Sulfonamides are a class of synthetic drugs, which share structural similarity with PABA (p-aminobenzoic acid)[1]. Sulfonamides and their N-derivatives are being constantly investigated to explore their diverse applications[2]. In the history of human pathology, sulfonamides were the first chemotherapeutic agents, which are effective against bacterial infections. Although penicillin and other antibiotics have diminished the status of sulfonamides[3], but still these drugs are used effectively against the infections of ophthalmic, urinary and gastrointestinal tract[4]. In humans, the sulpha drugs and their salt[5] are still of first choice along with ampicillin and gentamycin, against the infections caused by Escherichia coli.

Sulphamethoxazole (SMX) and trimethoprim (TMP) are co-administered to suppress the growth of Enterobacteriaceae and Gram-negative bacteria. TMP selectively inhibits dihydrofolate reductase (DHFR) and the inhibition of this enzyme leads to the depletion of tetrahydrofolate (THF) and the formation of 5, 10-methylenetetrahydrofolate (5, 10-MTHF). The formation of 5, 10-MTHF is the bottleneck of the folate salvage pathway and it is recycled into THF for the purines and pyrimidines synthesis in bacteria. TMP inhibits DHFR and reduces the folate salvage pathway of bacteria. Sulfonamides compete with p-aminobenzoic acid (PABA) for DHFR, which blocks the formation of tetrahydrofolate. Sulfonamides and sulfa drugs are of synthetic origin and are used as anti-infective drugs, which act by competitive inhibition of DHFR. The sulfonamides are competitive inhibitors of DHFR, which are used as anti-infective drugs in human and animal chemotherapy. Sulphadiazine and sulphadimidine are extensively used as antibacterial drugs and are also used for the treatment of urinary tract infections, skin infections and infections in the respiratory, gastrointestinal and genitourinary systems[6].

Sulphadiazine is a sulpha drug derived from para-aminobenzoic acid (PABA) and it is formulated as white or yellowish-white, granular, free-flowing powder. It shows the characteristic absorption maxima at 270 nm and 306 nm. Sulphadiazine is nontoxic and well absorbed from the gastrointestinal tract. The compound is used as a prophylactic drug in case of urinary tract infections, skin infections and infections in the respiratory, gastrointestinal and genitourinary systems[6].

Sulphadiazine has the following chemical structure:

\[ 
\text{Sulphadiazine} = \text{Para-aminobenzoic acid} + \text{Formaldehyde} + \text{Sodium chloride} 
\]

Sulphadiazine is well absorbed from the gastrointestinal tract and is excreted in urine. Sulphadiazine is metabolized in the liver and excreted in urine. It is used as an anti-infective drug in the treatment of urinary tract infections, skin infections and infections in the respiratory, gastrointestinal and genitourinary systems[6]. Sulphadiazine is a sulpha drug that is used as an anti-infective drug in the treatment of urinary tract infections, skin infections and infections in the respiratory, gastrointestinal and genitourinary systems. It is a synthetic drug that is derived from para-aminobenzoic acid and is formulated as a white or yellowish-white, granular, free-flowing powder. It shows the characteristic absorption maxima at 270 nm and 306 nm. Sulphadiazine is nontoxic and well absorbed from the gastrointestinal tract. It is used as a prophylactic drug in case of urinary tract infections, skin infections and infections in the respiratory, gastrointestinal and genitourinary systems[6].

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\]
formation of sulpha drugs, its quality and release in the systemic circulations play vital analytical task.

Use of nanotechnology in the current scenario of research and technology is inevitable. Various applications of nanotechnology have been utilised to fabricate nanomaterials useful in the mechanical, electromagnetic and chemical fields. In the pharmaceutical field, particularly in designing dosage forms, nanotechnology is often used to improve the efficacy of various water soluble and insoluble drugs and bioactive molecules through improving solubility, retention time and bioavailability. Mostly these studies are focused on the synthesis and applications of metallic, organic and polymeric nanoparticles while small organic molecules are comparatively less explored[7]. Now a days, nanoaggregates of drugs have emerged as potential candidates for modulation of electronic and molecular properties through molecular design[8,9].

Self-assembly of drug molecules to nanoaggregates is feasible in the aqueous environment through the amphiphilic and hydrophobic interactions[10-12], which result into numerous changes at physicochemical and biochemical level. These aggregations/interactions are one of the deciding factors in determining the destiny of a pharmaceutical preparation in vivo[13]. A small change in physiological conditions might result in the coexistence of many drug aggregates, which could alter biorecognition and transport behaviour of drug candidates. Combinations of such type could become a sensation and might show prominent therapeutic effects. However, estimation of accurate drug efficacy in these cases is a tedious job. The interactions with inter and intra molecules/receptors and transport across cell membrane depended greatly on the size, structure, surface charge and hydrophobicity of the aggregates. Studies related to self-assembled drugs and their in vitro and in vivo therapeutic efficacy are lacking. Polymer-drug nanoconjugates can provide prominent insights into the self-aggregation phenomenon of drugs. This process can further alter/modify aggregation phenomenon and hence the therapeutic effect of drugs. Past years had seen the efforts regarding co-delivery of polymer-drug nanoconjugates to desired target sites[13-15]. Many polymer-drug nanoconjugates have been designed targeting site-selective drug release and many of these are under clinical trials and some have received approval[15-20]. Polymer and antimicrobial drug nanoconjugates show improved solubility and therapeutic efficacy, as well as reduced side effects and multi-drug resistance in comparisons to pure antimicrobial drug[21-23]. Polymer carrier also promotes assembly of drugs and plays vital role in modification of drug nanoaggregates.

Polyethylene glycol (PEG) synthesized by the polymerisation of monomer ethylene glycol and is easily available in a wide range of molecular weights. The main utilisations of PEG in pharmaceutical preparations are; orally as a laxative, excipient in dosage formulations, and coating agent in capsule preparation[24]. PEG is extremely biocompatible and not a biodegradable polymer, which is excreted unchanged by the kidney. PEG is a hydrophilic polymer and can be used to stabilize nanoparticles in aqueous media, increase solubility and avoid aggregation by steric hindrance. As PEG does not have any ionic moiety, risk is almost negligible when it came in contact with charged biological molecules like DNA[25]. Immune system does not recognise PEG easily, so its retention time and in vivo circulation increases and the chances of PEG conjugated drug nanoaggregates to reach the target site becomes high. This is known as the stealth effect and makes PEG a suitable nano-drug carrier[26].

Taking cognizance of reported studies of both antimicrobial drugs and nanoaggregates, it was decided to conjugate both sulfonamide nanoaggregates and PEG to enhance the potential applications in antimicrobial chemotherapy. Initially, sulphanilamide (SM) from the class of sulphonamide antibacterial drugs was selected for evaluating potential applications in sustained drug delivery[27,28]. This is the first attempt by our group to conjugate drug nanoaggregates with PEG to evaluate drug delivery applications against four bacterial strains.

MATERIAL AND METHODS

SM (>99 %), PEG-6000 (AR grade) were purchased from Sisco Research Laboratories Pvt. Ltd., India and are used without further purification. AR grade DMSO was purchased from S. D. Fine-Chem Ltd., Mumbai, India.

Preparation of SM organic nanoaggregates (SMONAs):

SM is a sulfonamide and exhibit antibacterial action against various Gram-positive and Gram-negative bacterial strains by mimicking the action of PABA. The SMONAs were synthesized by reprecipitation method[29]. In this method about 1 ml of SM solution in DMSO was injected in to 50 ml of double-distilled water keeping constant rate of addition and stirring. It
was followed by sonication for about 20 min to produce SMONAs of desired size at ambient temperature. The concentration of injectable solution is the deciding factor for the size of nanoaggregates and here the concentration of SM was varied to get the desired size of SMONAs.

**Preparation of PEG-coated SMONAs:**

Nanoprecipitation is commonly applied to several polymeric systems. In the present case PEG was dissolved in DMSO. Under vigorous stirring this solution was poured into water already containing SMONAs, followed by sonication for 30 min. PEG acted as a stabilising agent for the SMONAs. The nucleation process of polymer immediately began when it came in contact with water and growth of nanoaggregates stopped when stability is reached. Excess organic solvent was then removed from the colloidal suspension by evaporation.

**Entrapment capacity of SM with PEG:**

Weighed amount of SM-loaded PEG was dissolved in methanol, sonicated for 5 min to break the PEG, diluted suitably and then analysed on a UV spectrophotometer at 260 nm\(^{30}\). The encapsulation capacity was determined by the following Eqn., entrapment capacity = actual weight of drug/theoretical weight of drug×100.

**Determination of antibacterial activity:**

*In vitro* antibacterial studies\(^{31}\) of SM, SMONAs and PEG-coated SMONAs were carried out against Gram-positive and Gram-negative strains by disk-diffusion assay. Minimum inhibitory concentration (MIC) values were determined using turbidimetry method\(^{32}\).

**Characterization:**

Techniques such as UV/Vis spectroscopy, fluorescence spectroscopy, dynamic light scattering (DLS), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to characterise the SMONAs and PEG-coated SMONAs.

**UV/Vis spectroscopy:**

The UV-absorption studies of the SM, SMONAs and PEG-coated SMONAs were performed on a Shimadzu (UV-1800) spectrophotometer using 1 cm wide quartz cells. The range of 200-500 nm was examined and baseline correction was applied for each spectrum.

**Fluorescence spectroscopy:**

Steady-state fluorescence was measured using a PerkinElmer Luminescence spectrometer LS-55 using pyrene as an external fluorescent probe (2×10\(^{-6}\) M) at an excitation wavelength of 280 nm at 25°. Temperature was controlled using a built-in temperature controller within ±0.1° by using circulating water bath. The spectra were recorded between 290 and 450 nm using an excitation and emission slit width of 2.5 nm, each.

**DLS:**

DLS measurements were made using a light scattering apparatus (Nano-ZS, Malvern Instruments) equipped with a built-in temperature controller having an accuracy of ±0.1°. A quartz cuvette with a path length of 1 cm was used for measurement and average of 10 measurements was considered as experimental data. Data were analysed using the standard algorithms and is reported with an uncertainty of less than 8 %.

**SEM:**

SEM measurements were performed using a Zeiss Ultra 55-Limited edition SEM. Solutions of SMONAs and PEG-coated SMONAs were placed on a thoroughly cleaned glass surface and dried for 24 h in air before imaging. Silver coating was given to samples before measurement.

**TEM:**

TEM measurements were performed on a JEM-2100 electron microscope at a working voltage of 200 kV. A drop of freshly prepared SMONAs and PEG-coated SMONAs were placed on a carbon coated copper grid (300 mesh), and the residual solution was blotted off. The samples were dried in air at room temperature for 24 h before measurements.

**RESULTS AND DISCUSSION**

The conversion of SM to SMONAs was examined by recording UV/Vis and fluorescence spectra. Formation of nanoaggregates in aqueous solution resulted in broadening of absorption bands accompanied with small red shifts in maxima positions and by decrease of extinction coefficients (hypochromism) as compared to the spectra of the SM in organic solvent at the same concentration (fig. 1a). The fluorescence spectrum of SM manifested the difference in the emission profile of SMONAs in aqueous system and organic solvent system (fig. 1b). The normalized intensity emission profile clearly showed that SMONAs displayed broad featureless emission centred at 350 nm, while SM dissolved in a suitable organic solvent showed fluorescence emission at the 275 nm wavelength. The emission profile SMONAs was recorded at different
dilutions of SMONAs. With increase in concentration of SM for the formation of nanoaggregates, the fluorescence intensity also increased, with no concentration-dependent shift observed in the emission profile (fig. 2). This is due to the fact that, decrease in concentration, did not affect the regular arrangement of nanoaggregates, which were uniformly dispersed in the aqueous phase.

DLS studies were conducted to determine the size of the SMONAs, which was found to be around 100 nm at 25 µM concentration (fig. 3a). SEM and TEM were also conducted to determine the size of the SMONAs (fig. 3b and 3c). SEM and TEM images indicated that variable-sized SMONAs were formed with average size of 100 nm.

The particle size, polydispersity index and zeta potential of SMONAs and PEG-coated SMONAs were also calculated for more clarity (Table 1). pH of the body fluids that usually come in contact with a pharmaceutical formulation changes from acidic to alkaline, depending upon the function of the organ involved. It is necessary to have the knowledge of the effect of pH during the stability studies, transport or release of any pharmaceutical preparation. This part of study explores the stability of SMONAs at different pH, using emission spectroscopy in aqueous medium as shown in fig. 4.

SMONAs showed an increase in emission maximum with increase in pH from 4 to 9, which indicated that greater disintegration of SMONAs occurred in alkaline media and hence would limit applications in drug delivery. This would provide rationale for the use of a capping/coating/stabilizing agent such as PEG-6000 on SMONAs.

Absorption and emission spectra confirmed the formation of PEG-coated SMONAs in an aqueous system (fig. 1). The decrease of extinction coefficients (hypochromic shift) was observed for PEG-coated SMONAs in comparisons to the spectra of the SM (fig. 1). The fluorescence spectra of PEG-coated SMONAs (fig. 1) showed the difference in the emission profile of PEG-coated SMONAs and the SMONAs.

Fig. 1: Absorption spectra and emission spectra of SM and SMONAs (a) Absorption spectra of SM (▬) and SMONAs (▬) (b) emission spectra of SM and SMONAs (c) absorption spectra of SMONAs (▬) and PEG-coated SMONAs (▬) (d) emission spectra of SMONAs and PEG-coated SMONAs. SM is sulphanilamide, SMONAs is Sulphanilamide organic nanoaggregates.
Formation of the PEG-coated SMONAs were further confirmed using DLS, SEM and TEM studies (fig. 3). DLS analysis showed (fig. 3) the formation of the PEG-coated SMONAs resulted in an increase in the size of the nanoaggregates from 100 nm to 197 nm. Interestingly, SEM images were recorded at different optical zoom and showed fern leaf like morphology (fig. 3). TEM studies showed similar morphology with average size of 200 nm (fig. 3). Further drug PEG entrapment capacity was also calculated\[30\], which was observed to be 82.1±2 % suggesting that SM was effectively capped by PEG.

PEG-coated SMONAs did not show any prominent change in emission profile with change in pH (fig. 4), which in turn indicated that there was no disintegration
of PEG-coated SMONAs with change in pH, as observed in the case of SMONA (fig. 4).

Keeping in view the lack of effect of pH on the disintegration of the PEG-coated SMONAs it was decided to extend studies further to determine the effect of biological ions, which might also be responsible for its disintegration. The interactions between SM and PEG-coated SMONAs with ions present in biofluids would help to understand their fate (stability and transport across biological barriers) and influence in the release of SM at the site of action.

Hence, the interactions of PEG-coated SMONAs with major biological ions and few organic acids present in biofluid, investigations were performed using emission spectroscopy at definite intervals of time. The results of the investigations revealed that different ions interacted differently in aqueous systems (fig. 5). The Mg$^{2+}$ ions showed maximum interaction in aqueous solutions of chlorides and sulphates (fig. 5a and 5b) while Na$^+$ ions exhibited maximum interactions in aqueous solutions of carbonates and biphosphates (fig. 5c and 5d) with PEG-coated SMONAs. Fig. 5e suggested that L-ascorbic acid showed greater interactions with PEG-coated SMONAs than nicotinic acid in aqueous system.

To further study the therapeutic effect of SM, SMONAs and PEG-coated SMONAs, these were screened on four different strains of Gram-positive and Gram-negative bacteria to understand antibacterial effects if any. The Gram-positive strain used was *Staphylococcus aureus* (MTCC-740, facultative anaerobic). The Gram-negative strains used were *Escherichia coli* (MTCC-119, facultative anaerobic), *Shigella flexneri* (MTCC-1457, facultative anaerobic) and *Pseudomonas aeruginosa* (MTCC-741, aerobic). Initially, MIC was monitored after 24 h of the treatment (fig. 6). In the case of aerobic Gram-positive bacteria *S. aureus*, MIC was 8 µg/ml for SMONAs followed by SM and PEG-coated SMONAs (MIC 12 µg/ml), which suggested that less concentration of SMONAs was required to inhibit the bacterial growth as compared to SM and PEG-coated SMONAs. For *P. aeruginosa*, MIC was 10 µg/ml for SMONAs followed by SM and PEG-coated SMONAs (MIC 15 µg/ml). In the case of *S. flexneri*, for SMONAs the MIC was 22 µg/ml followed by SM and PEG-coated SMONAs (MIC 44 µg/ml). Against *E. coli*, SMONAS showed an MIC at 15 µg/ml followed by SM and PEG-coated SMONAs (MIC 25 µg/ml, Table 2). From this discussion it could be concluded that the SMONAs exhibited more potent antibacterial effects as compared to parent SM or PEG-coated SMONAs. *S. aureus* was found to be most susceptible to the antibacterial effect of SMONAs. Further, to investigate the potential applications in drug delivery, MIC against different bacteria was determined at three definite intervals of time. It was observed that PEG-coated SMONAs showed promising antibacterial activities when observed after 72 h of treatment (fig. 6).

Furthermore, SEM images of *S. aureus* were also recorded before and after 48 h of the treatment (fig. 7a and 7b). Results of investigations revealed that before the treatment, bacterial size was found normal with no abnormalities in shape. However, after 48 h of exposure

### TABLE 1: DLS, PDI AND ZETA POTENTIAL OF SMONAS AND PEG-COATED SMONAS

<table>
<thead>
<tr>
<th>Particles</th>
<th>DLS diameter (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMONAs</td>
<td>100</td>
<td>0.1003</td>
<td>-11.8</td>
</tr>
<tr>
<td>PEG-SMONAs</td>
<td>197</td>
<td>0.1211</td>
<td>-6.76</td>
</tr>
</tbody>
</table>

![Graph](image1.png)  
![Graph](image2.png)  

Fig. 4: Effect of pH (a) on SMONAs (b) on PEG-coated SMONAs  
- SMONAs at pH 4;  
- SMONAs at pH 7;  
- SMONAs at pH 9

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to the treatments bacterial size and shape changed with some minor bleb formation on the surface of the bacteria which might be due to the disorganisation of the bacterial cytoplasm, which could lead to death of the bacteria.

In conclusion, interactions of SM and SMONAs with PEG-6000 were investigated. DLS, SEM and TEM techniques confirmed the formation of about 100 and 197 nm sized SMONAs of SM without and with PEG, respectively. In aqueous solutions of varying pH, SMONAs showed maximum interactions at pH 9, which could explain highest dissociation under basic pH. Therefore, to make these suitable for drug delivery, SMONAs were processed to PEG-coated SMONAs and were characterized using DLS, SEM and TEM. Effect of pH and electrolytes were also studied to determine various factors responsible for sustained release. Varying pH has no effect on the sustained

Fig. 5: Emission spectra of PEG-coated SMONAs with cations in different aqueous media
- PEG-coated SMONAs, (a) chlorides, - PEG-coated SMONAs+NaCl, - PEG-coated SMONAs+KCl, - PEG-coated SMONAs+MgCl2, - PEG-coated SMONAs+CaCl2. (b) sulphates, - PEG-coated SMONAs+Na2SO4, - PEG-coated SMONAs+K2SO4, - PEG-coated SMONAs+MgSO4, - PEG-coated SMONAs+CaSO4. (c) carbonates, - PEG-coated SMONAs+Na2CO3, - PEG-coated SMONAs+MgCO3, - PEG-coated SMONAs+CaCO3. (d) biphosphates, - PEG-coated SMONAs+Na2HPO4, - PEG-coated SMONAs+K2HPO4. (e) Organic acids, - PEG-coated SMONAs+LAA, - PEG-coated SMONAs+NA
Fig. 6: Comparison of Sustained release effect against different bacteria
(a) *S. aureus* (b) *P. aeruginosa* (c) *S. flexneri* (d) *E. coli*. ▬ SM, ▬ SMONAs, ▬ PEG-coated SMONAs

TABLE 2: *IN VITRO* ANTIBACTERIAL ACTIVITIES OF COMPOUNDS

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Treatment (h)</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>S. aureus</em> MTCC-740</td>
</tr>
<tr>
<td>SM</td>
<td>24</td>
<td>12</td>
</tr>
<tr>
<td>SMONAs</td>
<td>24</td>
<td>08</td>
</tr>
<tr>
<td>PEG-SMONAs</td>
<td>24</td>
<td>12</td>
</tr>
</tbody>
</table>

Fig. 7. SEM images of *S. aureus* at different intervals of time
(a) Before treatment, (b) 48 h incubation after treatment
release of PEG-coated SMONAs. Mg^{2+} showed maximum interactions with PEG-conjugated SMONAs in aqueous solutions of chlorides and sulphates while Na^{+} showed maximum interactions in aqueous solutions of carbonates and bicarbonates. L-ascorbic acid showed more interactions than nicotinic acid with PEG-conjugated SMONAs in aqueous solutions. Antibacterial activity of SM, SMONAs and PEG-coated SMONAs were carried out against *E. coli*, *S. flexneri*, *P. aeruginosa* and *S. aureus*. MIC profile indicated that the PEG-coated SMONAs showed higher antibacterial activity as compared to SM parent drug and SMONAs. Interestingly, highest activity was observed in the case of *S. aureus* (MIC 5). SEM studies were also conducted on the bacteria before and after the treatment which indicated that bacterial cell death was due to the accumulation of the cytoplasm leading to bleb formation. PEG-coated SMONAs showed highest antibacterial activity than that of SM through sustained release. PEG-coated SMONAs could find potential application in the development of sustained release antibacterial preparations.

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**Conflicts of interest:**

There are no conflicts of interest.

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