Fibroin-coated poly(ethylenimine)-docusate nanoparticles as a novel drug delivery system

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Poly(ethylenimine) (PEI), a polymer with monomers composed of amine groups bound to ethylene molecules, has gained increasing interest as a material for nanoparticulate drug delivery systems. However, its high toxicity due to excessive positive charge hinders the versatility of PEI in biomedical applications. Thus, this work aimed to develop and characterize novel PEI-based nanoparticles as a drug delivery system, with reduced surface charge, through polyelectrolyte complexation with a negatively charged compound. Among three candidates, fibroin, sodium docusate (DO) and alginate, DO yielded the best results. Further coating the PEI–DO nanoparticles with fibroin significantly reduced the system surface charge from +57.3 to +39.3 mV. The fibroin-coated PEI–DO nanoparticles were loaded with the model drug α-mangostin, which had a spherical shape with a hydrodynamic size of 260 nm, surface charge of +39.3 mV, entrapment efficiency of 94.6%, and drug loading capacity of 2.96%. The system increased the α-mangostin solubility up to 25 times and showed a fast drug released characteristic within 30 min. Finally, the developed nanoparticles significantly reduced the α-mangostin hematotoxicity. In conclusion, the novel fibroin-coated PEI–DO nanoparticles could be further studied as a potential drug delivery system.

Keywords: Drug delivery system, fibroin, nanoparticles, poly(ethylenimine), sodium docusate, α-mangostin.

RECENTLY, research on nanoparticles and their applications, especially in the biomedical field, has gained favourable attention. Due to their unique characteristics, including high surface-area-to-volume ratio, altered quantum features and the ability to encapsulate/adsorb various kinds of molecules, nanoparticles have been extensively utilized as drug delivery systems in disease prevention, diagnosis and treatment[1,2]. Nanomedicine could be categorized into three different types; polymer-based, lipid-based and inorganic nanoparticles. Among them, research is mainly focused on polymeric nanoparticles because of their simplicity in preparation, inexpensive and abundant materials and structure modifiability[3].

One of the interesting materials is poly(ethylenimine) (PEI), a polymer with monomers composed of amine groups bound to ethylene molecules. As a water-soluble polycationic substance, PEI and its modified moieties could form non-covalent polyelectrolyte nano-complexes with negatively charged DNA/RNA, and are widely used biologically in cell culture and DNA transfection[4,5]. Moreover, PEI-based nanoparticles have been used as a drug delivery system, carrying doxorubicin (for cancer)[6], *Punica granatum* peel extract (for oral infection)[7] and insulin (for diabetes)[8].

Nevertheless, PEI-based nanoparticles commonly possess unfavourable side effects of cellular toxicity due to their destabilization or disruption effect on the plasma membranes[4], possibly caused by the high positive surface charge[9]. Since limited approaches have been explored to decrease its positive charge density, research on PEI to reduce its toxicity is necessary. Techniques based on chemical modifications such as acetylation, carboxyalkylation, conjugation and PEGylation could alter the PEI structure and affect its transfection ability[9]. Therefore, polyelectrolyte complexation of PEI with other negatively charged molecules could be a beneficial approach due to its ease of manufacture and up-scaling, simple equipment, environment-friendly nature (i.e. no organic solvents) and versatility. To the best of our knowledge, this technique was reported with only dextran sulphate as a negatively charged molecule[7,8,10]. Hence, further studies are necessary.

The present study aimed to find the most suitable candidate to form polyelectrolyte nano-complex with PEI, to reduce its surface positive charge density and preserve its ability to carry drugs. To this end, various (poly)anionic compounds were selected for screening, including silk fibroin, sodium docusate (DO) and sodium alginate. Fibroin is a natural negatively charged protein extracted from *Bombyx mori* silkworm that has been utilized for numerous biomedical applications[11–14]. DO, a mild laxative and stool softener, is a negatively charged molecule used clinically to treat constipation. Alginic acid, a negatively charged polysaccharide found in the cell walls

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of brown algae, has been commonly used in the pharmaceutical industry[15]. The optimal formulation was further incorporated with a model drug, α-mangostin, and its properties such as particle size, shape, zeta potential, drug entrapment efficiency, loading capacity, aqueous stability, solubility, release profiles and hematotoxicity were studied.

Materials and methods

Materials

PEI (branched, MW = 25,000 Da) and standardized α-mangostin were purchased from Sigma-Aldrich, Singapore. DO and sodium alginate were purchased from Merck, Singapore. *B. mori* silkworm cocoons were collected from Bodin Thai Silk Khorat Co, Ltd, Thailand. Sheep whole blood was supplied by Nanomed Co Ltd, Thailand. Other chemicals were of analytical grade or higher.

PEI-based nanoparticles formulation

Three negatively charged compounds, including fibroin, DO and alginic acid were polyelectrolyte complexed with PEI. DO and sodium alginate were purchased in their pure forms. Fibroin was extracted from silkworm cocoons following standardized protocol[16–18].

For formulation, 1 ml of 1% PEI aqueous solution (pH 7.0), was added into 2 ml solution of either 1% fibroin or DO or alginic acid. The mixtures were stirred at 250 rpm for 30 min, followed by centrifugation at 31,514 *g* for 30 min (Mikro 220R, Hettich, Germany) to precipitate the nanoparticles. For the washing step, the particles were washed thrice by redispersing in de-ionized (DI) water followed by centrifugation at 31,514 *g* for 30 min. The final products were kept in the dispersion form for further experiments. Formulations were freshly prepared prior to all experiments. Finally, the formulations were optimized by varying: (i) the PEI pH (6.0 and 7.0), (ii) the volume of negatively charged compound solution (0.2–2.0 ml) and (iii) the manufacturing time (0–240 min).

Drug-loaded PEI-based nanoparticles

The optimal formulation was chosen to encapsulate the model drug α-mangostin. To this end, 0.2 ml of α-mangostin solution in DMSO (2.5 mg/ml) was added simultaneously into the mixtures of 1% PEI and 1% negatively charged compound. The particles were then formulated following the procedure described in the 'PEI-based nanoparticles formulation' section.

Particle characterization

Particle size, polydispersity index and zeta potential: Dynamic light scattering (DLS) and phase analysis light scattering (PALS) techniques (ZetaPALS® analyser, Brookhaven Instrument Corporation, USA) were utilized to determine the mean particle size and polydispersity index, and zeta potential respectively. All measurements were done in triplicate, following a previously described procedure[12].

Particle morphology: The morphology of optimal formulation was determined using transmission electron microscopy (TEM, Tecnai G TF20, 200 kV, Philips, USA). Briefly, 20 µl of sample dispersed in water was dropped onto a carbon-coated 300-mesh copper grid, negatively stained with 10 µl uranyl acetate 2% (w/v), air-dried and observed under TEM nitrogen atmosphere.

Drug entrapment efficiency and loading capacity: To calculate the entrapment efficiency (EE%), 1 ml of the nanoparticles dispersion was centrifuged at 31,514 *g* for 40 min. The precipitated particles were then extracted with EtOH thrice, and the ethanolic phase was measured using UV–VIS spectrophotometry (Genesis 10S, Thermo Fisher, USA) at λ_{max} = 320 nm, and calculated based on α-mangostin standard curves in EtOH. EE% was determined using eq. (1) below, with the total α-mangostin amount being 0.5 mg. To obtain the drug loading capacity (DL%), the nanoparticles were freeze–dried using a lyophilizer (Heto PowerDry LL3000, Thermo Fisher, USA, at –55°C). Then, 10 mg of freeze–dried nanoparticles was extracted thrice with 5 ml EtOH, followed by centrifugation. The α-mangostin in the supernatant was determined by UV–VIS spectrophotometry and DL% was calculated using eq. (2) below.

$$\text{EE\%} = \frac{\alpha\text{-Mangostin amount in ethanolic phase}}{\text{Total } \alpha\text{-mangostin amount}} \times 100, \tag{1}$$

$$\text{DL\%} = \frac{\alpha\text{-Mangostin amount in ethanolic phase}}{\text{Total nanoparticles amount}} \times 100. \tag{2}$$

Drug release profiles: The α-mangostin release profile from the PEI-based nanoparticles was determined using the shaker method at 37° ± 0.5°C. The dissolution medium composed of PBS (pH 7.4) + 0.1% Tween 80. A fixed amount of particles containing 1 mg of α-mangostin were dispersed in 20 ml of the release medium and shaken at 250 rpm for 24 h. At each time point, 1 ml of the sample was withdrawn and fresh buffer refilled. Then, the sample was centrifuged at 31,514 *g* for 5 min and the supernatant was measured using UV–VIS spectrophotometry at 320 nm. The percentage of α-mangostin release was calculated as follows.

$$\%\ \text{Cumulative release} = \frac{C_t V_0 + V\sum_1^{t-1} C_i}{M_0 - \sum_1^{t-1} M_i} \times 100, \tag{3}$$

where C_t and C_i are the concentration of released α-mangostin at time point t and i respectively, V_0 the total

volume of the dissolution buffer (20 ml), V the sample volume withdrawn at each time point (1 ml), M_0 the initial amount of α-mangostin (1 mg) and M_i is the total amount of α-mangostin withdrawal at time point i.

Hematotoxicity test

The hemolysis action (i.e. the ability to lyse red blood cells) of the unloaded and α-mangostin-loaded PEI-based nanoparticles was *in vitro* examined using sheep blood. Briefly, erythrocytes were collected by centrifugation of sheep whole blood at 2432 *g* for 5 min, followed by washing twice by re-dispensing the cells in PBS, and reconstituted in PBS at a concentration of 1% w/v (1% hematocrit). Then, formulations and the free drug at concentrations of 2.5, 5.0, 10, and 20 μg/ml, equivalent to α-mangostin, were incubated with 1 ml of the prepared erythrocytes at 37°C for 30 min. Finally, the mixtures were centrifuged at 2432 *g* for 5 min, and the haemoglobin presented in the supernatants was measured using UV–VIS spectrophotometry at 540 nm. The percentage of hemolysis was calculated as follows.

$$\%\text{Hemolysis} = \frac{A_t - A_n}{A_p - A_n} \times 100, \quad (4)$$

where A_t, A_n and A_p are the absorbance values of the test samples, negative control (PBS), and positive control (water) respectively.

Statistical analysis

Experiments were conducted and analysed in triplicate and the results were expressed in terms of mean ± SD. Student's *t*-test was used to identify the difference between samples, if any, with P values <0.05 for significant figures.

Results and discussion

PEI-based nanoparticles formulation

In search for a potential candidate to form polyelectrolyte nano-complex with PEI, three compounds were selected, including a protein (fibroin), a small molecule (DO) and a polysaccharide (alginic acid). Only PEI–DO could form nanoparticles at the given formulating conditions, whereas PEI–fibroin mixtures formed microparticles, and PEI–alginic acid yielded a gel system. This might be due to the large molecular structures of both fibroin and alginic acid, thus enlarging the polyelectrolyte complexes between them and PEI. Consequently, large-sized microparticles and/or gel matrix was formed rather than small-sized nanoparticles. Therefore, in this study, small, negatively charged molecule (DO) was beneficial for complexation with PEI to yield nanoparticles.

We then optimized the formulation processes by varying numerous factors and determined their effects on the system turbidity, particle size, polydispersity index (PI) and zeta potential. First, the effects of PEI pH and DO content on the particle properties were examined (Table 1). All 12 formulations possessed nano-sized particle (~220–350 nm) with a narrow size distribution (PI < 0.3) and positive surface charge of 65–85 mV. Additionally, the more the DO content, the more the system turbidity, indicating increased nanoparticle formation via PEI and DO complexation. In terms of pH, PEI solution at pH 7.0 generally yielded smaller particles than that at pH 6.0 (220 versus 330 nm). This could be due to the nature of the PEI molecule, which is inherently a polycation with a pI value of 10–11. Thus, at a lower pH of 6.0, PEI gains more positive charge (33% protonated nitrogens) compared to that at a higher pH of 7.0 (23% protonated nitrogens)[19]. Therefore, looser and large PEI–DO complexes were formed at pH 6.0 than at pH 7.0. Conclusively, due to its small size, low zeta potential the second formulation, and high turbidity formulation was chosen for further analysis.

The mixing time in the optimal formulation was then varied from 0 to 240 min. The most suitable mixing time was 30 min, as the particles were not completely formed before 30 min (i.e. dispersion turbidity was not at its peak), and no significant difference in particle properties was noted after 30 min (Table 2). In conclusion, the optimal PEI–DO formulation, composed of 1.0 ml 1% PEI (pH 7.0), 0.5 ml 1% DO, 1.5 ml DI water, at a reaction time of 30 min, was chosen to load the α-mangostin.

Alpha mangostin loaded PEI–DO nanoparticles

The optimal PEI–DO formulation was utilized to encapsulate the α-mangostin and characterize its properties. The PEI–DO nanoparticles could successfully encapsulate the α-mangostin, with an EE% of 76.1 ± 4.1 and DL% of 2.45 ± 0.14, while retaining its particle size similar to the unencapsulated one. Nevertheless, its high zeta potential of +57 mV was considered not ideal and thus, could be improved. To this end, we further modified the PEI–DO nanoparticle surface by coating with 0.2% fibroin solution for 2 h. The coating process was successful, as the TEM photomicrographs demonstrated clear fibroin coating surrounding the PEI–DO particle boundary (Figure 1 *b*), which was not presented in the uncoated particles (Figure 1 *a*).

Interestingly, the fibroin-coated PEI–DO nanoparticles had similar size compared to the uncoated ones; yet they significantly reduced the surface zeta potential (+39.3 mV versus +57.3 mV respectively) and increased EE% from 76.1 to 94.6 and DL% from 2.45 to 2.96. This might be

Table 1. Effects of PEI pH and DO content on the properties of PEI–DO nanoparticles

1% PEI pH 7.0 (ml)	1% PEI pH 6.0 (ml)	1% DO (ml)	DI water (ml)	Turbidity	Size (nm)	PI	Zeta potential (mV)
1.0		0.2	1.8	+++	227 ± 12	0.14 ± 0.01	+75.4 ± 3.5
1.0		0.5	1.5	++++	231 ± 30	0.13 ± 0.01	+69.2 ± 4.1
1.0		0.8	1.2	++++	218 ± 15	0.10 ± 0.01	+65.4 ± 3.0
1.0		1.2	0.8	++++	219 ± 13	0.16 ± 0.02	+73.6 ± 2.8
1.0		1.5	0.5	++++	218 ± 12	0.19 ± 0.02	+72.8 ± 4.3
1.0		2.0	0.0	++++	213 ± 10	0.14 ± 0.01	+70.1 ± 2.7
	1.0	0.2	1.8	+	320 ± 21	0.14 ± 0.01	+83.2 ± 2.9
	1.0	0.5	1.5	++	330 ± 35	0.13 ± 0.01	+80.7 ± 2.1
	1.0	0.8	1.2	+++	345 ± 32	0.08 ± 0.01	+81.1 ± 3.0
	1.0	1.2	0.8	+++	346 ± 30	0.12 ± 0.01	+79.6 ± 2.7
	1.0	1.5	0.5	+++	335 ± 26	0.14 ± 0.01	+78.8 ± 3.2
	1.0	2.0	0.0	++++	320 ± 30	0.12 ± 0.01	+76.5 ± 2.6

PEI, Poly(ethylenimine); DO, Sodium docusate; PI, Polydispersity index.

Table 2. Time reaction of PEI–DO nanoparticles, with 1 ml PEI 1% (pH 7), 0.5 ml DO 1% and 1.5 ml DI water

Time (min)	Turbidity	Size (nm)	PI
0	++	221 ± 15	0.16 ± 0.01
5	++	222 ± 20	0.17 ± 0.01
10	++	220 ± 13	0.18 ± 0.02
20	++±	223 ± 25	0.17 ± 0.01
30	+++	225 ± 30	0.16 ± 0.01
45	+++	230 ± 15	0.18 ± 0.02
60	+++	225 ± 21	0.17 ± 0.01
90	+++	218 ± 10	0.15 ± 0.01
120	+++	218 ± 20	0.16 ± 0.01
180	+++	220 ± 24	0.17 ± 0.01
240	+++	220 ± 12	0.16 ± 0.01

due to the negative charge of fibroin (i.e. carboxylic groups located on the acidic amino acids), which could form additional ionic interaction with the positively charged PEI on the particle surfaces, thus coating the particles and decreasing PEI charge. Moreover, fibroin could form additional hydrophobic interactions and/or hydrogen bonding with α-mangostin, further entrapping it in the particle fibroin shell. This, together with the entrapped drug in the PEI–DO matrix, significantly enhanced the drug EE% and DL%. Additionally, as PEI–DO nanoparticles are charged moieties, they easily attract charged ions and polar molecules present in water (i.e. water itself) to form an additional boundary on their surfaces. Thus, their hydrodynamic size (200–260 nm), determined by the DLS method, was significantly larger than that of their counterparts observed by the TEM method (100–120 nm; Figure 1).

Drug release study

Figure 2 demonstrates the drug release profile of the α-mangostin-loaded, fibroin-coated PEI–DO nanoparticles, in which a burst release characteristic is observed with nearly 100% drug released within 30 min. This can be explained as follows. First, the drug solubility was 25-fold increased from 0.4 ± 0.2 μg/ml of the free α-mangostin to 116.2 ± 8.3 μg/ml of the α-mangostin-loaded, fibroin-coated PEI–DO nanoparticles. Since a drug release profile of nanoparticles is strongly affected by their solubility in the release medium[12,13], a higher solubility correlates with a faster release. Secondly, as previously discussed, most of the drug molecules were bound to the fibroin in the particles surface through weak interactions (i.e. hydrogen bonding, hydrophobic interactions). Thus, the drugs were rapidly released from nanoparticles. The results are beneficial for biomedical applications in which immediate release of drugs is required.

Hematotoxicity study

Positively charged particles are usually bound to negatively charged surfaces of cells, especially the red blood cells, which could cause severe hemolysis. Additionally, α-mangostin, a polyphenolic non-ionic surfactant, might destroy the cell membranes by disrupting the lipid–protein interface[20,21]. Therefore, we tested the cytotoxicity of the blank and α-mangostin-loaded, fibroin-coated PEI–DO nanoparticles, in comparison with the free drug, in sheep red blood cells (Figure 3). Obviously, α-mangostin hemolysis action was logarithmically correlatd with its concentration, in which 50% hemolytic concentration was 16.26 μg/ml, in agreement with a previous study[12]. Interestingly, when being encapsulated in the fibroin-coated PEI-DO nanoparticles, its toxicity at the high concentration of 20 μg/ml was reduced by five times, to approximately 20%. It is worth mentioning here that no significant difference in terms of hemolysis action was found between the blank and α-mangostin-loaded, fibroin-coated PEI-DO nanoparticles, at all the studied concentrations, regardless of drug amount. Thus, the 20% hemolysis did not come from the α-mangostin, but from the fibroin-coated PEI–DO nanoparticles. It could be concluded that the system decreased α-mangostin

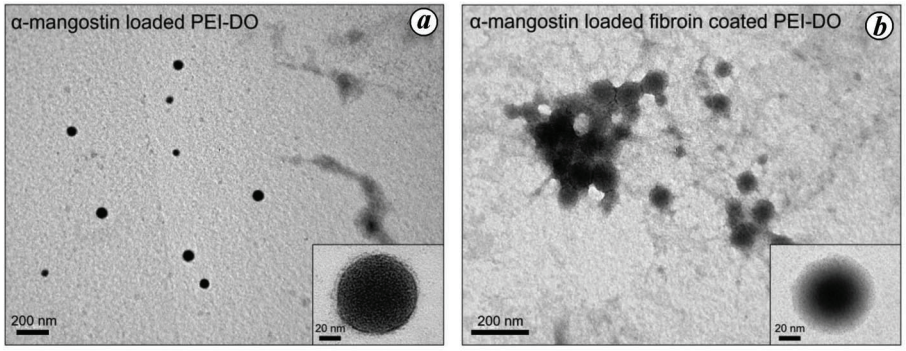

Figure 1. Transmission electron photomicrographs of (***a***) α-mangostin loaded poly(ethylenimine)–sodium docusate (PEI–DO) nanoparticles and (***b***) α-mangostin-loaded fibroin coated PEI–DO nanoparticles. Scale bar: big – 200 nm and small – 20 nm.

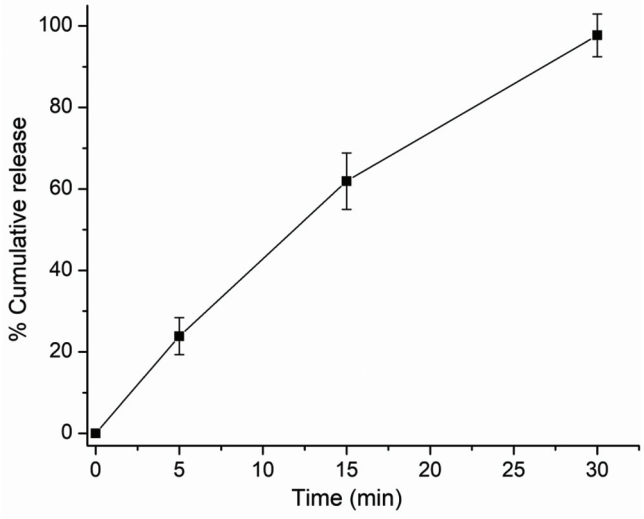

Figure 2. Cumulative drug release profile of α-mangostin loaded fibroin coated PEI–DO nanoparticles (n = 3).

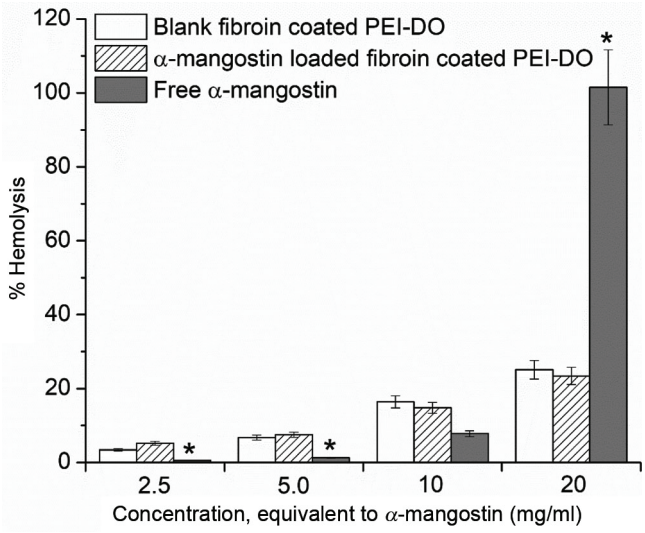

Figure 3. Hemolysis activity of blank and α-mangostin loaded fibroin coated PEI–DO nanoparticles in comparison with the free drug at various concentrations. *Significant differences with other formulations at the same concentration, $P < 0.05$.

hematotoxicity to nearly 0%. This was due to the nanoparticles ability to entrap, protect and alter the α-mangostin polymorph of the nanoparticles.

Compared to the free PEI, which had a hemolysis of 92.67% ± 1.34% at a concentration of 1 mg/ml (ref. 22), our systems (at similar PEI concentration) were 4.5-fold lower in hematotoxicity (20%). This might be attributed to the reduced zeta potential, which made the particles less toxic. A hemolysis of 20%, although high, might not be a real issue in *in vivo* studies due to the high volumes of systemic blood, and small nanoparticle doses, dependent on the applications. Nevertheless, these issues require further research to improve the particle properties.

Conclusion

This study examined the potential negatively charged entities to polyelectrolyte complex with PEI, for the manufacture of novel nanoparticulate drug delivery systems. Among three candidates, viz. fibroin, DO and alginic acid, DO was the best option. The optimal fibroin-coated PEI–DO nanoparticles, loaded with the model drug α-mangostin, had a spherical shape with a size of 260 nm, surface charge of +39.3 mV, EE% of 94.6 and a DL% of 2.96. The system could increase the α-mangostin solubility up to 25 times, and rapidly released the drug within 30 min. Finally, these particles reduced the α-mangostin hematotoxicity from 100% to nearly 0%. In conclusion, the novel, fibroin-coated PEI-DO nanoparticles could be further studied as a potential drug delivery system.

Conflict of interest: The authors declare that they have no conflict of interest.

Statement of human and animal rights: This article does not contain any studies with human participants or animals performed by any of the authors.

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Received 5 June 2021; revised accepted 28 July 2021

doi: 10.18520/cs/v121/i6/775-780