**Functionalized Folic Acid with Chitosan and PAMAM Dendrimers for Delivery of DNA and RNA**

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**Abstract**

In this review, we explore the potential applications of functionalized folic acid-polymer nanoparticles in nucleic acids delivery. Folic acid-chitosan (carbohydrates) and folic acid-PAMAM (dendrimers) of different polymer sizes were used to conjugate with DNA and tRNA in vitro. Thermodynamic analysis showed that conjugation of DNA and tRNA to nanoparticles occurred via hydrophilic, hydrophobic, H-bonding and van der Waals contacts. Major alterations of DNA and RNA morphology occurred by nanocarrier interaction. These results indicate that functionalized folic acid-nanoparticles can deliver DNA and RNA to target sites.

**Keywords:** DNA, tRNA, Gene delivery, Folic acid-polymer, Thermodynamics, Transmission electron microscopy

**Introduction**

The conjugation of polymers with multiple targeting ligands has become a popular approach for targeted gene and drug delivery [1,2]. Functionalized polymer–drug conjugates are increasingly used to obtain biodegradable, targeted tools to further enhance localized gene and drug delivery systems [1-3]. Folic acid (FA)-conjugated biodegradable polymers were tested as effective gene and drug delivery tools [4-7]. Folate receptors are cellular markers highly expressed in various cancer cells and on the surface of activated macrophages [8-12]. Chitosan (Ch), (1–4)-2-amino-2-deoxy-β-D-glucan, is a polysaccharide obtained from alkaline hydrolysis of chitin, one of the most abundant natural amino polysaccharides extracted from the exoskeleton of crustaceans and insect, from fungal cell walls, etc. [13,14]. There are amine groups (-NH₂) and hydroxyl groups (-OH) along the chitosan chain, which can be used as cross-linkable functional groups to react with cross-linking agents for in-situ chemical cross-linking [13]. In addition, these amino groups can be protonated below pH 6.3 and hence chitosan can interact with DNA/RNA phosphate groups in an electrostatic manner. Dendrimers are highly branched three-dimensional molecules, with defined molecular weight and a large number of controllable peripheral functionalities [15,16]. Polyamidoamines (PAMAMs) were historically the first class of dendrimers to be synthesized [17]. The peripheral –NH₂ groups of PAMAM can also interact with DNA/RNA phosphate groups by electrostatic forces [18]. The interaction of charged amino and imino groups with DNA/RNA phosphate groups is known to provoke DNA/RNA condensation to nanoparticles [19,20]. Since DNA/RNA transport through the cell membrane is an inefficient process, their condensation to nanoparticles is important to facilitate DNA delivery.

The application of dendrimers in gene delivery has been recently reviewed [21]. The bundling and aggregation of DNA by PAMAM is known [22-26]. Conjugated folic acid with synthetic and natural polymers has been extensively used in gene and drug delivery systems [27]. A gene delivery
system based on folic acid-polyethylene glycol (PEG)-chitosan-PAMAM was used for cancer cell targeting [28]. The conjugation of DNA with chitosan and folic acid was recently reported [29,30]. Chitosan-based formulation for the delivery of DNA and RNA is known [31]. Chitosan is widely used as a gene delivery vehicle due to its ability to condense DNA, facilitate transport and subsequently release plasmid DNA, allowing gene expression [32,33]. The fabrication and structural characterization of the functionalized folic acid-PAMAM and folic acid-chitosan complexes have been recently reported [34,35].

Due to the major applications of folic acid-polymer conjugates in gene and drug delivery systems, we are reviewing recent studies on the encapsulation of DNA and tRNA by functionalized folic-acid-polymer conjugates here. In this review, the loading efficacies of DNA and tRNA by folic acid-PAMAM (G3 and G4) and folic acid-chitosan (15 and 100 kDa) nanoparticles are reported, using spectroscopic, thermodynamic and transmission electron microscopy (TEM) image analysis. Thermodynamic analysis of the complexation process can provide the necessary physical chemical data on the stability of nanoparticles with potential biotechnological applications.

Stability of DNA and RNA Conjugates with Folic Acid-polymer Nanoparticles

DNA and tRNA interactions with folic acid-PAMAM and folic acid-chitosan complexes induced major alterations of the folic acid-polymer absorption spectra. The observed changes were used to calculate the binding constants of DNA and tRNA complexes with folic acid-polymer nanoparticles. The ultraviolet (UV) spectra of DNA and tRNA with polymer nanoparticles are shown in Figures 1-4. DNA and tRNA complexation occurred with an increase in the folic acid-polymer absorption band at 260 nm. The DNA and tRNA binding constants were calculated as previously reported [36] and the results are shown in Figures 1-4 and Table 1. These results showed that more stable DNA/tRNA-FA-polymer conjugation occurred as FA-PAMAM and FA-Ch sizes increased (from G3 to G4 and chitosan-15 to chitosan-100 kDa), with an order of stability of FA-PAMAM-G4 > FA-PAMAM-G3 as well as FA-Ch-100 > FA-Ch-15 (Table 1). The increased stability of FA-PAMAM-G4 is related to the presence of additional terminal charged -NH_2 groups on PAMAM-G4 (64 groups) as compared to PAMAM-G3 (32 groups) and that of FA-Ch-100 compared with FA-Ch-15, as these charged amino groups are involved in biomolecular interactions. Evidence

Figure 1: UV-visible spectra of folic acid-PAMAM and their DNA conjugates (pH 7.2) with FA-PAMAM-G3 (A) and FA-PAMAM-G4 (B), with polymer 60 µM (a) and its DNA complexes (b-m) with DNA at 1, 3, 5, 10, 20, 30, 40, 50, 60, 70 and 80 µM. Inset: plot of 1/(A-A_0) vs (1/ DNA concentration) and binding constants (K) for DNA-acid-PAMAM conjugates.
Figure 2: UV-visible spectra of folic acid-PAMAM and their tRNA conjugates (pH 7.2) with FA-PAMAM-G3 (A) and FA-PAMAM-G4 (B) with polymer 60 µM (a) and its tRNA complexes (b-m) with tRNA at 1, 3, 5, 10, 20, 30, 40, 50, 60, 70 and 80 µM. Inset: plot of 1/(A-A₀) vs (1/ tRNA concentration) and binding constants (K) for tRNA-acid-PAMAM conjugates.

Figure 3: UV-visible spectra of folic acid-chitosan and their DNA conjugates (pH 7.2) with FA-chotsan-15 (A) and chitosan-100 kDa (B) with folic acid-chitosan at 60 µM (free acid) (a) and its DNA complexes (b-i) for chitosan-15 at 1, 5, 10, 20, 30, 40, 50 and 60 µM and (b-i) for chitosan-100 kDa at 1, 5, 10, 20, 30, 40, 50 and 60 µM. Inset: plot of 1/(A-A₀) vs (1/ DNA concentration) and binding constants (K) for DNA-acid-chitosan conjugates.
regarding hydrophobic, hydrophilic or H-bonding interactions comes from the thermodynamic analysis of DNA/tRNA-FA-polymer conjugates, as discussed in the next section.

**Thermodynamics of DNA/tRNA Binding to Folic Acid-polymer Nanoparticles**

Based on thermodynamic analysis, the $\Delta G$, $\Delta H$ and $\Delta S$ of the nature of DNA/tRNA-FA-polymer interactions can be determined [37,38]. The thermodynamic parameters for the interaction of DNA and tRNA with folic acid-polymer conjugates at 298.15 K are presented in Table 2. The negative sign of $\Delta G$ shows that the binding process between DNA/tRNA with FA-polymer conjugate is spontaneous. Furthermore, all the DNA/tRNA-FA-polymer nanoparticles have negative $\Delta H$, which means that the complex formation between DNA and tRNA and FA-polymer complex is an exothermic reaction. The negative $\Delta H$ and negative $\Delta S$ for DNA/tRNA-FA-polymer nanocarrier show that H-bonding and van der Waals interactions are prevailing in the complex formation (Table

![Figure 4: UV-visible spectra of folic acid-chitosans and their tRNA conjugates (pH 7.2) with acid-chitosan-15 (A) and acid-chitosan-100 kDa (B) with folic acid-chitosan at 60 µM (free conjugate) (a) and its tRNA complexes (b-m) for acid-chitosan-15 at 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µM and (b-m) for acid-chitosan-100 kDa at 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µM. Inset: plot of $1/(A-A_0)$ vs (1/ tRNA concentration) and binding constants ($K$) for tRNA-acid-chitosan conjugates.](image-url)
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| Complexes                          | $\Delta H^0$ (kJ.mol$^{-1}$$\pm$2) | $\Delta S^0$ (J.mol$^{-1}$$\pm$2) | $\Delta G^0$ (kJ.Mol$^{-1}$$\pm$2) |
|-----------------------------------|------------------------------------|-----------------------------------|------------------------------------|
| DNA-folic-acid-PAMAM-G3           | -14.56                             | -0.70                             | 14.35                              |
| DNA-folic-acid-PAMAM-G4           | -19.66                             | -17.59                            | -14.41                             |
| tRNA-folic-acid-PAMAM-G3          | -13.19                             | 4.37                              | -14.50                             |
| tRNA-folic-acid-PAMAM-G4          | -14.36                             | 0.92                              | -14.64                             |
| DNA-folic-acid-chitosan-15         | -12.00                             | 8.83                              | -14.63                             |
| DNA-folic-acid-chitosan-100        | -17.54                             | -9.43                             | -14.73                             |
| tRNA-folic-acid-chitosan-15        | -10.07                             | 13.97                             | -14.24                             |
| tRNA-folic-acid-chitosan100        | -14.72                             | -1.16                             | -14.37                             |

Table 2: Thermodynamic parameters for DNA/tRNA-folic-acid-polymer conjugates at 398.15 K.

The loading efficacy for DNA and tRNA to FA-polymer conjugates was determined, as previously reported [39]. The loading efficacy was estimated to be 35-50% (FA-PAMAM-G3 and FA-Ch-15), which increased to 50-55% (FA-PAMAM-G4 and FA-Ch-100), in DNA/tRNA-FA-polymer nanoconjugates (Table 1). This result shows the important role of polymer size in DNA and RNA-nanocarrier interactions.

**Effect of Folic Acid-polymer Conjugation on DNA and tRNA Morphology**

The morphological changes of DNA and tRNA by folic acid-polymer nanoparticles were monitored, using TEM. The TEM analysis of the free FA-PAMAM-G3 and FA-PAMAM-G4 and their DNA and tRNA conjugates in aqueous solution at pH 7.2 are shown in Figures 5 and 6. The TEM images of the free DNA and tRNA show major spherical aggregates, with particle sizes ranging from 3 to 10 nm with a mean diameter of 5 to 6 nm (Figures 6A and 6A), which is in agreement with literature reports [40-45]. Marked differences were also observed in the morphology of the FA-PAMAM aggregates. TEM images showed the appearance of irregular shaped aggregates dispersed in solutions of FA-PAMAM-G3 and FA-PAMAM-G4 (Figure 5B and 5C) [35]. Upon addition of DNA and tRNA to FA-PAMAM conjugates, DNA and tRNA aggregates became more evident in the TEM images (Figures 5 and 6D and E), revealing that the conjugation of DNA and tRNA by FA-PAMAM caused an increase in DNA/tRNA aggregation. The aggregate size analysis showed a major increase in the diameter of DNA and tRNA aggregates (Figures 5 and 6D and E). The DNA and tRNA aggregate formation were more pronounced in FA-PAMAM-G4 than that in FA-PAMAM-G3, indicating more perturbations of nucleic acid structures by higher generation PAMAM (Figures 5 and 6D and E). Similar structural changes were observed upon testosterone conjugation with DNA and tRNA [46,47].

In the presence of folic acid-chitosan nanoparticles, major changes were also observed in the TEM images of DNA and tRNA aggregates. The TEM images of DNA and tRNA, in the presence and absence of folic acid-chitosan conjugates in aqueous solution at pH 7.2, are shown in Figures 6 and 7. The TEM photographs of the free DNA and tRNA exhibit major spherical aggregates, with the particle size ranging from 3 to 10 nm with a mean diameter of 5 to 6 nm (Figures 6A and 7A), which is in agreement with other reports [34,48,49]. Marked differences were also observed in the morphology of the acid-chitosan conjugates.

TEM photographs showed the appearance of irregular shaped aggregates dispersed in solutions of Ch-15 and Ch-100 kD conjugated with folic acid (Figures 6B and 7B). The conjugated Ch-15 and Ch-100 with folic acid exhibit major changes in polymer morphology [34]. Upon addition of DNA and tRNA to folic acid-chitosan conjugates, DNA and tRNA aggregates became more evident in the TEM images (Figures 6D and 7E), revealing that the conjugation of DNA and tRNA by folic acid-chitosan nanoparticles caused an increase in the DNA and tRNA aggregation. The aggregate size analysis showed a major increase in diameter of DNA aggregates (Figures 6D and E). It is important to note
Figure 5: TEM images showing the morphology of DNA (A) with folic acid-PAMAM-G3 (B) and folic acid-PAMAM-G4 (C) and their DNA conjugates (D and E) at pH 7.2 at 24°C. The concentrations of DNA and folic acid–PAMAM were 60 µM in all samples.

Figure 6: TEM images showing the morphology of tRNA (A) with folic acid-PAMAM-G3 (B) and folic acid-PAMAM-G4 (C) and their tRNA conjugates (D and E) at pH 7.2 at 24°C. The concentrations of tRNA and folic acid–PAMAM were 60 µM in all samples.
that conjugation of folic acid-polymer nanocarrier also induced major morphological changes on DNA and tRNA structures (Figures 5-7).

**Conclusions and Outlook**

Based on thermodynamic analysis, the $\Delta G$, $\Delta H$ and $\Delta S$ of the nature of DNA/tRNA-FA-polymer interactions can be determined [37,38]. The thermodynamic parameters for the interaction of DNA and tRNA with folic acid-polymer conjugates at 298.15 K are presented in Table 2. The negative sign of $\Delta G$ shows that the binding process between DNA/tRNA with FA-polymer conjugate is spontaneous. Furthermore, all the DNA/tRNA-FA-polymer nanoparticles have negative $\Delta H$, which means that the complex formation between DNA and tRNA and FA-polymer complex is an exothermic reaction. The negative $\Delta H$ and negative $\Delta S$ for DNA/tRNA-FA-polymer nanocarrier show that H-bonding and van der Waals interactions are prevailing in the complex formation (Table 2). However, hyrophobic and H-bonding contacts are also observed in the case of negative $\Delta H$ and positive $\Delta S$ (Table 2).

The conjugation of polymer with multiple targeting ligands has become a popular approach for targeted gene and drug delivery [1-3]. Folic acid-conjugated with biodegradable polymers were tested as effective gene and drug delivery tools [4-12]. These multivalent polymers have great utility in controlled release and targeting studies of different bioactive molecules. Chitosan and PAMAM dendrimers and their functionalized folic acid nanoparticles were often used for drug and gene delivery [50-63]. DNA, RNA and drug bindings to functionalized folic acid-polymer nanocarrier occurred via hydrophilic, hydrophobic, H-bonding and van der Waals contacts. As polymer size increased, the stability and loading efficacy of nucleic acids and drug-polymer conjugation also increased. Major alterations of DNA and RNA morphology were observed upon nanocarrier complexation, as the condensation of DNA by these and other ligands facilitate cellular transport [64-67]. These results show that functionalized folic acid-polymer conjugates can be used to deliver DNA and RNA to target sites. Future research should be focused on the conjugation of polymers with multiple targeting ligands to develop effective functionalized nanocarrier for targeted gene and drug delivery systems.

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Figure 7: TEM images showing the morphology of DNA (A) with folic acid-chitosan-15 (B) and folic acid-chitosan-100 kDa (C) and their DNA conjugates (D and E) at pH 7.2 at 24°C. The concentrations of DNA and folic acid -chitosan were 60 μM in all samples.
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