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An Ion Pairing Approach to Enhance Oral Bioavailability of Alendronate

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Authors' contributions

This work was carried out in collaboration between all authors. Authors BNA carried out fabrication and physicochemical assessment of AL/PEI association complexes. Authors GMS and MAA participated in the design of this study. Author GMP conceived the study, coordinated experimental designs, and helped drafting the manuscript. All authors read and approved the final manuscript. This research was supported in part by a predoctoral fellowship from the Egyptian Ministry of Higher Education awarded to author BNA. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Aim: Alendronate (AL) is a nitrogen-containing bisphosphonate drug that exhibits limited oral bioavailability due to predominantly hydrophilic molecular properties. To enhance oral absorption of this important osteoporosis drug, a novel ion-pairing strategy using the cationic polymer polyethylenimine (PEI) was explored as an initial step of an alternate oral drug delivery strategy that attempts to prepare polymer-encapsulated ion pair nanoparticles.
Methodology: Electrostatically stabilized AL/PEI association complexes were fabricated by combining AL and PEI solutions prepared in 0.05 M acetate buffer, pH 5.0, at different AL/PEI

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charge ratios under stirring. The free fraction of AL after complexation with PEI was quantified spectrophotometrically at λ=300 nm using ferric chloride. Particle size distribution and zeta potential of ion pairs formed at different molar AL/PEI ratios were measured by dynamic laser light scattering.

Results: The complexation efficiency of PEI was low until an AL/PEI charge ratio of1:1.7. Increasing PEI concentrations effectively decreased the free fraction of AL implying formation of stable ion pairs between the negatively charged AL and the positively charged polymer. The lowest fraction of free AL was 18.7% measured at an AL/PEI charge ratio of 1:33. The mean hydrodynamic diameter of nanoassemblies decreased with increasing AL/PEI charge ratio reaching a limiting value of 71±1.4 nm at AL/PEI=1:33. Corresponding zeta potential measured for these association complexes was +37±2.8 mV.

Conclusion: AL/PEI charge ratio greater than 1:1.7 facilitates effective formation of electrostatically stabilized ion pairs that carry a significant positive surface charge indicative of substantial colloidal stability in aqueous solution. The small size of AL/PEI complexes fabricated at 1:33 favors these ion pairs for subsequent encapsulation into biocompatible polymers suitable for oral drug delivery.

Keywords: Polyethyleneimine; particle size; zeta potential; nanoassembly.

ABBREVIATIONS

AL = Alendronate; DLS = Dynamic Laser light Scattering; PEI = Polyethyleneimine; ZP = Zeta Potential; PDI = Polydispersity Index; RT = Room Temperature; mPas = one millipascal-second.

1. INTRODUCTION

Alendronate (AL), a synthetic analog of pyrophosphate, is a hydrophillic, amphiprotic drug that is therapeutically used in the prevention and treatment of bone diseases, including post-menopausal osteoporosis and Paget's disease [1-3]. Consistent with the mechanism of action of other bisphosphonates, clinical benefits of AL are attributed to increase in bone density, bone mineralization, and bone strength mediated by inhibition of both osteoblast apoptosis and osteoclast activity [4].

Poor oral absorption of bisphosphonates appears a direct consequence of the polar physicochemical properties of these molecules resulting in unfavorable transcellular permeation across epithelial cell barriers. At physiological pH in the small intestine, bisphosphonates are negatively charged resulting in human oral bioavailability less than 1% [5]. Oral administration is the preferred route of administration for pharmacologically active agents due to higher patient acceptability. Therefore, more efficient oral delivery systems of AL must be developed to augment oral drug bioavailability. Various prodrug strategies have been employed for improving drug permeation across the intestinal mucosa. However, reversible covalent modification of bisphosphonates is technically challenging and results in a new molecular entity that requires extensive safety assessment prior to initiating clinical efficacy evaluation [6].

Ion pairing, in contrast, is a non-covalent drug delivery approach that attempts to increase apparent lipophilicity of an ionizable hydrophilic drug molecule by forming electrostatically-stabilized association complexes with lipophilic counter ions [6]. The ionic interaction establishes a temporary opportunity to overcome unfavorable physicochemical, pharmaceutical, and biopharmaceutical properties without the need for covalent modification of the active moiety. Consequently, these association products have been explored for various drug delivery applications [7]. The ion pairing strategy involves simple mixing of complementary ions in solution. However, physicochemical properties vary depending on molar ratio of counter ions, pH, and salt concentrations [6].

The overall objective of this research is to prepare lipophilic AL-excipient complexes suitable for encapsulation into biocompatible polymers (e.g., PLGA or lipids) that can be administered orally as nanoparticle formulations. The results presented in this manuscript summarize physicochemical properties of electrostatically stabilized association complexes between AL and polyethylenimine (PEI) fabricated at different charge ratios to select the

most favorable ion pair complex for subsequent encapsulation studies.

2. MATERIALS AND METHODS

2.1 Materials

Alendronate sodium (Fig. 1.), acetic acid glacial and sodium acetate trihydrate were obtained from Sigma Aldrich, USA. Polyethyleneimine, branched (average M_N=60,000) (Fig. 2.) and perchloric acid 70% solution in water were purchased from Acros Organics, USA. Ferric chloride hexahydrate was obtained from Fisher Scientific, USA. Tables 1 and 2 summarize most relevant physicochemical properties and functional groups contributing to ionic interaction between alendronate and polyethyleneimine at pH = 5.

2.2 Methods

2.2.1 Formation of ion pairs between AL and PEI

Different molar concentrations of PEI ranging from $5.7x10^{-8}$ to 0.0114 mM were dissolved in 1% acetic acid solution at room temperature (RT). Three mL were combined drop-wise with an equivalent volume of an alendronate sodium solution prepared in 0.05 M acetate buffer, pH 5.0. At this pH, each molecule of AL carries two negative charges and each molecule of PEI carries 17,406 positive charges. Complex characterization was performed after 1 min stirring at RT.

2.2.2 Determination of free alendronate

Since alendronate has no chromophore suitable for UV/Vis analysis, free alendronate was quantified using the colored ferric/AL complex formed in 0.01 M perchloric acid solution that allows spectrophotometric determination at λ=300 nm (Beckman DU 7400). Briefly, an iron (III) chloride standard solution (5 mM) was prepared by dissolving ferric chloride hexahydrate in 0.01 M perchloric acid. Aliquots (0.4 mL) of the prepared AL-PEI complexes were combined with 0.15 mL of ferric chloride solution, and the volume was completed to 3 mL with perchloric acid solution. The analytical method was fully validated for linearity, accuracy and precision.

2.2.3 Particle size and zeta potential measurements

Particle size distribution and zeta potential (ZP) of prepared complexes were measured by dynamic laser light scattering (DLS) using the Zetasizer 3600 (Malvern Instruments, Worcestershire, UK) equipped with a 4mW helium/neon laser (λ=633nm) and a thermoelectric temperature controller. For zeta potential measurements, disposable folded capillary cuvettes were used. Air bubbles were removed from the capillary before measurement. All measurements were carried out in triplicate and are reported as mean ± SD.

3. RESULTS

3.1 Measurement of Complexation Efficiency

Complex formation between AL and PEI (average M_N=60,000) was carried out in a mixture comprised of 0.05 M acetate buffer and 1% acetic acid solution at RT. The slow addition of PEI to the AL solution induced a gradual decrease in free AL until a plateau value of 18.7% was reached at a charge ratio of AL/PEI= 1:33. Fig. 3 shows the free fraction of AL in % as a function of the charge ratio between AL and PEI.

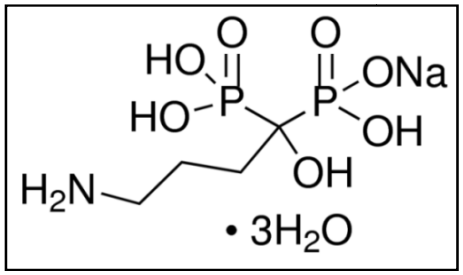

Fig. 1. Molecular structure of alendronate sodium trihydrate

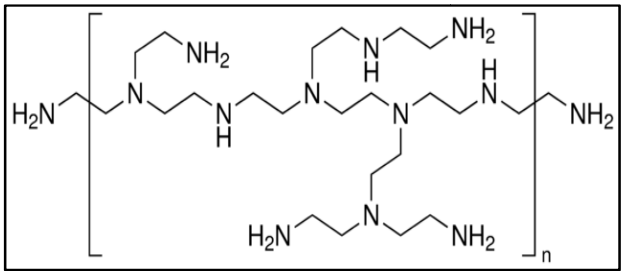

Fig. 2. Molecular structure of polyethyleneimine

Table 1. Relevant physicochemical properties of alendronate sodium and polyethyleneimine

Alendronate sodium trihydrate	Polyethyleneimine, branched
Physical state: white crystalline nonhygroscopic powder [8].	Physical state: Colorless to Light Yellow viscous liquid [10].
Dissociation constants: pKa (strongest acidic) is 1.33 and (strongest basic) is 11.82 [9].	Dissociation constants: pKa differs according to pH ranging from (5.95 at pH 6 to 8.17 at pH 9) [11].
Solubility characteristics: highly soluble in water (10 mg/mL), very slightly soluble in ethanol and practically insoluble in chloroform [8].	Solubility characteristics: soluble in water due to the presence of charged amine groups[10].
Partition and distribution coefficients: log P (octanol/water): - 4.49 [8].	Viscosity (mPa.s): 18000 – 40000[10].

Table 2. Functional groups involved in the interaction of alendronate and polyethyleneimine at pH=5

Functional groups for Alendronate
Two phosphonate groups : 2[PO(OH)(O^-)]
Functional groups for polyethyleneimine
Primary amino group: R1(NH2)
Secondary amino group: R1(NH)R2
Tertiary amino group: R1(N)R2R3

There was a noticeable decrease of free AL at the charge ratio of AL/PEI=1:0.000165 and remained at almost constant value until the charge ratio reached 1:1.7. Then, free AL decreased sharply to 62.54% and 81.29% at the charge ratio of 1:16.5 and1:33, respectively. The pH of the complex aqueous solution was found to be around 5 for all the complexes, which favor the interaction between AL and PEI due to substantial ionization of the two counter ions. In aqueous solution at pH 5, the predominant alendronate species has 2 negative charges, which are paired with polycationic PEI which is protonated at this pH [11].

3.2 Particle Size Measurement

The interaction between AL and PEI was investigated by DLS and zeta potential measurements to determine the particle size and charge of the complexes, respectively. Formation of AL/PEI complexes is predicted to involve the ionized cationic and anionic functional groups present at pH 5.0. The size of the electrostatically stabilized association complexes was substantially dependent on the drug/polymer ratio. Fig. 4 shows that the particle size of the formed complexes decreased sharply as the charge ratio of AL/PEI increased from 1:0.000165 to 1:0.17. Further increase in the charge ratio showed no change in particle size.

3.3 Zeta Potential Measurement

Table 3 shows that with increasing AL/PEI ratios the zeta potential increased suggesting gradual complexation of the negatively charged drug by the positively charged polymer.

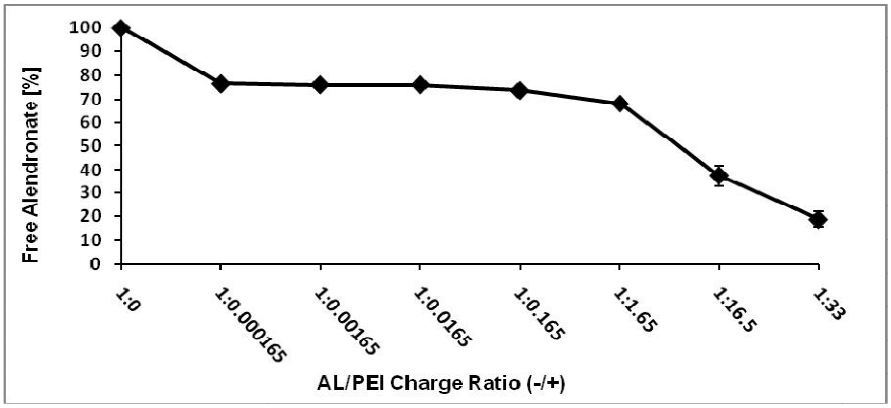

Fig. 3. Impact of different PEI concentrations on complexation with alendronate sodium in acetate buffer, pH=5. Data are shown as mean ± SD (n=3)

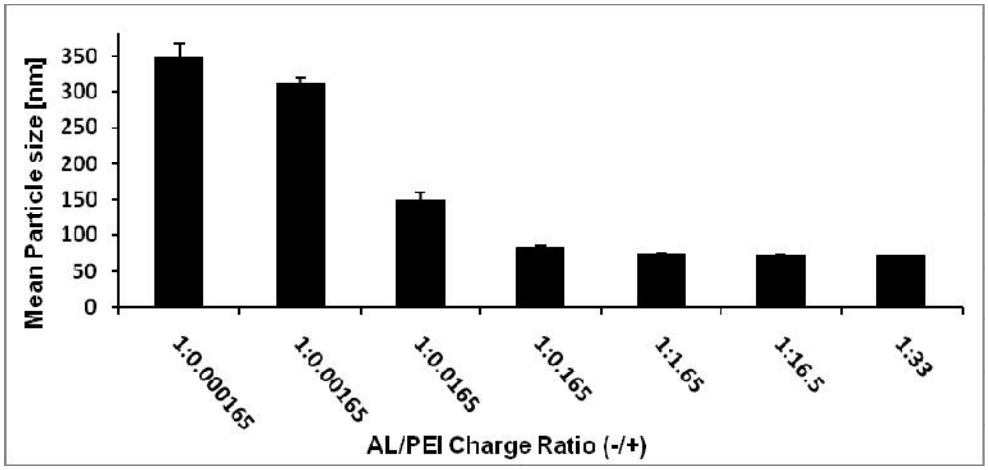

Fig. 4. Mean hydrodynamic particle diameter of PEI/alendronate associate complexes prepared in acetate buffer (pH=5) at different charge ratios. Data are shown as mean ± SD (n=3)

4. DISCUSSION

4.1 Complexation Efficiency

Formation of AL / PEI complexes is thermodynamically driven by ionic interactions between the cationic charges present in the polymer and the negatively charged phosphate groups of AL. Neves et al. [12] analyzed the molecular features underlying physiological interactions between the phosphate group of bisphosphonates and calcium ions of hydroxyapatite. Since the oxygen atoms of the phosphate groups contribute to a substantial negative charge density and the calcium ions are highly positively charged, it is hypothesized that the dominant intermolecular forces stabilizing these association complexes are electrostatic in nature [13]. The experimentally determined stoichiometric ratio at which AL binds to PEI differs from the theoretical ratio ($1:1.14x10^{-4}$), which corresponds to a charge ratio of 1:0.99. This could be attributed to the branched structure of PEI that may prevent uniform charge-charge interactions between cationic and anionic centers. These findings are in agreement with results reported earlier by Ikonen and colleagues [14] who investigated PEI/DNA and PLL/DNA complexes. In these systems, aggregation behavior drastically increased for polymer/DNA complexes fabricated at an N/P ratio where complexes have no net charge.

The authors explained the fact that the molar PEI/DNA ratio of these association complexes carrying no net charge was not close to unity because of the structure of PEI. In addition to primary amines, the polymer also contains secondary and tertiary amines and, thus, only a fraction of the nitrogens are protonated. Utsuno and Uludag [11] assessed the effect of different pH values on the binding affinity of PEI to DNA. This study identified a correlation between binding affinity and environmental pH value. PEI appeared to form complexes with DNA at pH 6 when compared to pH 7. On the other hand, more PEI molecules at pH 8 were needed to precipitate the DNA. Using conventional Henderson-Hasselbach relationship, the fractions of protonated nitrogen of PEI molecules at pH 6,7, and 8 were calculated revealing about 50% of nitrogens in PEI were protonated at pH 6, whereas only ~21% were protonated at pH 8.

Despite the relatively high concentration of PEI that was used in these experiments, the percentage of complexed AL was 81.29%. This can be attributed to the effect of buffer ions which might affect the complexation efficiency of PEI. The presence of such ions may cause partial dissociation of the AL/PEI ion pairs as acetate ions from the buffer may interchange AL from PEI binding sites. This might explain the presence of free AL in such a relatively high concentration of PEI. Similar result was also described by Ramírez-Rigo et al. [15] whostudied the interaction between the cationic polymer Eudragit E100 and enalapril maleate. They reported that the interaction between Eudragit and anionic drugs yields a high proportion of counter ionic condensation. The presence of non-electrolytes does not affect the equilibrium of

ion-pair formation, whereas the addition of ions to the system generates ion exchanges and regrouping of charges causing a partial dissociation of the ion pairs [15,16].

Analytical determination of free AL by spectrophotometry was performed in the presence of form AL-PEI complexes. To assess whether the presence of Fe (III) ions used for the spectrophotmetric quantitation of free AL negatively influences the AL-PEI complex, comparative experiments were performed using equilibrium dialysis (data not shown). In this method, free AL at equilibrium is physically separated from AL-PEI complexes by the semi-permeable dialysis membrane (MW cut-off: 6,000-8,000 Da) and can be quantified without interference with the complexes using the same UV method. Mass-balance calculations revealed a free fraction of 14.9% at a charge ratio of 1:33, which is comparable to the results summarized in Fig. 3. Consequently, it was concluded that presence of Fe (III) did not dramatically alter the complex formation between AL and PEI under given conditions allowing analytically valid determination of free AL without additional separation of the formed ion pair complexes.

4.2 Particle Size Measurement

The hydrodynamic diameter measured for these association complexes varied with the polymer/drug ratio. As the ratio increases, the particle size decreased. However, further increase in polymer concentration beyond a threshold value did not translate into additional particle size reduction. This phenomenon was attributed to the polymer conformation itself. The hyperbranched PEI has extended and flat side chains due to the condensed positive charges of the polymer [17]. Interaction with the negatively charged phosphate groups of AL may result in a partial collapse of the polymer chains due to charge neutralization and, hence, resulting in decreased chain extension. This collapse induced a reduction in polymer volume associated in coil size degree. It was then concluded that formed complexes are kinetically stable colloidal dispersions rather than thermodynamically stable systems [17].

Similar results were also reported by Hinrichs et al. [18]. They studied the interaction between poly (DMAEMA-co-NIPAAm) and plasmid by DLS and zeta potential measurements to determine the size and charge of the complexes, respectively. At polymer/plasmid weight ratios smaller than two, relatively large aggregates with a polydispersity index (PDI) larger than 0.3 were formed, whereas at polymer/plasmid weight ratios larger than two, the particles size and PDI were 150–200 nm and 0.1–0.2, respectively. The formation of large aggregates at a low poly DMAEMA/plasmid ratio can be ascribed to cross-linking of plasmid by the polymer. At higher poly DMAEMA/plasmid ratios, enough poly DMAEMA was present to maximally cover the plasmid with polymer by which crosslinking was prevented. As a consequence, the plasmid structure is condensed to small particles.

Formation of ion pair association complexes is primarily driven by electrostatic attraction between oppositely charged functional groups. At pH = 5, the negatively charged AL is predicted to experience electrostatic attraction to the hyperbranched PEI. As a consequence, compact monodisperse ion complexes where AL is surrounded by positively charged PEI strands. Using experimental technologies such as ellipsometry, X-ray and atomic force microscopy, it could be explored whether these interactions result in weaker intra-molecular electrostatic repulsion between PEI strands, which will affect polymer charge density and conformation [17].

Generally, it was found that particle size will affect the mechanism by which particles are absorbed from the small intestine. Particles under 50 nm permeate by kneading between epithelial cells, particles between 50 nm and 500 nm are absorbed by endocytosis, and large particulates under 5 μm can be taken up by lymphatic uptake through M cells of Peyer's patch [19]. As shown in particle size analysis, the formed complexes are quite heterogeneous in particle size ranging from 71–349 nm. It is expected that the smallest particles will to be absorbed through gut wall predominantly via passive diffusion. However, utilization of physiologically available transports mechanisms for colloidal material (e.g., pinocytosis or clathrine-medited endocytosis) may become available in the future by selectively targeting M cell in Peyer's patches.

4.3 Zeta Potential Measurement

As summarized in Table 3, zeta potential of association complexes increases as the concentration of PEI increases. However, once the drug/polymer ratio reaches 1:1.65 charge ratio the zeta potential remained almost constant. These results are similar to those reported by

Hinrichs and co-workers [18], who found that with increasing poly DMAEMA/plasmid ratios the zeta potential gradually increased until a plateau value of around +26 mV was reached at a polymer/plasmid weight ratio of 2–4. The increased zeta potential with increased polymer/plasmid ratios can be ascribed to a gradual interaction of the negatively charged plasmid with the positively charged polymer. Above a certain copolymer/plasmid ratio, the zeta potential remains constant. This indicates that the extra amount of polymer will be present as free polymer.

This dominant positive zeta potential may have an effect on the stability of the formed complexes that will be studied later. This result was similar to that found in the complex formed between Eudragit E100, a cationic polymer, and enalapril maleate, an anionic drug. It was found that the high and positive zeta potentials indicated significant electrostatic repulsions between the colloidal particles that correlate with observed physical stability [15].

Table 3. Influence of alendronate/PEI charge ratio on size and zeta potential of the electrostatically stabilized associate complexes formed in 0.05 M acetate buffer at pH=5

AL/PEI charge ratio (-/+)	Size [nm]	Zeta potential [mV]
1:0.000165	349.0±17.9	-0.22±0.2
1:0.00165	203.9±11.6	-0.08±0.3
1:0.0165	149.7±9.3	14.50±0.6
1:0.17	82.4±2.9	20.80±1.3
1:1.7	74.9±1.4	31.80±1.2
1:16.5	72.9±0.4	35.20±3.6
1:33	71.0±1.4	37.90±2.8

Data are shown as mean ± SD (n=3)

5. CONCLUSION

The results obtained from this study demonstrate increasing complexation efficiency of AL in the presence of increasing PEI up to a charge ratio of 1:33. Particle size analysis showed decreased particle size with increasing charge ratio between alendronate and PEI. Particle size decreased from 349 nm to a limiting value of 71 nm at a charge AL/PEI ratio = 1:33. Consequently, it is conceivable that association complexes may be small enough to boost oral absorption with a significant contribution of transcellular transport mechanisms specialized for colloidal material. Future studies will have to address whether AL effectively dissociates from the ion pair complex after absorption across the intestinal mucosa in order to exert its desired pharmacological effect.

COMPETING INTERESTS

The authors declare that they have no competing interests.

REFERENCES

1. Sultana S, Ali R, Talegaonkar S, Ahmad FJ, Mittal G, Bhatnagar A. *In vivo* lung deposition and sub-acute inhalation toxicity studies of nanosized alendronate sodium as an antidote for inhaled toxic substances in Sprague Dawley rats. Environmental Toxicology and Pharmacology. 2013;36:636.
2. Walash MI, Metwally ME, Eid M, El-Shaheny RN. Validated spectrophotometric methods for determination of Alendronate sodium in tablets through nucleophilic aromatic substitution reactions. Chemistry Central journal. 2012;6:25.
3. Gupta VK, Jain R, Sharma S, Agarwal S, Dwivedi A. Quantitative determination of alendronate in human urine. International Journal of Electrochemical Science. 2012;7:569.
4. Fulya K, Nevin C. Bisphosphonates and Alendronate. FABAD J. Pharm. Sci. 2006;31:31-42.
5. Lin JH. Bisphosphonates: A review of their pharmacokinetic properties. Bone. 1996;18:75-85.
6. Suresh PK. Ion-paired drug delivery: An avenue for bioavailability improvement. J. Biomed. Res. 2011;3:70–76.
7. Kindermann C, Matthee K, Strohmeyer J, Sievert F, Breitkreutz J. Tailormade release triggering from hot-melt extruded complexes of basic polyelectrolyte and poorly water-soluble drugs. Eur. J. Pharm. Biopharm. 2011;79:372-381.
8. Gennady A, Jasmina N, Anna T. Profiles of drug substances, excipients, and related methodology. Apotex Inc., Toronto, Ontario, Canada. 2013;38. ISSN: 1871-5125.
9. Milan M, Zuzana F, Lucie N, Tomas P. Thermodynamic dissociation constants of alendronate and ibandronate by regression analysis of potentiometric data. Journal of Chemical and Engineering Data. 2011;3848:3854.

10. Specification Sheet of Poly (ethyleneimine) solution. Available:http://www.sigmaaldrich.com/catalog/product/aldrich/181978
11. Utsuno K, Uludag H. Thermodynamics of Polyethylenimine-DNA Binding and DNA Condensation. Biophysical Journal. 2010;99:201–207.
12. Neves M, Ganoa L, Pereira L, Costa MC, Costa MR, Chandia M, Rosado M, Fausto R. Synthesis, characterization and biodistribution of bisphosphonates Sm-153 complexes: Correlation with molecular modeling interaction studies. Nuclear Medicine and Biology. 2002;29:329-338.
13. Fry AJ. Computational studies of ion pairing 9. The "steric" effect of tetraalkylammonium ions with electrochemically generated anions is not steric. Electrochemistry Communications. 2013;35:88-90.
14. Ikonen M, Murtomaki L, Kontturi K. Controlled complexation of plasmid DNA with cationic polymers: Effect of surfactant on the complexation and stability of the complexes. Colloids and Surfaces B: Biointerfaces. 2008;66:77-83.
15. Ramirez-Rigo MV, Olivera ME, Rubio M, Manzo RH. Enhanced intestinal permeability and oral bioavailability of enalapril maleate upon complexation with the cationic polymethacrylate Eudragit E100. European Journal of Pharmaceutical Sciences. 2014;55:1-11.
16. Kindermann C, Matthee K, Sievert F, Breitkreutz J. Electrolyte-stimulated biphasic dissolution profile and stability enhancement for tablets containing drug-polyelectrolyte complexes. Pharm. Res. 2012;29:2710-2721.
17. Bain CD, Claesson PM, Langevin D, Meszaros R., Nylander T, Stubenrauch C, Titmuss S, Von Klitzing R. Complexes of surfactants with oppositely charged polymers at surfaces and in bulk. Advances in Colloid and Interface Science. 2010;155:32-49.
18. Hinrichs WLJ, Schuurmans-Nieuwenbroek NME, Van de Wetering P, Hennink WE. Thermosensitive polymers as carriers for DNA delivery. Journal of Controlled Release. 1999;60:249-259.
19. Lee HE, Lee MJ, Park CR, Kim AY, Chun KH, Hwang HJ, Oh DH, Jeon SO, Kang JS, Jung TS, Choi GJ, Lee S. Preparation and characterization of salmon calcitonin–sodium triphosphate ionic complex for oral delivery. Journal of Controlled Release. 2010;143:251-257.

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