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Short communication

Evaluation of polyamidoamine (PAMAM) dendrimers as drug carriers of anti-bacterial drugs using sulfamethoxazole (SMZ) as a model drug

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Abstract

Sulfamethoxazole (SMZ), a sulfonamide with well-known anti-bacterial properties, is not freely soluble in water and causes problems in its clinical applications. In the present study we investigated the potential of ethylenediamine (EDA) core polyamidoamine (PAMAM) dendrimers as drug carriers of SMZ by aqueous solubility, *in vitro* release as well as anti-bacterial activity studies. Results showed that the aqueous solubility of SMZ was approximately proportional to dendrimer concentration (a 40-fold increase in solubility in 10 mg/ml G3 PAMAM dendrimer solutions compared with that in double-distilled water at 37 °C). The *in vitro* release of SMZ in the presence of PAMAM dendrimers was significantly slower compared to pure SMZ dissolved in ethanol. Microbiology studies showed that PAMAM dendrimers could increase the anti-bacterial activity of SMZ (a 4- or 8-fold increase in the anti-bacterial activity of SMZ in dendrimer solution compared to pure SMZ dissolved in dimethylsulfoxide (DMSO) or 0.01 M NaOH solution). The *in vitro* release behavior and anti-bacterial activity studies indicated that PAMAM dendrimers might be considered as potential drug carriers of sulfonamides with a sustained release behavior under suitable conditions.

Keywords: Polyamidoamine dendrimers; PAMAM; Drug carrier; Anti-bacterial drugs; Sulfamethoxazole

1. Introduction

Bacterial infections remain major causes of morbidity and mortality in hospitals around the world [1]. A new report estimated that *Staphylococcus aureus* (*S. aureus*) infections alone resulted in 9.5 billion dollars in extra hospital charges and nearly 12,000 inpatient deaths per year [2]. Sulfonamides, the development of which is a fascinating and promising area in medicinal chemistry, are widely used in various bacterial infections including enteric and urinary tract, and respiratory tract [3]. They are preferred due to the ease of administration and wide spectrum of anti-bacterial activity. However, the clinical use of sulfonamides is limited mostly due to their extremely low solubility in water, rapid elimination in blood, low level of association to plasma proteins and several side effects, which are characterized by fever, skin rash, hepatotoxicity, lymphadenopathy and hematological disorders [4]. The poor solubility of sulfonamides restricts their use in topical and parenteral applications. As poor solubility is generally related to a low bioavailability, this presents a major challenge during drug formulation. In order to improve the solubility of sulfonamides in water, cyclodextrin–sulfonamides complexes were prepared to enhance dissolution and absorption rate [5,6]. However, high costs and nephrotoxicity on parenteral administration limit

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the use of cyclodextrins. Moreover, the aqueous solubility of the commonly used cyclodextrin is insufficient to stabilize drugs at therapeutic doses [7].

Dendrimers are hyperbranched, monodisperse, three-dimensional macromolecules, having defined molecular weight and host–guest entrapment properties. They allow the precise control of size, shape and placement of functional groups and combine typical characteristics of small organic molecules and polymers that result in special physical and chemical properties [8–11]. Accordingly, dendrimers have attracted increasing attention for their applications in many fields. Among them the use of dendrimers as a drug carrier in delivery systems has been of great interest.

Polyamidoamine (PAMAM) dendrimers with an ellipsoidal or spheroidal shape is one of the most studied starburst macromolecules. Due to specific synthesis, PAMAM dendrimers have some interesting properties, which distinguish them from classical linear polymers, *e.g.* PAMAM has a much higher amino group density comparing with conventional macromolecules, a third generation PAMAM prepared from ammonia core has 1.24×10^{-4} amine moieties per unit volume (cubic Angstrom units) in contrast to the 1.58×10^{-6} amine moieties per unit volume of a conventional star polymer [11]; also, PAMAM dendrimers possess empty internal cavities and many functional end groups, which are responsible for high solubility and reactivity. These specific properties make dendrimers suitable for drug delivery systems [12–15]. Drugs or other molecules can either be attached to dendrimers' end groups or encapsulated in the macromolecule interior [16]. The high density of amino groups and special structure in PAMAM dendrimers may be expected to have potential applications in enhancing the solubility of the low aqueous solubility drugs and as delivery systems for bioactive materials [17]. Drugs bound to dendrimers are at early stages of development and data on them are limited. Several authors reported on the encapsulation of non-steroidal anti-inflammatory drugs (NSAIDs) and anti-cancer drugs in dendrimers [18,19]. However, to our knowledge there are no studies devoted to the solubilization of anti-bacterial drugs in the presence of dendrimers. Here, we focus on using PAMAM dendrimers as potential drug carriers, which are emerging as a promising group of safer and perhaps more effective alternatives to traditional sulfonamides.

The aim of the present work was (1) to investigate the potential of PAMAM dendrimers (G2–G4) as solubility enhancers of sulfonamides as exemplified by SMZ; (2) to study the effect of concentration, generation, ion concentration and temperature on the solubility of SMZ; and (3) to investigate the *in vitro* release behavior and anti-bacterial activity of SMZ in the presence of PAMAM dendrimers.

2. Experiments

2.1. Materials

SMZ was purchased from Shouguang Fukang Pharmacy Factory (Shandong, China); ethylenediamine, methyl acrylate, methanol, and DMSO (HPLC grade) were obtained from Shanghai Chemical Co. (Shanghai, China). For both solubility and *in vitro* release behavior studies, double-distilled water was used.

2.2. Synthesis of PAMAM dendrimers

PAMAM dendrimers were synthesized according to Ref. [20]. Ethylenediamine (10.0 g, 0.166 mol) was dissolved in 100 ml methanol in a 1 L-round-bottomed flask. Methyl acrylate (94.6 g, 0.751 mol) was added at 40 °C and the system stirred for 24 h under nitrogen. Excess methyl acrylate was removed under vacuum at room temperature. A Michael addition between the amine and the acrylate yielded a product bearing four terminal methyl ester groups, defined as the G0.5 PAMAM. Subsequently, ethylenediamine (120 g, 2.00 mol) was dissolved in methanol and added to the G0.5 PAMAM and, after stirring for 48 h under nitrogen and removing excess reactants by vacuum distillation, a product bearing four terminal amino groups were obtained, defined as the G1 PAMAM. By repeating the above cycle, higher generation PAMAM dendrimers (up to G5) were synthesized. Purity of the amine-terminated PAMAM dendrimers was characterized via FT-IR, ^{1}H and ^{13}C NMR and Element analysis. The results agreed well with that reported in the literature [11].

2.3. Solubility test

The solubility of SMZ was determined using the equilibrium solubility method. Excess drugs were added to 500 μl of each test solution to ensure the drug solution reached saturation. The solution was mechanically shaken for 24 h at 37 °C and then the solutions were centrifuged at 10,000 rpm for 3 min. The saturated solutions were then diluted to a proper concentration (500×). Three repeats were conducted.

2.4. UV–Vis spectroscopy

SMZ in ethanol solution or distilled water gives maximum absorbance in UV region at its characteristic wavelength (265 nm for SMZ). A calibration curve of SMZ was prepared at different SMZ concentrations. Perkin–Elmer UV–Vis spectrometer was used to estimate the amount of drug incorporated in the dendrimer. The drug–dendrimer solution was diluted by the same distilled water. Since the dendrimers in the diluted solutions give no absorbance at 265 nm, the absorbance obtained from SMZ–dendrimer solution would be solely from SMZ. This absorbance was correlated with the calibration curve and amount of SMZ was determined [21].

2.5. In vitro release studies

In vitro release behavior of SMZ in the presence of G3 PAMAM dendrimers was investigated [22]. The SMZ was dissolved in dendrimer solutions and diluted by distilled water to a final concentration of 4 mg/ml. Pure SMZ was dissolved in-methanol (4 mg/ml) and used as control. This solution (1 ml in

volume) was transferred to a dialysis bag (M.W. cut off = 1000) immediately. The dialysis bag was placed in a 50 ml beaker containing 40 ml distilled water. The outer phase was stirred continuously. After a scheduled interval of time for 12 h, 100 μl of sample was withdrawn from the outer phase, and the outer phase was again replenished with 100 μl distilled water. The absorbance of the outer phase was monitored at 265 nm using a spectrophotometer in order to characterize the concentration of SMZ.

2.6. Anti-bacterial activity test

The compounds were tested against *Escherichia coli (E. coli)* for their anti-bacterial activities, using a common Luria-Bertani liquid medium micro-dilution method as described in Ref. [32]. Before the anti-bacterial tests, the drug formulations in the presence/absence of dendrimer were prepared as follows: SMZ was dissolved in 10 mg/ml G3 PAMAM dendrimer to a concentration of 2 mg/ml, while pure SMZ was dissolved in DMSO and 0.01 M NaOH at the same drug concentration. G3 dendrimer, DMSO and 0.01 M NaOH solutions were also evaluated in the absence of SMZ. When conducting the anti-bacterial activity studies, 50 μl of Luria-Bertani liquid medium was firstly distributed from the second to the 10th well of a 96-well plate. Then, 100 μl test solutions prepared as above were added to the first test well of each line, and 50 μl of scalar dilution was transferred from the second to the 10th well. Finally, 50 μl of a microbial suspension ($\sim 10^6$ colony forming units, CFU/ml), obtained from an overnight growth at 37 °C, was added to each well of the plate. The final concentration of these samples used to evaluate the anti-bacterial activity was from 2 mg/ml (second well) to 0.0078 mg/ml (tenth well). The plates were incubated for 18 h at 37 °C and examined by measuring the optical density in a spectrophotometer (630 nm). The concentration of each test compound, in which the O.D. 630 nm value is lower than 0.1, was taken as its Minimal Inhibitory Concentration (MIC).

3. Results and discussion

3.1. The effect of dendrimer concentration and generation on solubility of SMZ

The effect of PAMAM dendrimer concentration on solubility of sulfonamides was measured at 37 °C, and the results are shown in Fig. 1. It was observed that the extremely low water solubility of SMZ has been significantly improved by PAMAM dendrimers (a 40-fold increase in solubility in 10 mg/ml G3 PAMAM dendrimer solutions compared with that in double-distilled water). The apparent solubility of SMZ increased linearly as a function of PAMAM dendrimer solution over the whole concentration range. The increase of solubility of extremely low water solubility of SMZ was presumably contributed to the internal cavities that are available to encapsulate SMZ molecules. Due to the specific and interesting property of PAMAM dendrimers, the cavities in PAMAM

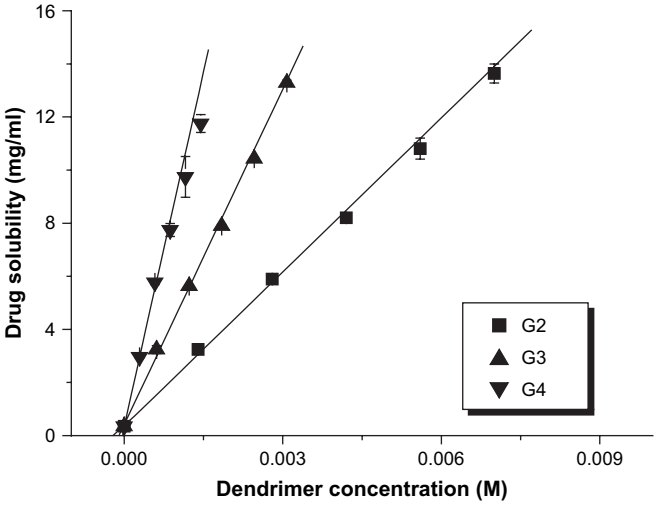

Fig. 1. Solubility of SMZ in the presence of increasing concentration of PAMAM dendrimers.

dendrimers can keep small guest molecules inside and make dendrimers suitable for enhancing the solubility of drug molecules such as SMZ molecules in aqueous solutions [23]. Also, there are tertiary amines in these internal cavities, which could interact with the atoms of the SMZ molecules by hydrogen bond formation [24]. Therefore, PAMAM dendrimers possess open and internal cavities and many functional terminal groups, which are responsible for high solubility and reactivity. These specific properties make dendrimers suitable for drug delivery systems.

The effect of various generations of PAMAM dendrimers (G2–G4) on the process was investigated. The results are also shown in Fig. 1, from which it is clear that the solubility of SMZ was affected by the generation of PAMAM dendrimer. The solubility of SMZ in higher generation PAMAM solution was in fact higher than those in lower ones. The solubility of hydrophobic compounds in dendrimer solutions likely depends on the dendrimer generation (size) [25]. Since the number of cavities and tertiary amines in the dendrimer increases with generation size, at a given pH condition, higher generation dendrimer has a tendency to entrap more hydrophobic compound inside than lower ones. In this way, we could explain why higher generation dendrimers could enhance the solubility of SMZ more efficiently than lower ones.

3.2. The effect of ion concentration on solubility of SMZ in the presence of PAMAM dendrimers

Fig. 2 shows the effect of salt concentration (sodium chloride in this study) on solubility of SMZ in the presence of PAMAM dendrimers. Interestingly, high ion concentration could inhibit the inclusion of SMZ in the cavities of PAMAM dendrimers. Take a fourth generation PAMAM dendrimer (G4) with a molecular weight of 6900 Da and 32 amino groups in the outer shell for example, the approximate number of SMZ molecules associated with each G4 PAMAM dendrimer without any sodium chloride is calculated to be 30 according

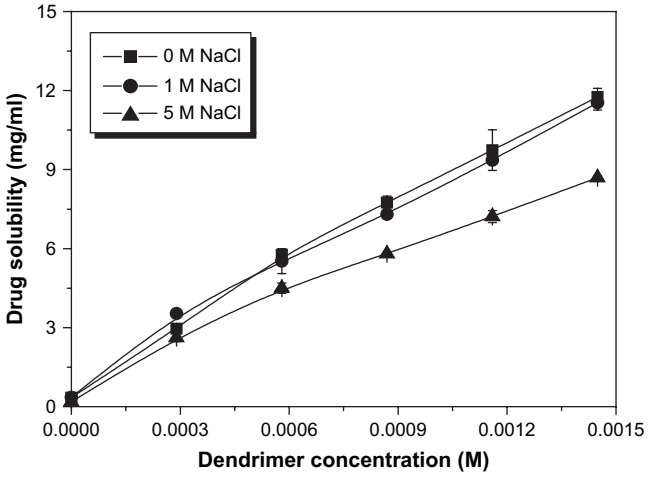

Fig. 2. Solubility of SMZ in the presence of G4 PAMAM dendrimers at various salt concentrations.

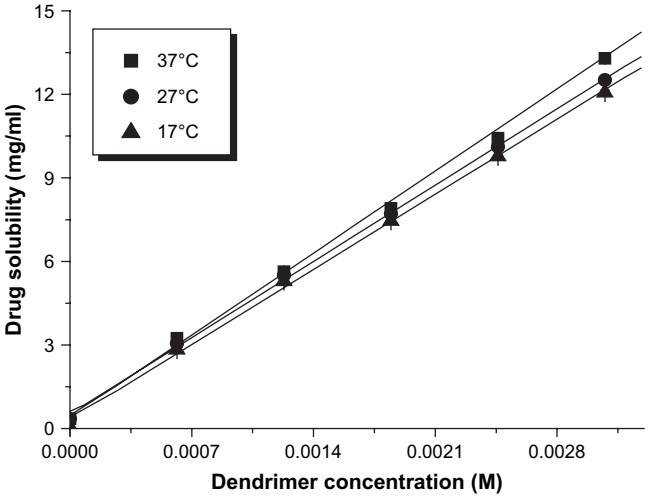

Fig. 3. Solubility of SMZ in the presence of G3 PAMAM dendrimers at various temperatures.

to the slope of the line in Fig. 2; when the salt concentration is 1.0 M and 5.0 M, this number is 27 and 22, respectively. The enhanced SMZ solubility by dendrimers was slightly greater compared to the ones in the presence of sodium chloride with the same concentration of PAMAM dendrimers. Welch and Muthukumar [29] reported that varying the salt concentration in the solvent could change the shape of the intramolecular density profile of dendrimers in solution. Also they found that a reversible between a 'dense core' and a 'dense shell' dendritic structure could be observed as the ionic strength is cycled from high to low. Combining the simulation results of Ref. [29] and the solubility results of this study, it is proposed therefore that conformation of the PAMAM dendrimer in solution can be tailored by varying the ion concentration in the solvent. It was reported that the nature of the intramolecular density profile and the position of the terminal groups are critical in utilizing dendrimers as hosts in controlled release systems [26]. Ideally, the branches of dendrimers would be highly extended at each generation of growth, with branch termini lying at the periphery of the molecule. Several theoretical studies have addressed the possibility of this occurring in flexible dendritic systems [27,28]. Furthermore, Welch and Muthukumar [29] using Monte Carlo simulations reported that the density profiles of synthetic systems are tunable from that of the dense core to that of the dense shell by manipulation of the salt concentration or pH in aqueous solutions. Our data from this study, consistent with earlier data, suggest that large changes in molecular conformation of PAMAM dendrimers are realizable. This is an important factor controlling the release of drug molecules in a variety of environments.

3.3. The effect of temperature on solubility of SMZ in the presence of PAMAM dendrimers

Fig. 3 shows the effect of temperature on SMZ in the presence of G3 PAMAM dendrimer. No significant difference in the solubility of SMZ in the presence of PAMAM dendrimers can be observed from the curves at different temperatures. Milhem et al. [30] reported that the amount of ibuprofen dissolved in PAMAM dendrimers was inversely proportional to temperature. It is interesting that such result was not observed in our experiments. In most cases, the effect of temperature had no significant impact on the dendrimers' host ability.

3.4. In vitro release behavior

After the anti-bacterial drug SMZ was equably dissolved in G3 PAMAM dendrimer, the solutions were filtered through a 0.45 μm HA filter (Millipore). UV–Vis absorbance measurements were carried out for the characterization of precise SMZ concentration in the dendrimer solution. The maximum number of SMZ numbers associated with G3 PAMAM dendrimer was approximately 14 mol/mol of dendrimer. The *in vitro* release behavior of SMZ from the PAMAM dendrimer solution was examined in distilled water at room temperature [22]. The results are shown in Fig. 4. After 1 h, 35.6% of the pure drug is released, whereas only 4.8% is released from the SMZ-G3 dendrimer

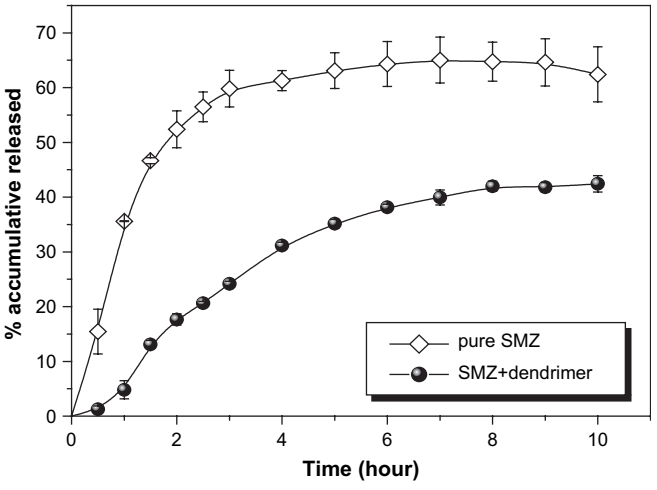

Fig. 4. *In vitro* release of SMZ in G3 PAMAM dendrimer solution compared with the pure SMZ release behavior.

solution. Ten hours after the system started 62.4% release was obtained for the pure drug while 42.4% was obtained for SMZ-G3 PAMAM solution. The release of SMZ from the drug–dendrimer solution was significantly slower compared to pure SMZ.

3.5. Anti-bacterial activities

The anti-bacterial activity of SMZ, dendrimer and SMZ–G3 dendrimer is present in Fig. 5. The results indicated that the three compounds display anti-bacterial activity against *E. coli* at proper concentrations. Interestingly, when equal amounts of free SMZ and SMZ–G3 dendrimer are considered (the actual amount of SMZ in the dendrimer solution was equal to the free drug used), SMZ–G3 dendrimer is definitely more potent than free SMZ dissolved in DMSO or 0.01 M NaOH solution (a 4- or 8-fold increase in anti-bacterial activity). As pure G3 PAMAM dendrimer displayed anti-bacterial activity against *E. coli* (O.D. 630 nm < 0.1) at a much higher concentration (2.5 mg/ml, data not shown), the enhanced anti-bacterial activity should not be contributed to dendrimer itself. It was well-known that PAMAM dendrimers with primary amine surface functional groups could penetrate through cell membrane. We could presume that the enhanced anti-bacterial activity was contributed to the dendrimers, which might favor the interaction of the drug with its target or help SMZ with penetration through the bacterial membrane. The precise reason for this increased activity is at present unclear. Although further investigations are necessary in this respect, the *in vitro* results are very promising as they indicate that appropriate complexation with dendrimer can increase the effectiveness of SMZ while it was used as an anti-bacterial drug. Such a development would increase the clinical use of SMZ.

4. Conclusion

Although dendrimer drug delivery is in its infancy, it offers several attractive features. It provides a uniform platform for drug attachment that has the ability to bind and release drugs through several mechanisms [31]. Our work demonstrated that encapsulation of SMZ into dendrimers led to sustained release of the drug *in vitro* and an increased anti-bacterial activity. We are in the process of conducting pre-clinical testing to evaluate the potential of dendrimers as carrier for SMZ and other anti-bacterial drugs. Although toxicity problems may exist, modification of the structure of dendrimers should resolve this issue.

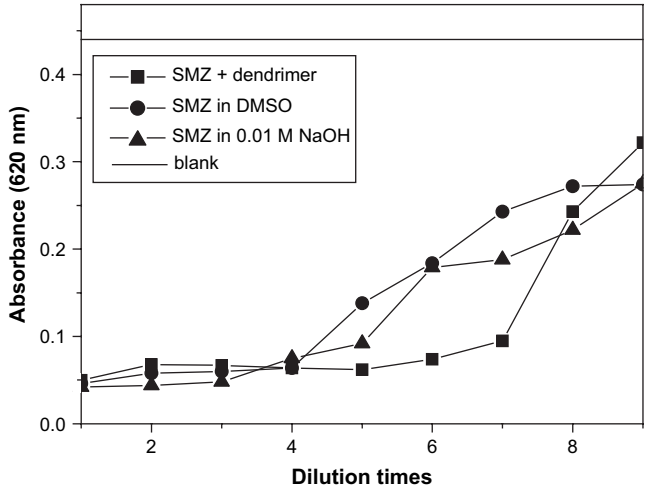

Fig. 5. Anti-bacterial activity behavior of different formations of SMZ (■ SMZ in dendrimer solution; ● SMZ in DMSO; ▲ SMZ in 0.01 M NaOH).

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