INTRODUCTION

Urinary tract infections (UTIs) are some of the most common bacterial infections, affecting 150 million people each year worldwide. As resistance to antibiotics by bacteria has become more common, there is a need to lower dosages and reduce side effects using alternative treatments such as targeted drug delivery with nanoparticles (Foxman, 2014). Due to a small size (<200 nm) and large surface area, nanoparticles can pass through the cell walls of the living cells and efficiently deliver drugs to the cells, resulting in a reduction of side effects.
effects and cost of specific medications. To be used as a platform for targeted delivery of drugs, nanoparticles must also be functionalized, have a low toxicity profile and must be biocompatible (Faraji & Wipf, 2009; Thanh & Green, 2010). Nanodiamond particles (NDPs) offer several medically significant advantages, such as chemical inertness, optical transparency and biocompatibility (Mochalin, Shenderova, Ho, & Gogotsi, 2012; Osawa & Ho, 2012), whereas their processing remains simple and inexpensive (Danilenko, 2004, 2005). Also, the low toxicity (Iyer et al., 2018; Schrand et al., 2007) and high cellular uptake of NDPs make them ideal carriers for some protein-based and chemotherapeutic drugs (Chen et al., 2010; Chow et al., 2011; Shimikunas et al., 2009). NDPs functionalized with low toxicity polyethyleneimine (PEI) have been used for DNA, siRNA (Chen et al., 2010) and gene delivery (Zhang et al., 2009). In addition, diamond nanoparticles show multiplicity of stable optical emission wavelengths offering a strong possibility that one or more of optical emissions can be used for optical transduction based biosensing applications.

In a previous study, for the first time we demonstrated our successful approach of purification of both 6 and 25 nm NDPs, functionalization of the surface of 25 nm NDPs with PEI and loading amoxicillin on NDPs (Rouhani et al., 2016). In another study, we showed that plain acid-treated NDPs are non-toxic and can get internalized into human bladder cells line (Iyer et al., 2018). Whereas the aim of the drug delivery system is to release the drug at the desirable medium, it is essential to investigate whether any part of the drug carrier such as PEI is released with amoxicillin or not. PEI is not traceable with UV-Vis spectroscopy, but Ungaro, De Rosa, Miro, and Quaglia (2003) showed that adding copper sulphate to PEI forms cuprammonium complex that can be detected by UV-Vis spectroscopy. However, for tracing PEI in the presence of the amoxicillin copper sulphate or copper nitrate cannot be used due to the overlap of the peaks with the amoxicillin. To resolve this, for the first time, we developed a technique to trace the PEI during amoxicillin release by using copper oxide particles. In the current study, we are presenting our systematic study on quantifying the amount of PEI and amoxicillin load and release on 6 and 25 nm NDPs at different pH and temperature. We also demonstrated the effect of PEI and surface charge on the amount of drug loading and released from NDPs.

2 | MATERIALS AND METHODS

Monocrystalline NDPs with an average size of 25 nm and detonation synthesized NDPs with the average size of 6 nm were purchased from Advanced Abrasive Corporation and Nanostructured and Amorphous Materials Inc., respectively. Polyethyleneimine (PEI) solution (50% w/v solution of PEI in water) was purchased from Fluka. Copper (II) oxide (97%) was purchased from Alfa Aesar. Agilent Varian 680-IR Fourier Transform Infrared (FTIR) spectrometer with PIKE VeeMAX II reflection setup, Perkin Elmer high-resolution Lambda 1050 UV–Vis NIR spectrophotometer and Malvern Nano S90 Dynamic Light Scattering (DLS) were used as characterization instruments.

2.1 | Quantitative analysis of loaded amoxicillin

First, the UV–Vis spectroscopy calibration curve of the amoxicillin was made to determine the instrumental limitations and response to sets of known concentrations. A set of amoxicillin samples was made at various concentrations from 0.0001 to 3.2 mg/ml, and the UV–Vis response at each concentration was recorded. This experiment was replicated thrice, and the measured data were fit using linear regression. The linear fit was used to predict the unknown concentration of amoxicillin in an endotoxin-free water solvent.

To quantify the amounts of adsorbed amoxicillin on the surfaces of the nanodiamond loaded with PEI (ND-PEI) particles, the initial concentration of amoxicillin (C0), the concentration of non-adsorbed amoxicillin after mixing with NDPs (Ceq) and the concentration of the excess of the amoxicillin during washing steps (Cwash1, Cwash2) were measured by monitoring the absorption spectrum of amoxicillin by UV-Visible spectrophotometer (Lambda 1050 Perkin Elmer). The absorbance peak of amoxicillin in solution was measured at 272 nm for each trial and was correlated to the amoxicillin concentration by Beer’s Law:

\[
A = -\log \left( \frac{l}{l_0} \right) = \varepsilon c
\]

where \(\varepsilon\) is the molar absorption coefficient of the material, \(C\) is its concentration, and \(l\) is the length of the beam’s path.

2.2 | Drug release of ND-PEI-amoxicillin

The release of amoxicillin from the nanodiamond loaded with PEI and amoxicillin (ND-PEI-amoxicillin) particles was studied at the storage temperature of 4°C, room temperature of 25 and 37°C, which is the human body temperature, for different pH values, pH 4 (citrate buffer), pH 7 (PBS buffer), pH 8 (sodium bicarbonate buffer) and endotoxin-free water (pH 6.5). 1 mg/ml of the drug-loaded particles with 5 ml of desired media was mixed, centrifuged at 14,385 g for 30 min; then, the absorbance of the supernatant was read at 272 nm with UV–Visible spectrophotometer. The sample was incubated at the desired temperature, and the samples were taken at different times of 1–190 hr to study the drug release.

2.3 | Quantitative analysis of PEI loading on NDPs and PEI release

For detecting PEI in the presence of the amoxicillin, copper sulphate or copper nitrate cannot be used due to the overlaps of their peaks with amoxicillin. We developed a technique in which copper oxide particles were used. First, to generate a UV–Vis spectroscopy calibration curve of the PEI, a set of PEI samples was made with a range of concentrations from 0.01 to 0.001 mg/ml, and each was mixed with 0.05 mg of copper oxide particles. Due to the formation of the
blue cuprammonium complex (Cu(NH$_3$)$_4$)$^{2+}$) when copper ions (Cu$^{2+}$) are added to PEI, the UV–Vis spectrum at each concentration was detected. The measurement was replicated thrice; then, the data were fitted linearly, and the obtained calibration function was used to predict the unknown concentration of PEI in an endotoxin-free water medium. To study the interaction of the amoxicillin with CuO, 0.05 mg of CuO was added to the solution of the 2.5 mg/ml of amoxicillin, and then, the UV–Vis spectrum of the sample was studied.

To quantify the amounts of adsorbed PEI on the surfaces of the ND-PEI, the initial concentration of PEI (C$_0$), the concentration of non-adsorbed PEI after mixing with NDPs (C$_{eq}$) and the concentration of the excess of the PEI during washing steps (C$_{wash1}$, C$_{wash2}$) were measured by monitoring the absorption spectrum of PEI-CuO by UV–Visible spectrophotometer. The absorbance peak of PEI-CuO in solution was measured at 630 nm for each trial and was correlated to the PEI concentration by Beer’s Law. To study the PEI release from ND-PEI or ND-PEI-amoxicillin, the supernatant of each sample at various pH was collected, added to CuO solution and studied with UV–Vis spectrophotometer.

3 | RESULTS AND DISCUSSIONS

3.1 | Characterization of loaded NDPs with PEI and amoxicillin

Nanodiamond particles were purified and loaded with PEI and amoxicillin using methods described in our previous paper (Rouhani et al., 2016). Results of further study on loading amoxicillin on acid-treated purified 25 nm NDPs that have a strong carboxyl group on its surface at 1,800 cm$^{-1}$ (Figure 1ai) and 6 nm NDPs having carboxyl group with a lower intensity (Figure 1bi). Figure 1ai,bi show the FTIR spectra of acid-treated NDPs coated with PEI in which carboxyl group of NDPs (at 1,800 cm$^{-1}$) is shifted to 1,660 cm$^{-1}$ due to the hydrogen bond between the amine group of PEI at 1,580 cm$^{-1}$ and C=O on NDP surface. The FTIR spectrum of acid-treated ND/PEI particles loaded with amoxicillin shows the amoxicillin β-lactam peak at 1,770 cm$^{-1}$ and the sharp feature at 3,650 cm$^{-1}$ that is due to the bond between PEI NH stretching and amoxicillin carboxylic acid group confirming amoxicillin loading on ND/PEI particles (Figure 1aiii,biil).

Zeta potential measurement was used to find the surface charges on the NDPs before and after coating with PEI. Table 1 summarizes the findings on zeta potential and particle size during different stages of coating/loading. ND-PEI is dispersed in DI water to remove the excess of PEI. The particles are collected by centrifugation and the supernatant, which contains excess of the PEI is removed. This step is called wash 1 and wash 2. Then, the collected ND-PEI is dispersed in DI water for measurement of surface charge and particle size by DLS.

The zeta potential of the acid-treated 25 nm NDPs mixed in distilled water was −50 mV (pH 7). After coating NDPs with PEI, the zeta potential of the particles mixed with PEI was +55 mV (pH 7), which shows that the surface of NDPs is covered with PEI. After two times of washing to remove the excess of PEI, the zeta potential of the collected particles was +45 mV (pH 7). The change in the zeta potential of NDPs from −50 mV to +45 mV for the ND-PEI particles confirms the successful coating of PEI on NDPs. Hydrodynamic particle size measurements by dynamic light scattering (DLS) technique showed that the size of the NDPs dispersed in distilled water was 57 nm, and size of the ND-PEI particles was 160 nm.

FIGURE 1 FTIR spectra of (a) (i) acid-treated NDPs 25 nm and (b) (i) acid-treated NDPs 6 nm with carboxyl group at 1,800 cm$^{-1}$, (a) (ii) and (b) (ii) acid-treated and PEI-loaded ND/PEI particles showing the carboxyl group of NDPs (1,800 cm$^{-1}$) is shifted to 1,660 cm$^{-1}$ due to the hydrogen bond between amine of PEI and C=O of NDPs surfaces, and (a, b) (iii) acid-treated and PEI/amoxicillin-loaded ND/PEI/amoxicillin particles after washing. Amoxicillin β-lactam peak and the feature at 3,650 cm$^{-1}$, which is due to bond between PEI NH stretching and amoxicillin carboxylic acid group (COOH), confirming the successful loading of amoxicillin to the surface of ND/PEI particles (Rouhani et al., 2016)
Table 2 summarizes the findings on hydrodynamic size and zeta potentials of NDPs after loading with amoxicillin. The zeta potential of the ND-PEI nanoparticles mixed in distilled water was +45 mV (pH 7). After loading NDP-PEI nanoparticles with amoxicillin, the zeta potential of the mixture was +70 mV (pH 7), and after two times of washing to remove the excess of amoxicillin, the zeta potential of the collected particles was +66 mV (pH 7). The zeta potential of ND-PEI particles changed from +45 mV to +66 mV for ND-PEI-amoxicillin. This change suggests that amoxicillin molecules are loaded on ND-PEI particles and create sites of positive charges. The hydrodynamic size of the collected ND-PEI-amoxicillin particles mixed in DI water was 180 nm.

In case of 6 nm NDPs, the zeta potential of the bare particles was +42 mV, for ND-PEI was +60 mV and for ND-PEI-amoxicillin was +75 mV. Due to the agglomeration of the bare and amoxicillin coated 6 nm particles, the hydrodynamic size of the particles was not reliable and varied from 50 to 1,000 nm.

3.2 Quantitative analysis of loaded amoxicillin on NDPS

Figure 2a shows the calibration curve of the amoxicillin in endotoxin-free water. Based on the linear fit to the experimental data, absorbance intensities of the initial pure amoxicillin solution in endotoxin-free water and the supernatants after mixing and washing were calculated. The obtained amoxicillin concentrations were called C₀, CSUP, Cw1, and Cw2, respectively. Figure 2b shows the UV–Vis spectrum of the initial pure amoxicillin solution, the supernatant after mixing the ND-PEI particles with amoxicillin, and the supernatants after two steps of washing. Table 3 summarizes the typical results for quantification of the loaded amoxicillin on 1 mg ND-PEI.

The results of systematic quantification of loaded amoxicillin show that 1 g ND-PEI adsorbed up to 114.50 mg amoxicillin. However, this loading created aggregates of ND-PEI-amoxicillin, which hid some unbound amoxicillin molecules in the centre of the aggregated structure whereas other amoxicillin molecules formed a hydrogen bond with the ND-PEI. To release the excess of amoxicillin, two steps of washing were performed. After wash 1, 53.31 mg amoxicillin was loaded on 1 g of ND-PEI, and after wash 2, only 5.18 mg amoxicillin was detected in the supernatant. The difference between the concentration of the initial amoxicillin and the sum of the Csup, Cw1 and Cw2 was 48.124 mg, which suggested that the amount of amoxicillin successfully bonded to the surface of the 1 g ND-PEI is 48.24 mg. Similarly, it was found that for 6 nm NDPs, the amount of loaded amoxicillin was 19 mg on 1 g ND-PEI. Due to the positive surface charge of the 6 nm NDPs and the agglomeration, less PEI and amoxicillin was loaded on the 6 nm NDPs compared to the 25 nm.

3.3 Drug release from ND-PEI-amoxicillin particles

Amoxicillin release from 1 mg NDPs-PEI-amoxicillin particles was studied at 37°C (simulated human body temperature), 25°C (room temperature) and 4°C (storage temperature) in media with pH 4, 7 and 8 for 200 hr. First, the calibration curve of amoxicillin in citrate buffer (pH 4), phosphate buffer saline (PBS, pH 7) and bicarbonate buffer (pH 8) was generated. Then, the effect of temperature and pH on amoxicillin degradation was studied. Finally, the amoxicillin release from ND-PEI-amoxicillin at various pH and temperatures was measured and analysed. The results of these experiments are discussed in the following sections.

3.3.1 Effects of temperature on amoxicillin degradation

Figure 3 shows the UV–Vis spectrum of the amoxicillin dissolved in endotoxin-free water with a final concentration of 2.5 mg/ml at storage temperature (4°C), room temperature (25°C) and body temperature (37°C) kept for 7 days. The UV–Vis spectrum of the sample kept at 4°C does not show any change. The sample kept at room temperature

### TABLE 1 Zeta potential and hydrodynamic particle size of NDPs, ND-PEI particles

| pH     | Zeta potential (mV) | Hydrodynamic particle size (nm) |
|--------|---------------------|--------------------------------|
| 7      | −50                 | 57                             |
| 7      | +55                 | 210                            |
| 7      | +50                 | 170                            |
| 7      | +45                 | 160                            |
| 7      | +45                 | 160                            |

### TABLE 2 Zeta potential and hydrodynamic particle size of NDPs, ND-PEI and ND-PEI-amoxicillin

| pH     | Zeta potential (mV) | Hydrodynamic particle size (nm) |
|--------|---------------------|--------------------------------|
| 7      | −50                 | 60                             |
| 7      | +45                 | 160                            |
| 7      | +75                 | 200                            |
| 7      | +70                 | 180                            |
| 7      | +66                 | 180                            |
| 7      | +66                 | 180                            |
shows a weak signature peak at 350 cm$^{-1}$ after 5 days, and the one kept at 37°C, shows the new feature at 350 cm$^{-1}$ after 2 days.

The appearance of the peak at 350 cm$^{-1}$ is due to the amoxicillin degradation. The mechanisms of amoxicillin degradation due to the temperature and pH are explained in the following sections.

### 3.3.2 | Effects of pH on amoxicillin degradation

Figure 4a,b shows the absorbance spectrum of amoxicillin dissolved in citrate buffer (pH 4) at room temperature 25°C and body temperature 37°C for 7 days. The acidic medium of the citrate buffer resulted in amoxicillin degradation after 2 days at room temperature and body temperature. A strong feature at 350 cm$^{-1}$ was observed along with the change in the colour of the solution, which turned to yellow. Figure 4c,d shows the UV–Visible spectrum of the amoxicillin dissolved in PBS (pH 7) at room temperature 25°C and body temperature 37°C. As can be seen, amoxicillin is stable at room temperature for 7 days. But it shows features related to its degradation at temperature of 37°C after 3 days.

Figure 4e,f shows the effect of basic medium of bicarbonate sodium (pH 8) on amoxicillin degradation. The feature assigned to amoxicillin degradation at 350 cm$^{-1}$ appeared after 1 day for both room and body temperature. The inset of Figure 4f shows the increase of the peak at 350 cm$^{-1}$ with time as a signature of more amoxicillin degradation. Note that the intensity of the peak at 271 cm$^{-1}$ which has been used for amoxicillin calibration is not stable after amoxicillin degradation and cannot be used to calculate the amoxicillin concentration.

Figure 5a–c shows the FTIR spectra of the amoxicillin samples dissolved in endotoxin-free water, citrate buffer, PBS and bicarbonate sodium buffer for 7 days at temperature of 37°C. For the samples at pH 4 and 7, the peak related to the β-lactam at 1,778 cm$^{-1}$ is obvious. However, for the sample dissolved in acidic pH 4, the relative intensity of the β-lactam peak to amide II peak at 1,450 cm$^{-1}$ is lower than the one in pH 7. For the sample dissolved in basic buffer (pH 8), there is no signature of the β-lactam.

The β-lactam peak in amoxicillin structure is susceptible to acid and base catalysed hydrolysis of the four membered β-lactam ring. Amoxicillin is more stable at lower pH conditions because under these conditions, the amine group of amoxicillin is protonated, so the free electron pairs of the nitrogen is utilized. At higher pH (pH 8 and above), the amine group is deprotonated, and a pair of free electrons is available for nucleophilic attack of β-lactam. This will generate a tricyclic compound identified by Bundgaard (1977), as illustrated in Figure 5d. Whereas the main amoxicillin absorbance peaks detected by UV–Visible spectrophotometer are related to thiozole ring (240 cm$^{-1}$) and phenol (271 cm$^{-1}$), the peak observed at 350 cm$^{-1}$ is related to the new ring which appears in the structure of the tricyclic compound.

### Table 3

Typical results for quantification of the loaded amoxicillin on 1 mg 25 nm ND-PEI

|                  | Initial amoxicillin | Supernatant | Wash 1  | Wash 2  |
|------------------|---------------------|-------------|---------|---------|
| Concentration (mg/ml) | 2.50114            | 2.38692     | 0.06104 | 0.00504 |
|                  | 2.50125            | 2.3868      | 0.06115 | 0.00521 |
|                  | 2.50346            | 2.38661     | 0.06137 | 0.00531 |
| Average concentration (mg/ml) | 2.50128            | 2.386778    | 0.06191 | 0.005187|
| Standard deviation     | 0.000133           | 0.000129    | 0.000136| 0.000111|
According to Zia, Shalchian, and Borh (1977), amoxicillin's maximum stability has been measured at 35°C and pH of 5.77 for 55 hr.

Our study on amoxicillin degradation shows that body temperature (37°C) and basic pH (pH 8) increased the rate of amoxicillin degradation. The combined effect of the temperature 37°C and pH 8 resulted in complete disappearance of the β-lactam ring after 1 day. On the other hand, partial degradation of amoxicillin in acidic pH (pH 4) or neutral pH (pH 7) at body temperature 37°C occurred after 2 days. These findings helped to design the drug release experiments and interpret the results more accurately.

### 3.3.3 Effects of pH and temperature on amoxicillin release from ND-PEI-amoxicillin

Figure 6a shows the amoxicillin release from ND-PEI-amoxicillin at room temperature for different values of the solvent pH. Up to 55% of the loaded drug is released in acidic media (pH 4). Whereas the drug release in PBS (pH 7) is 25%, in endotoxin-free water (pH 6.5) is 14% and for the higher pH such as pH 8, it is 8%. Figure 6b shows drug release at body temperature 37°C in which 85% of the loaded drug is released in acidic media (pH 4). Increase in temperature from 25 to 37°C increased the drug release in different media by almost twice.

To compare the effect of the temperature on drug release in media with different pH values, plots of drug release in each media are compared (Figure 6c–f). The amount of release after 1 hr at body temperature 37°C at pH 4 was equal to the release after 5 hr at room temperature 25°C. It seems that by increasing the temperature, the movement rate of the ions in the solution, and their interaction with the surface of the loaded particles increased and resulted in more amoxicillin release. Effect of the temperature on the faster release of the drug can be seen for the samples at higher pH. It should be noted that our study on drug release at a temperature slightly higher...
FIGURE 4  Effects of temperature and pH on amoxicillin degradation. Results show that body temperature (37°C) and basic pH (pH 8) increased the rate of amoxicillin degradation. Degradation of amoxicillin in acidic pH (pH 4) or neutral pH (pH 7) at body temperature occurred after 2 days. UV-Vis feature at 350 cm⁻¹ is a signature of the amoxicillin degradation.
than the body temperature (40°C) did not show any significant difference with that of the body temperature (37°C).

Drug release from 6 nm NDPs shows the same trend as for the 25 nm NDPs at various values of pH and temperature.

Studies on amoxicillin solubility in different solvents with varying values of pH showed that due to the zwitterion effect at pH < 9, the ionic charges of the carboxylic acid (negative pK$_a$ = 2.87) and the amine (positive pK$_a$ = 7.28) will counteract each other and cause the antibiotic to be less soluble (Saesmaa & Tötterman, 1990). Hence, the mechanism of the drug release can be explained by accounting for the role of the PEI under conditions of varying pH and temperature. Branched PEI has a primary, secondary or a tertiary amine connected by an ethylene group. As every third atom is amino nitrogen that can be protonated, PEI has a high cationic charge density. Nitrogen presence at every third backbone atom results in a wide range of pK$_a$ values and high buffering capacity. On the other hand, PEI is a hydrophobic polymer due to its ethylene rich backbone. Competition between interchain and intrachain interactions results in the extension of a hydrophobic polyelectrolyte backbone. The charge repulsion among the amine groups in the same chain (intrachain) favours chain extension, whereas the charge repulsion between amine groups of the two branch of PEI (interchain) may compact the molecule. The balance between interchain and intrachain repulsion changes due to the solution condition (pH, salt and concentration; Reed, Ghosh, Medjahdi, & Francois, 1991). For example, solutions with high or low pH will increase the charges on the PEI, whereas solutions with salts (like PBS) screen the electrostatic interaction and reduce charge repulsion. For low ionic strength solutions, in which the concentration of ions is low, the attractive hydrophobic interaction among polymer sections is cancelled by the electrostatic repulsion resulting in an extended polymer configuration. The presence of salts in the solvent screens the electrostatic repulsion, so the polymer behaves like a neutral polymer. Weak intrachain repulsion and strong interchain repulsion result in the collapse of the hydrophobic polymer (Ulrich, Laguecir, & Stoll, 2005). For PEI, the charged groups are located on the backbone, separated by two ethylene groups and form close spacing of charges. The effects due to the close spacing of charges in PEI are as follows: each amine protonation will increase both the charge repulsion and free energy of protonation in the neighbour amine. This effect has been studied extensively. For example, a single-chain simulation of the intrarepulsion forces in PEI shows that the amine protonation occurs in steps (Ziebarth & Wang, 2010). Also, the experimental tests on PEI titration showed that the two and three neighbour influence on amine protonation is required.

**FIGURE 5** FTIR spectrums of amoxicillin dissolved in different buffers with (a) pH 7, (b) pH 4 and (c) pH 8 for 7 days at temperature of 37°C, and (d) the amoxicillin degradation diagram. The combined effect of the temperature 37°C and pH 8 resulted in complete disappearance of the β-lactam ring and amoxicillin degradation after 1 day. Partial degradation of amoxicillin in acidic pH (pH 4) or neutral pH (pH 7) at body temperature occurred after 2 days.
Drug release of the ND-PEI-amoxicillin at (a) room temperature and pH 4, 7, 6.5 and 8, (b) body temperature and pH 4, 7, 6.5 and 8, (c) comparison of the release at pH 4 at room and body temperature, (d) pH 6.5 at room and body temperature, (e) pH 7 at room and body temperature and (f) pH 8 and room and body temperature. Body temperature and acidic pH results in higher drug release.
Almost 100× more free energy is required for 50% protonation of amines in the backbone of the PEI than in its non-polymeric component such as dimethyl-amine (Sun, Tang, Uludağ, & Cuervo, 2011). The extension or aggregation of the polymer backbone will affect the neighbourhood charge repulsion. Backbone extension depends on the balance between interchain and intrachain charge repulsion. Aggregation occurs when intrachain repulsion is lowered or as interchain repulsion is increased (Curtis et al., 2016). Overall, the PEI charge increases with a decrease in pH, and the charge repulsion is reduced by the addition of salt (PBS) or by increasing the pH, as shown in Figure 7.

From the preceding discussion, the mechanism of the drug release in acidic pH can be explained based on the effect of pH and salt on PEI configuration. When ND-PEI-amoxicillin particles were added to the citrate acid buffer (pH 4), most of the amide groups on PEI were protonated with [H⁺] as it is described in the following reaction.

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\text{Polyethyleneimine protonation at pH 4 resulted in having positive charges on the polymer chain, which increased the intracharge repulsion. As shown in Figure 7, the increase in intracharge repulsion results in backbone extension, thereby forming an open-chain configuration. Physically adsorbed amoxicillin molecules are released in the media, whereas the chemically conjugated ones (hydrogen bonded) are released with proton and then released in the media. In case of adding ND-PEI-amoxicillin to PBS (pH 7) which contains several salts, positive and negative ions of salts act as screening charges between amine groups of PEI and as a result lower the intracharge repulsion and form a closed chain of neutral atoms. For ND-PEI-amoxicillin added to sodium bicarbonate buffer (pH 8), pH and salt ions (here Na⁺) are increased. As a result, the intrachain repulsion decreases, and closed chain of PEI contains neutral atoms. Physically adsorbed amoxicillin molecules in the closed chain of PEI are trapped and are not exposed to the media to be released. To investigate this theory, the hydrodynamic particle size of ND-PEI-amoxicillin in various pH was measured. Results showed that whereas NDPs loaded with PEI and amoxicillin have size of 180 nm in endotoxin-free water (pH 6.5), their size of particles changed into 644 nm at citrate buffer (pH 4), 164 nm at PBS buffer (pH 7) and 94 nm at bicarbonate sodium (pH 8). These results confirm the expansion of the PEI in acidic media, which results in higher drug release, as well as the PEI collapse or closed chain in PBS or basic pH that results in less drug release.

3.4 Quantification of PEI loading on NDPs and PEI release

As PEI does not have any signature in UV–Vis range, its spectrophotometric quantification is based on the formation of the blue cuprammonium complex (Cu(NH₃)₄²⁺) when copper ions (Cu²⁺) are...
added to PEI. Cuprammonium has two absorbance peaks at 270 and 630 nm. Figure 8 shows the UV–Vis spectrum of the copper oxide separately added to the PEI and amoxicillin.

As can be seen, PEI/CuO shows two peaks at 270 and 630 nm due to the formation of the cuprammonium, and amoxicillin/CuO shows two peaks, one at 270 nm which is the characteristic feature of amoxicillin and a new peak at ~300 nm due to the presence of copper ions in the structure of the amoxicillin.

As the cuprammonium absorption at 270 nm is close to that of the amoxicillin (272 nm), we chose to calibrate the PEI concentration based on the peak at 630 nm. Figure 9 shows the UV–Vis spectrum and calibration curves of the PEI/CuO. As can be seen, the intensity of the peak at 630 nm is decreasing by decrease in the concentration of the PEI (Figure 9a,b).

Figure 9c shows the calibration curve of the PEI in endotoxin-free water. The linear fit of the experimental calibration data was used to calculate the absorbance intensities of the initial PEI and the supernatants after mixing and washing. The calculated PEI concentrations were called $C_0$, $C_{sup}$, $C_{w1}$ and $C_{w2}$, respectively.

The results of the systematic quantification of loaded PEI show that 1 g ND adsorbed up to 1.64 mg PEI. To remove the excess of PEI, two steps of washing were performed. After wash 1, 0.042 mg PEI was removed, and after wash 2, no PEI was detected in the supernatant. The difference between the concentration of the initial PEI and the sum of the $C_{sup}$, $C_{w1}$ and $C_{w2}$ was 1.60 mg, which suggested that the amount of PEI successfully bonded to the surface of the 1 g ND is 1.60 mg. For 6 nm NDPs, the amount of loaded PEI was found to be 0.33 mg on 1 g ND. Due to the positive surface charge of the 6 nm NDPs and particle agglomeration, less PEI was loaded on 6 nm NDPs compared to 25 nm.

To investigate PEI release from ND-PEI in different pH, first, we separately studied the UV–Vis spectra of CuO in citrate buffer (pH 4), PBS (pH 7) and bicarbonate sodium (pH 8). We noticed that CuO mixed in PBS (pH 7) and bicarbonate sodium (pH 8) do not show any feature in the UV–Vis spectrum as well as in the endotoxin-free water. However, CuO addition to citrate buffer (pH 4) creates a peak at 780 nm, which is due to formation of the $Cu^{2+}$ - citrate dimer complexes in the acidic solutions (Figure 10a).

**FIGURE 9** (a and b) UV–Vis spectrum of PEI at various concentrations and (c) calibration curves of the PEI/CuO. As the cuprammonium absorption at 270 nm is close to that of the amoxicillin (272 nm), we chose to calibrate the PEI concentration based on the peak at 630 nm.

**FIGURE 10** UV–Vis spectra of the (a) CuO and, (b) PEI/CuO in citrate buffer (pH 4), PBS (pH 7) and bicarbonate sodium (pH 8). Results show that in the presence of PEI and at pH 7 and 8, a peak at 630 nm appears due to the formation of the cuprammonium. With NO PEI, at pH 4, a broad peak was formed at 730 nm due to the presence of both cuprammonium and $Cu^{2+}$ - citrate dimer complex.
In another experiment, PEI/CuO was added separately to the PBS (pH 7), bicarbonate sodium (pH 8) and citrate (pH 4) buffer. UV–Vis spectrum shows at pH 7 and 8, a peak at 630 nm appears due to the formation of the cuprammonium. At pH 4, a broad peak was formed at 730 nm due to the presence of both cuprammonium and Cu\(^{2+}\) - citrate dimer complex. (Figure 10b).

Figure 11 shows the UV–Vis spectrum of the supernatant of ND-PEI. Note that ND-PEI particles were added to different types of buffers. As can be seen for 25 nm, ND-PEI added to buffers in pH 4, 7 and 8 there is no peak at 630 nm as a signature of the PEI presence in the supernatant. The peak is at 780 nm is due to the interaction of the citrate buffer (pH 4) with the Cu ions. For 6 nm ND-PEI traces of PEI release into the supernatant can be seen for all buffers.

The difference between the amounts of PEI loaded on 6, and 25 nm NDPs and its release can be explained by noticing the difference between the surface of the 6 and 25 nm NDPs. The 25 nm NDPs has negative surface charge and carboxyl group on the surface,

Figure 11 UV–Vis spectrum of the CuO added to the supernatant of (a) 25 nm ND-PEI and (b) 6 nm ND-PEI. For 25 nm ND-PEI added to buffers in pH 4, 7 and 8, there is no peak at 630 nm as a signature of the PEI presence in the supernatant. The peak is at 780 nm is due to the interaction of the citrate buffer (pH 4) with the Cu ions. For 6 nm ND-PEI traces of PEI release into the supernatant can be seen for all buffers.

The difference between the amounts of PEI loaded on 6, and 25 nm NDPs and its release can be explained by noticing the difference between the surface of the 6 and 25 nm NDPs. The 25 nm NDPs has negative surface charge and carboxyl group on the surface,

Figure 12 UV–Vis spectrum of the supernatant of (a) 25 nm ND-PEI-amoxicillin and (b) 6 nm ND-PEI-amoxicillin in different buffers. For the 25 nm ND-PEI-amoxicillin, the features at 320 nm are related to the interaction of amoxicillin and CuO, and the one at 780 nm is due to the formation of Cu\(^{2+}\) - citrate dimer complex in acidic pH. As can be seen, there is no feature at 730 or 630 nm, so there is no PEI release for the 25 nm ND-PEI-amoxicillin at different pH. For the 6 nm ND-PEI-amoxicillin, features at 630 and 730 nm indicate PEI release for all pH values. Results show that PEI is released from 6 nm ND-PEI-amoxicillin in various pH, but there is no PEI release from 25 nm ND-PEI-amoxicillin in different pH.
which resulted in forming covalent bond between 25 nm NDPs and PEI. Because of the strong bond between 25 nm NDPs and PEI, no PEI release was detected in the supernatant, whereas 6 nm NDPs has positive surface charge with both NH and carboxyl groups on the surface resulting in less PEI loading with weak hydrogen bond leading to PEI release into the supernatant.

ND-PEI-amoxicillin was added to different types of buffers, and the supernatant was taken for analysis. Figure 12a shows the UV–Vis spectrum of 25 nm ND-PEI-amoxicillin supernatants, which were mixed in various buffers, then added to CuO. The features at 320 nm are related to the interaction of amoxicillin and CuO, and the one at 780 nm is due to the formation of CuO- citrate dimer complex in acidic pH. As can be seen, there is no feature at 730 or 630 nm, so there is no PEI release for the 25 nm ND-PEI-amoxicillin at different pH. Figure 12b shows the results for 6 nm ND-PEI-amoxicillin in which UV–Vis features at 630 and 730 nm indicate PEI release for all pH values. For 6 nm ND-PEI-amoxicillin, the peak is at 320 nm in out of scale due to high intensity of the released amoxicillin, but the peak shoulder can be seen. Overall, it can be concluded that PEI is released from 6 nm ND-PEI and ND-PEI-amoxicillin in various pH, but there is no PEI release from 25 nm ND-PEI or ND-PEI-amoxicillin in different pH.

4 | CONCLUSIONS

A study on the loading of PEI and amoxicillin on NDPs as a vehicle for drug delivery was conducted. The surface charges of the NDPs before and after loading with PEI and amoxicillin confirmed the loading of both. The amount of loaded amoxicillin was calculated to be 48.24 mg on 1 mg of 25 nm NDPs and 19 mg on 1 mg 6 nm NDPs. The result of the amoxicillin release showed that the highest release occurred at body temperature and acidic pH in which almost 85% of the loaded drug was released during 24 hr. The drug release at acidic pH was due to the presence of PEI. The benefit of drug release at acidic pH was considered beneficial when ND-PEI-amoxicillin could be internalized in the cell and passed through the lysosome, which has an acidic pH where the drug can be released inside the cell.

A new method to detect the amount of loaded and released PEI in the presence of amoxicillin was developed for the first time. It was based on the formation of cuprammonium complex when copper ions were added to PEI solution. The amount of PEI successfully bonded to the surface of the 1 g of 6 and 25 nm ND found to be 0.33 and 1.60 mg, respectively. It was also found that whereas no PEI was released from 25 nm NDPs in acidic pH, there were traces of PEI released for 6 nm NDPs. The difference between the amounts of PEI loaded on 6 and 25 nm NDPs was explained by noticing the difference between surfaces of the 6 and 25 nm NDPs. The 25 nm NDPs had negative surface charge and carboxyl group on the surface, which led to forming covalent bonds between 25 nm NDPs and PEI. Because of the strong bond between 25 nm NDPs and PEI, no PEI was released and not detected in the supernatant. Whereas 6 nm NDPs had positive surface charge with both NH and carboxyl groups on the surface resulting in less PEI loading with weak hydrogen bond leading to PEI release into the supernatant. Overall, it can be concluded that PEI was released from 6 nm ND-PEI and ND-PEI-amoxicillin in various pH, but there is no PEI release from 25 nm ND-PEI or ND-PEI-amoxicillin in various pH. These studies showed that purified and PEI-functionalized 25 nm NDPs are promising carrier for the antibiotic amoxicillin as it can bind to the drug, carry it to the media with acidic pH and release it.

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