/pDNA and A3V/pDNA complexes was probably owing to the lower cell toxicity of AV copolymer and optimum AV polyplexes based on MTT test results.

The highest transfection efficiency was observed in the group treated with DNAfectamin as positive control. The manufacturer claims that the DNAfectamin consists of polycations and liposomes to enhance its efficiency. Thus, the findings suggest that the higher number of transfected cells in DNAfectamin treated group compared to polyplexes was possibly due to its different composition and subsequently its different pathway of cellular uptake, endosomal escape and cell trafficking (Pichon et al.,

2010; Zarei et al., 2021). Moreover, it has been demonstrated that, the liposomal reagents exhibit superior transfection efficiency compared to non-liposomal ones in some specific cell lines including HeLa, HEK293, HepG2, MCF-7, MDA-MB-231 (Chong et al., 2021a, 2021b). For instance, polylactic-*co*-glycolic acid (PLGA)/cetylated PEI/hyaluronic acid nanoparticles (PCPH NPs) exhibited lower transfection efficiency than Lipofectamine 2000 in HEK293 cells (Zhu et al., 2016). Furthermore, poly-L-lysine–b–poly[N-(2-hydroxypropyl)-methacrylamide)] (poly[HPMA]) block copolymer polyplexes and fuGeneHD (liposomal reagent) transfected 12 % and 70 % of HEK393 cells, respectively (Tappertzhofen et al., 2015). In addition, it has been demonstrated that transfection efficiency of non-viral carriers is dependent on cell type and the superior transfection of the positive control over polyplexes in HeLa cell line did not necessarily imply its surpassing intrinsic capability in gene transfection in all other cell types (Breunig et al., 2007).

4. Conclusions

Copolymerization of 2-aminoethyl methacrylate and 1-vinylimidazole at different molar ratios was successfully carried out by radical polymerization without employing complicated methods like RAFT polymerization. All resultant polycations were capable of pDNA condensation and the prepared polyplexes exhibited great stability against heparin, serum and DNase I. AV/pDNA, A2V/pDNA and A3V/pDNA polyplexes presented different degrees of transfection efficiency. Furthermore, AV polymer had lower cytotoxicity than other copolymers and the AV/pDNA polyplexes showed higher cell transfection efficiency compared to A2V/pDNA and A3V/pDNA polyplexes indicating the great potential of AV copolymer to be used as a novel non-viral gene carrier in future. One of the strengths of the present study is employing 1-vinyl imidazole along with 2-aminoehtyl methacrylate to produce a novel biocompatible copolymer capable of forming a stable complex and preventing unwanted DNA dissociation in neutral or physiologic pHs unlike 1-vinylimidazole homopolymer. Also, the resultant polyplexes are physiologically resistance against enzyme digestion, and serum polyanions. In addition, some of the limitations of this study that are suggested to be considered in further studies include: 1) evaluating the transfection efficiency of the copolymer polyplexes in other cell lines, 2) addition of targeting agents to the polyplex structure to enhance cellular uptake and subsequently transfection efficiency of the nanocarriers and 3) assessment of *in vivo* transfection level of the copolymer polyplexes.

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CRediT authorship contribution statement

Sahel Soghrati: Formal analysis, Investigation, Methodology, Software, Writing – original draft. **Jaleh Varshosaz:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition, Conceptualization. **Mahboubeh Rostami:** Data curation, Formal analysis, Methodology. **Mina Mirian:** Formal analysis, Methodology. **Fariborz Sharifianjazi:** Project administration. **Ketevan Tavamaishvili:** Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data will be made available on request.

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