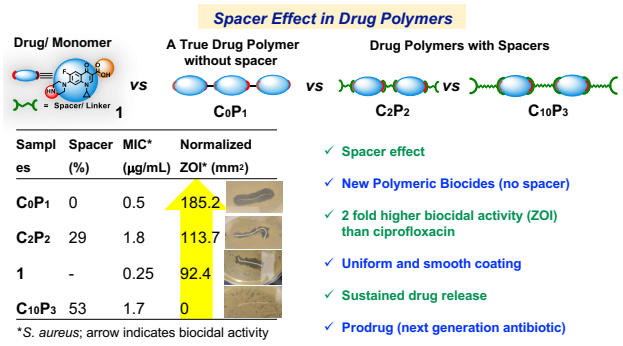
Unravelling the effect of non-drug spacers on a true drug-polymer and a comparative study of their antimicrobial activity

Shaifali Sartaliya, Vijayendran Gowri, Vianni Chopra, Himadri Shekhar Roy, Deepa Ghosh, Govindasamy Jayamurugan*

Institute of Nano Science and Technology, Knowledge City, Sector 81, SAS Nagar, Manauli PO, Mohali, Punjab 140306, India.

E-mail: jayamurugan@inst.ac.in

Keywords: Drug-polymers; Polymeric Biocide; Suture coating; Antimicrobial activity; Ciprofloxacin



Abstract: Several studies have been conducted on polymerisation of drug units using spacers or other polymeric units. In order to study the importance of spacers in drug polymers, we designed polymers with and without spacers. As a proof of concept, herein, we present a comparative study on the efficacy of antibacterial activity using a polymeric biocide (PB) **C_0P_1** having no spacer (0%) and two other PBs with varied spacer content (**C_2P_2:**29%, **$C_{10}P_3$:**53%). We considered **C_0P_1** as a potential new type of PB generated from a widely used fluoroquinolone antibiotic, ciprofloxacin 1, by a simple self-condensation activation with thionyl chloride. Monomer **2** (formylated methyl ester of **1**) was polymerised with ethylenediamine (C_2) and 1,10-diaminodecane (C_{10}) to provide **C_2P_2** and **$C_{10}P_3$**, respectively. The trend for minimum inhibitory concentration study against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) was observed as **1**>**C_0P_1**>**C_2P_2**=**$C_{10}P_3$**>>**2**. Further, after coating on nylon threads, the non-spacer polymer **C_0P_1** showed enhanced zone of inhibition (ZOI) than monomer **1** as well as the spacer polymers owing to its superior coating ability and sustained drug release capabilities. Thus, this study clearly states that the bio-efficacy of a drug-polymer could be retained and enhanced in the absence of non-bioactive spacer units.

1. INTRODUCTION

The current COVID-19 pandemic reaffirmed that prevention from pathogenic microorganisms (PM) is a serious challenge modern science faces to safeguard millions of lives worldwide.[1–7] The possible causes for infections by PM is mainly due to healthcare-associated infections and unhygienic environments.[8,9] The ongoing antimicrobial crisis has increased the gap between existing antimicrobials and resistant microbial strains.[10–13] Since discovering new antimicrobials is expensive and time-consuming, an alternate approach would be favorable to improve the antimicrobial effect of existing antibiotics. One such approach is polymerizing existing antimicrobials to obtain antimicrobial polymers (AMP) with enhanced activity.[14,15] The previously reported AMPs are generally grouped into three categories such as a) biocidal polymer (BP), b) polymeric biocide (PB), and c) biocidal releasing polymer (BRP) (Figure 1). They involve antimicrobial polymers consisting of various biocide units i) polymers holding the biocide as terminal projecting part, ii) biocide unit linked to a polymeric backbone, and iii) physical encapsulation of biocide encapsulated nanocomposite/polymer cluster.[15–23] Interestingly, all cases known so far contain at least a significant volume of non-drug spacer units present in addition to the biocidal unit.

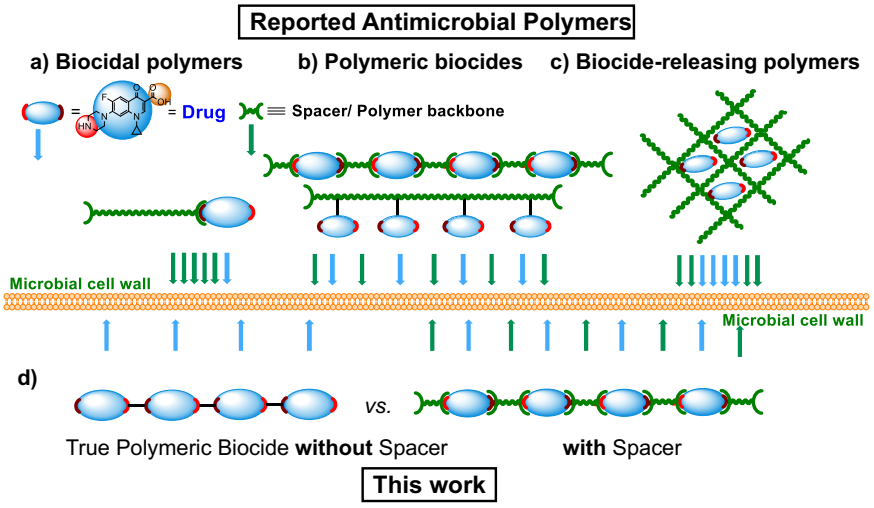


Figure 1. Schematic representation of types of previously known AMPs and a new kind of true PB without the spacer (blue and green arrows indicate releasing of biocidal- and spacer-units, respectively.

The polymerisation of medicinally essential molecules like drugs and antimicrobial agents to obtain PBs has been used as prodrugs to prevent infections by coating medical devices, hospital surfaces, etc.[24–26] PB has recently earned increasing attention from academic and industrial research. Usually, they perform better than their monomer analogs by possessing

enhanced antimicrobial activity, reduced toxicity, minimized environmental hazards, sustained-release activity, and addressing antimicrobial resistance.[27–31]

One of the drug/antibiotic molecule widely used for synthesizing PB is the second-generation fluoroquinolone 1-cyclopropyl-6-fluoro-4-oxo-7-piperazine-1-ylquinoline-3-carboxylic acid (ciprofloxacin) because of its broad-spectrum activity and relatively low cost.[26,32] For example, Woo *et al.* synthesised a biodegradable polymer of ciprofloxacin using 1,6-hexanediisocyanate (HDI), polycaprolactone diol (PCL) as the non-drug-polymer backbone as a spacer. However, the degradation products, *i.e.,* ciprofloxacin and ciprofloxacin bonded to fragments of PCL and HDI had exhibited both active and inactive forms, respectively.[33] This study suggests that releasing the actual drug unit upon degradation without conjugating the non-drug counterpart is pertinent. Similarly, Parwe *et al.* synthesised with increasing ciprofloxacin content in conjugated polylactic acid (PLA) based nonwoven nanofiber mat using telechelic (two-, three-, four-, six-arm, and star-shaped) PLA polymer as a starting material.[34] They observed that the ciprofloxacin release rates from the PLA conjugate nonwoven nanofiber mat could be controlled by the drug loading content and the release medium, indicating that drug content plays a critical role in achieving the higher antibacterial activity. Boyer, Wong, and co-workers have demonstrated that sequence effect in synthetic AMPs could tune the antimicrobial activity.[35] Further, Dizman et al. have prepared homopolymer with a new methacrylate monomer containing norfloxacin as a pendant group by free radical solution polymerisation and found that the resulting drug-polymer was stable even at high temperature than the monomer.[36] However, all these examples demonstrate clearly that non-drug content has significant consequences on the activity of the drug. Therefore, to the best of our knowledge, far less attention has been given to develop self–polymerised/homo-polymerised drugs/biocide monomers, at least of the molecules comprising AB monomer, as shown in Figure 1d. Hence, we investigated this study to find answers to the questions such as i) whether the non-drug spacers used to polymerize the biocide monomer would enhance or reduce the efficacy compared to the drug-polymer *per se*, *i.e.,* without a spacer, ii) can we self-polymerize the currently used antimicrobial drug, and iii) since the new polymeric biocide having no spacer is structurally different from its monomer, can it be regarded as a prodrug and in turn whether this would lead to new drug discovery. Upon screening the literature on the drug's list, we envisaged that ciprofloxacin, a drug molecule with a secondary amine and a carboxylic acid as end functional groups, can be self-sufficient for polymerisation without the need for linkers/ spacers or any polymeric unit.[37,38]

While the self-condensed polymer of ciprofloxacin is unprecedented, the dimer of acetylated ciprofloxacin was reported by Turos and co-workers in a patent and demonstrated its activity against drug-resistant bacteria (Faculative Intracellular Bacteria); however, there was no mention of polymerisation by covalent bonding or self-condensation reaction.[39] Similarly, Fisher and co-workers have also demonstrated that dimers of ciprofloxacin derivatives synthesised using aryl and alkyl linkers effectively target the gyrase enzyme in streptococcus pneumonia.[40] Mesallati and Tajber reported that it is possible to increase the solubility of ciprofloxacin through polymer assisted amorphous salt solid dispersions *via* non-covalent interactions.[41] On that account, covalently linked homopolymer of ciprofloxacin or even an oligomer of ciprofloxacin without any linker has not been reported yet. Although several reports have incorporated the spacers with antimicrobials,[42–44] the concept of the spacer effect on the activity of drug-polymer has not been investigated in detail. Here we tried to examine the same and found that the non-drug spacer length in drug-polymer is inversely proportional to the biocidal activity of polymer, at least in the present case.

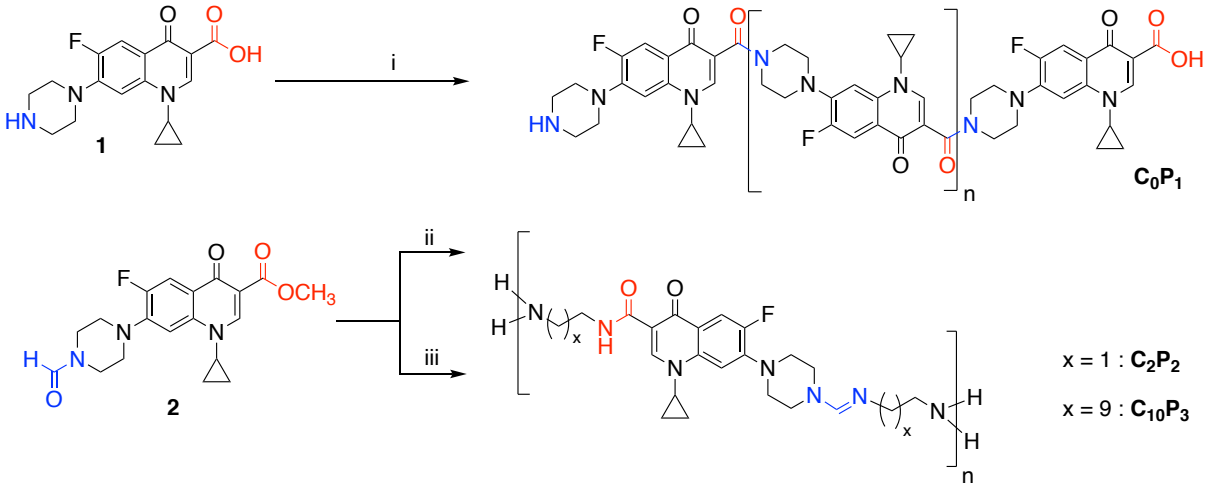
Here we report the synthesis of a new kind of PB for the first time, *i.e.,* $\mathbf{C_0P_1}$ ciprofloxacin polymer having no spacer unit *via* self–condensation polymerisation. For comparison purposes, we have also synthesised two other polymers $\mathbf{C_2P_2}$ and $\mathbf{C_{10}P_3}$, with increased spacer content using *N*-formylated **1** (**2**) and bis–amines. These three polymers were studied for their antibacterial activity against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*), both solution and in the suture coating, and the results are discussed below.

2. RESULTS AND DISCUSSION

Synthesis and Characterisation of Polymeric Biocides and their Monomers. Three polymeric biocides such as $\mathbf{C_0P_1}$, $\mathbf{C_2P_2}$, and $\mathbf{C_{10}P_3}$ were designed with the varied ratio of non-drug content of 0, 29, and 53%, respectively, to assess the role/importance of spacer/non-drug content in drug-containing polymers. The general scheme for the synthesis of these three polymers is shown in Scheme 1. The synthesis of $\mathbf{C_0P_1}$ polymer having no spacer was achieved using commercially available ciprofloxacin **1** *via* self-condensation between carboxylic acid and secondary amine activated by $SOCl_2$ and further heating the reaction mixture at 150 °C in DMSO and Et_3N (1 equivalent (equiv.)) for 48 h to afford $\mathbf{C_0P_1}$ in 55% yield. Whereas to synthesize spacer polymers $\mathbf{C_2P_2}$ and $\mathbf{C_{10}P_3}$ with varying spacer lengths, ciprofloxacin **1** was derivatised to obtain **2** (*N*-formyl and methyl ester functionalised ciprofloxacin) by following reported protocols,[44] to couple *via* imine and amide formation reactions in one pot with *bis*-

amines such as ethylenediamine (C_2) and 1,10-diaminodecane (C_{10}), respectively. The alkyl chains C_2 and C_{10} were chosen because they have marked mass differences and minimal/no antimicrobial activity. For instance, 1,4-diaminobutane is known to have significant antimicrobial activity and toxicity.[45]

Scheme 1. Synthesis of polymers of ciprofloxacin 1 with- and without-spacers[a]



[a]Reagents and conditions: (i) 1. $SOCl_2$, CH_2Cl_2, 40 °C, 6 h, Et_3N, 2. DMSO (10 mL), 150 °C, 48 h, 55% (**C_0P_1**); (ii) ethylenediamine (2 equiv.), CH_3OH, 50 °C, 72 h, 54% (**C_2P_2**); (iii) 1,10-diaminodecane (2 equiv.), CH_3OH, 50 °C, 96 h, 27% (**$C_{10}P_3$**).

The synthesised polymers were characterised by Fourier-transform infrared (FT-IR), nuclear magnetic resonance (NMR), and size exclusion chromatographic (SEC) techniques. The FT-IR spectra of polymer **C_0P_1**, its precursor **1** and the spacer polymers **C_2P_2**, **$C_{10}P_3$,** and their precursor **2** were provided in Figure 2 for comparison purposes. The newly formed peak at 1680 cm^{-1} corresponding to amide carbonyl (C=O stretching) and the reduction in peaks at 3388 and 3239 cm^{-1} corresponding to carboxylic acid (O–H stretching) and amine (N–H stretching), respectively in **C_0P_1** indicate the formation of amide bonds. The spectra of **C_2P_2** and **$C_{10}P_3$** spacer polymers featuring a new merged peak for imine (C=N) and amide (C=O) at ~1660 cm^{-1} and disappearance of the characteristic carbonyl peak for ester and N-CHO at 1724 and 1614 cm^{-1}, respectively, suggest the formation of both amide and imine bonds. More importantly, this indicates that the terminal groups are amine in both cases.

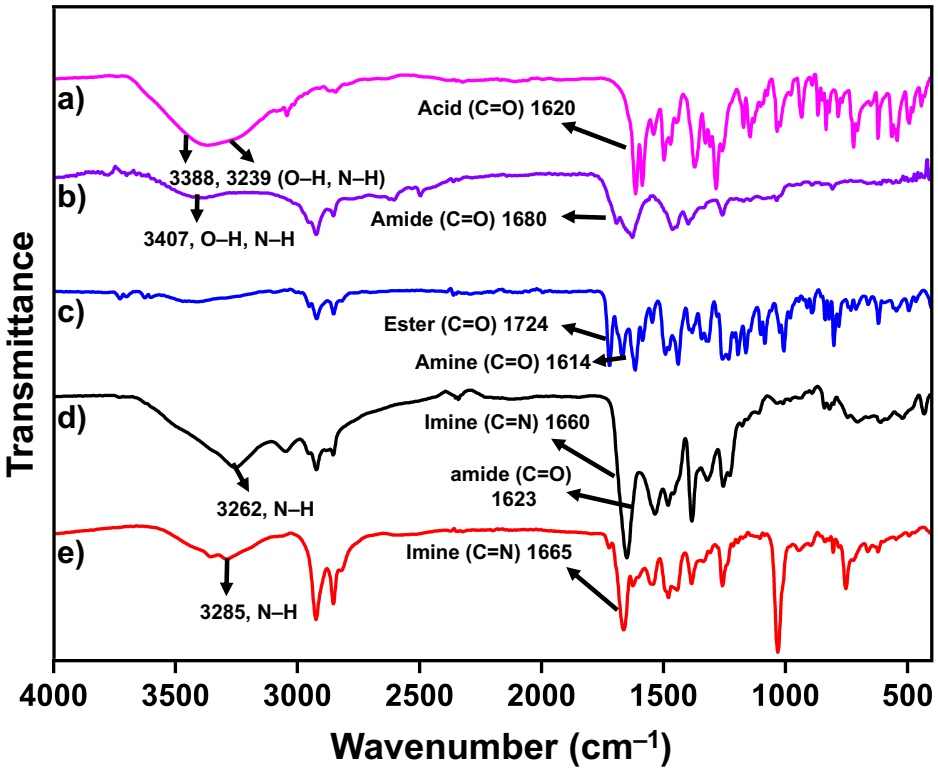


Figure 2. FT-IR spectra of a) monomer **1**, b) non-spacer polymer $\mathbf{C_0P_1}$, c) monomer **2**, spacer polymers d) $\mathbf{C_2P_2}$, and e) $\mathbf{C_{10}P_3}$.

The formation of all polymers was further characterised by NMR spectroscopy and the overlay of ^{1}H NMR spectra provided in Figure 3. The peaks were assigned based on the literature values as well as COSY and ROESY spectra (Section B, Supporting Information).[46–48] ^{1}H NMR spectrum of $\mathbf{C_0P_1}$ in Figure 3a shows not only the characteristic signals with quite broadened nature, which is probably due to large-molecular-weight as corroborated by SEC data (*vide infra*). In contrast, $\mathbf{C_{10}P_3}$ polymer showed quite sharp signals relative to $\mathbf{C_0P_1}$ and $\mathbf{C_2P_2}$ owing to the low molecular weight nature (Figure 3c). The complete absence of methyl ester signal at ~3.9 ppm and the simultaneous appearance of amide (9.92–9.99 ppm) and imine (~8.0–8.12 ppm) protons signals in $\mathbf{C_2P_2}$ and $\mathbf{C_{10}P_3}$ with almost equal intensities are affirmative of formation of alternative amide and imine bonds in both the polymers. Interestingly, the absence of additional methylene protons corresponding to amide-amide or imine-imine coupling suggests the observation of orthogonal reactions for both amine ends. The quite downfield shifted amide protons at 9.95 ppm indicate the presence of an H-bonded network. While increasing oligomeric size due to a higher H-bonded network, it has been observed that the amide protons tend to shift towards the downfield region.[49] It was observed that, unlike $\mathbf{C_0P_1}$, the $\mathbf{C_2P_2}$ had shown partial hydrolysis due to weak imine bond,

thus producing aldehyde and ethylene diamine at 8.11 and 2.63 ppm, respectively. Since the IR spectrum did not feature N-CHO stretching at 1614 cm^{-1}. We infer that partial hydrolysis is because of the water content present in DMSO-d_6 solvent and rested for 24 h. Further, the minimal water content in **$C_{10}P_3$** did not result in considerable hydrolysis, though detectable hydrolysed products were present (Figure 3c).

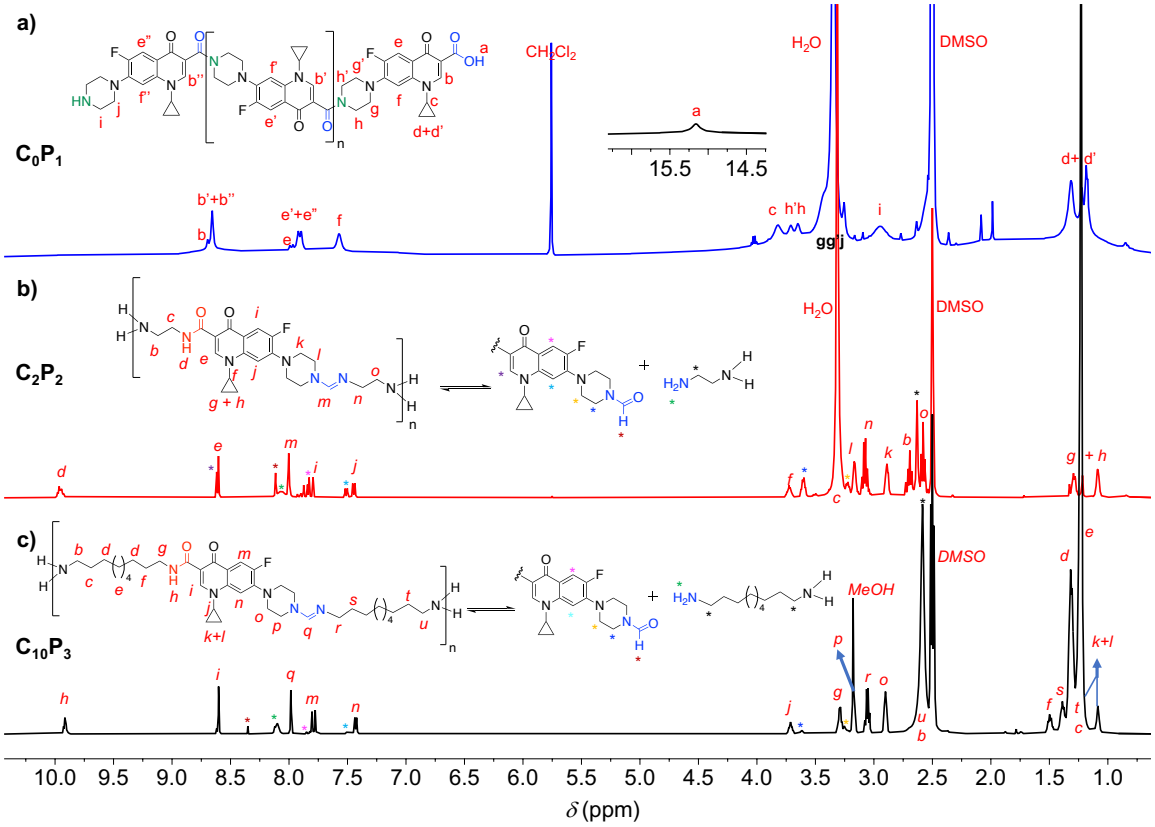


Figure 3. ^{1}H NMR spectra of polymers a) **C_0P_1** (500 MHz), b) **C_2P_2** (400 MHz), and c) **$C_{10}P_3$** (500 MHz) in DMSO-d_6 at 298 K.

Size exclusion chromatography (SEC) was performed, and the results are provided in Table 1 to support the observation of different molecular weights ranges of the polymers as observed by NMR studies. Though **C_2P_2** and **$C_{10}P_3$** are soluble in many organic solvents (like MeOH, CH_3CN, THF, DMF and DMSO, etc.), **C_0P_1** solubility is limited with DMF and DMSO probably due to high molecular weight nature. Hence, the SEC was accomplished using DMF as solvent at 50 °C for all polymers. The SEC analysis revealed that the average molecular weight (M_w) of polymers **C_0P_1**, **C_2P_2**, and **$C_{10}P_3$** were 973.27, 9.61, and 3.74 kDa, respectively. Further, the **C_0P_1** polymer is 42 and 128 fold larger in size than **C_2P_2** and **$C_{10}P_3$**, respectively. The polydispersity index (PDI) values for **C_0P_1**, **C_2P_2** and **$C_{10}P_3$** were found to be 2.72, 1.13 and 1.29, respectively, indicating the spacer polymers exhibit narrow molecular weights as compared to **C_0P_1**.

Table 1. Molecular weights (M_w) and polydispersity index (PDI) of polymers as determined by SEC analysis using DMF at 50 °C and calibrated with linear polystyrene standards.

Polymer	RT (min)[a]	M_n (Kg/mol)[b]	M_w (Kg/mol)[c]	M_w/M_n (PDI)
C_0P_1	09.72	357.9	973.27	2.72
C_2P_2	11.34	8.5	9.61	1.13
$C_{10}P_3$	11.41	2.8	3.74	1.29

[a]RT = retention time, [b]M_n = number average molecular weight (Da), [c]M_w = weight average molecular weight (Da).

2.2. Antibacterial Activity. The antimicrobial activity study was performed against Gram-negative and Gram-positive strains such as Escherichia coli (*E. coli*) and Staphylococcus aureus (*S. aureus*), respectively. The experimental details are described in material and methods (Section C, Supporting Information).[50,51] The screening was done to estimate drug activity changes to unravel the role of spacer (non-drug unit) upon introducing different spacers in drug-polymers. First, a solution-based minimum inhibitory concentration (MIC_{90}) determination study was performed *via* the turbidity assay method (Table 2). Different doses of treatment were used from the stock solutions obtained from 0.1 to 10 μg/mL of all compounds in DMSO. Since the ciprofloxacin monomer is responsible for the antibacterial activity, for a fair comparison, to maintain the number of drug units same for all polymers, the monomer unit in **C_0P_1** was normalized with the repeating unit in spacer polymers for **C_2P_2** and **$C_{10}P_3$** (Table 2). To do that, the normalization factor (*NF*) was introduced to calculate the molecular weight difference ratio in the repeating unit drug *vs* non-drug content. The NF values were calculated to be 1, 1.4, and 2.1 for polymers of **C_0P_1, C_2P_2,** and **$C_{10}P_3$**, respectively. The MIC values for **C_0P_1** PB exhibited 50% reduced activity than the ciprofloxacin **1** against both *E. coli* (**1**: 0.5 mg/mL, **C_0P_1**: 1 μg/mL) and *S. aureus* (**1**: 0.25 μg/mL, **C_0P_1**: 0.5 μg/mL). This reduction in activity could be attributed to the end-group modification of ciprofloxacin and the slow hydrolysis nature of **C_0P_1** with stronger tertiary amide bonds as interlinking functional groups. However, it was found that the 0% spacer polymer, *i.e.,* **C_0P_1**, showed ~1.8 times increased biocidal activity against *E. coli* whereas ~3.6 times against *S. aureus* as compared to the spacer polymers of **C_2P_2** and **$C_{10}P_3$**.

Table 2. Minimal inhibitory concentration (MIC in μg/mL) of compounds (**C_0P_1, C_2P_2, $C_{10}P_3$, 1, 2, and 3**) against panels of Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacterial strains.

entry	Samples	Repeating unit molecular formula	Repeating unit/ monomer molecular weight (Daltons)	Normalization factor (*NF*)[a]	*E. coli*		*S.aureus*	
					MIC (μg/ mL)	Normalized MIC[b] (μg/ mL)	MIC (μg/ mL)	Normalized MIC[b] (μg/ mL)
1	**1**	$C_{17}H_{18}FN_3O_3$	331.4	1.0	0.5	0.5	0.25	0.25
2	**C_0P_1**	$C_{17}H_{16}FN_3O_2$	313.3	1.0	1.0	1.0	0.5	0.5
3	**C_2P_2**	$C_{22}H_{26}FN_7O_2$	439.5	1.4	2.5	1.8	2.5	1.8
4	**$C_{10}P_3$**	$C_{38}H_{58}FN_7O_2$	663.9	2.1	4.0	1.9	3.5	1.7
5	**2**	$C_{19}H_{20}FN_3O_4$	373.4	1.0	NA[c]	NA[c]	NA[c]	NA
6	**3**	$C_{18}H_{18}FN_3O_4$	359.4	1.0	2.0	2.0	1.5	1.5

[a] Normalization Factor (*NF*) = molecular weight of monomer unit of **C_2P_2** or **$C_{10}P_3$**/molecular weight of monomer of **C_0P_1**; [b]Normalized MIC = MIC/*NF*; [c] NA = No activity up to 10 μg/mL addition.

While similar activity trends were observed for both *E. coli* and *S. aureus*, interestingly, in solution, both **C_2P_2** and **$C_{10}P_3$** have shown similar activity of 1.7-1.9 (MIC) for *S. aureus* and *E. Coli*. In contrast, **C_0P_1** has shown significantly increased 0.5 (MIC) activity for *S. aureus* over 1.0 (MIC) for *E. Coli*. This may be because **C_0P_1** contains 100% drug content, whereas the spacer polymers include additional alkyl chains, thus featuring the significantly higher hydrophobic character, which helps the spacer polymers penetrate the hydrophobic regions of bacterial cell wall mediated by hydrophobic effect, thus facilitating membrane disruption. Similar hydrophobic effect observation has been reported, for e.g., Yao and co-workers have observed that *S. aureus* has a higher hydrophobic surface than *E. coli* which helps in the degradation of the hydrophobic compound diethylphthalate.[52] The starting material monomer **2** having formyl and ester functional groups was tested as a positive control sample and found no activity. We inferred that upon hydrolysis of **C_0P_1**, the polymer catalysed either by acid or enzyme, and the hydrolysed product would be the monomer **1**, which makes the **C_0P_1** regarded as a prodrug. Whereas for the spacer polymers, if both amide and imine hydrolysis occurs, the end hydrolysed product would be **3**, *i.e., N*-formyl and carboxylic acid functionalised ciprofloxacin. For this purpose, compound **3** was synthesised by following the reported

protocol for formylation norfloxacin,[53] which was adopted for **1** and characterised.[54] Upon testing the monomer **3**, found that it retained similar moderate activity against *E. coli* (2.0 μg/mL) and slightly better activity against *S. aureus* (1.5 μg/mL) compared to the spacer polymers $\mathbf{C_2P_2}$ and $\mathbf{C_{10}P_3}$, indicating the end functional group do have a role in biocidal activity as observed for monomer **2** (entry 6, Table 2). Overall, according to this study, the trend observed was **1**>$\mathbf{C_0P_1}$>**3**>$\mathbf{C_2P_2}$=$\mathbf{C_{10}P_3}$>>**2**. These results show that the increasing amount of non-drug spacer content in the polymeric biocides reduces the biocidal activity, at least in the present case.

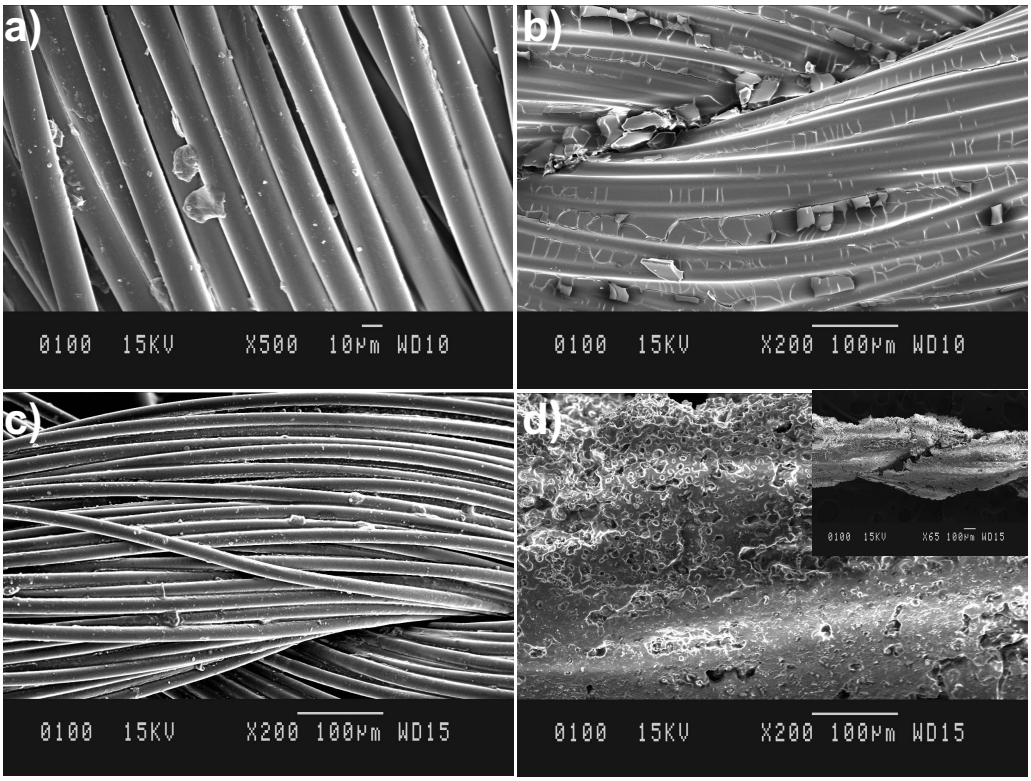


Figure 4. SEM images of nylon sutures coated with a) ciprofloxacin **1**, b) $\mathbf{C_0P_1}$, c) $\mathbf{C_2P_2}$, and d) $\mathbf{C_{10}P_3}$ (inset: zoom scale of X65 showing inter twinning of yarn).

After successfully demonstrating antibacterial activities in solution for polymers with varied spacers, we then tested its antibacterial effect on surgical devices since medical device-based infections majorly (60–70%) belong to hospital-acquired infections.[55,56] Post-surgical incisions can attract bacteria, in particular, via suture materials that can lead to bacterial colonization. To test the efficacy of our polymerised biocides, we coated nylon thread-based sutures with solutions of polymers and monomers and used them as samples for disc diffusion assay. Scanning electron microscopy (SEM) was performed for ciprofloxacin and polymers coated sutures to investigate the coating nature (Figure 4). SEM images of **1** and $\mathbf{C_2P_2}$ show

smooth individual nylon fibers indicating either an insubstantial coating layer or imperfect coating nature.

In contrast, a thick coating layer was observed on the $\mathbf{C_0P_1}$ and $\mathbf{C_{10}P_3}$, wherein individual nylon fibers were not visible. However, $\mathbf{C_0P_1}$ polymer with no spacer showed a pretty smooth and continuous surface, while $\mathbf{C_{10}P_3}$ polymer displayed a porous and rough texture. Further, breakage in the coating was also observed between the inter twinning of yarn in spacer polymer $\mathbf{C_{10}P_3}$ (Figure 4d inset). The SEM study suggests that the polymer $\mathbf{C_0P_1}$ has better coating efficiency and a uniform coating on the nylon sutures, indicating that $\mathbf{C_0P_1}$ is more favorable than spacer polymers $\mathbf{C_2P_2}$ and $\mathbf{C_{10}P_3}$ as well as ciprofloxacin itself. A more uniform coating of $\mathbf{C_0P_1}$ on nylon thread is possibly due to its high viscous nature owing to its larger molecular weight (973.27 kDa). Thus, it appears promising for further coating applications in medical devices.

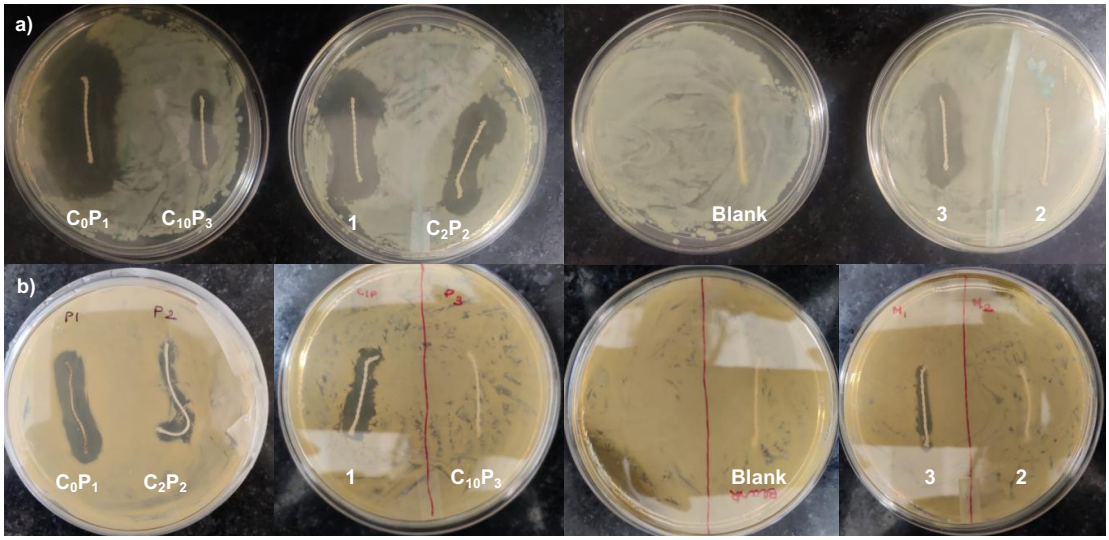


Figure 5. Antimicrobial activity of polymers and monomers coated nylon thread against a) *E. coli*. b) *S. aureus.*

Since *S. aureus* is the common bacteria that infect post-surgical procedures[57,58] and ciprofloxacin **1** is a broad-spectrum antibiotic, the latter derivatives were tested against both *E. coli* and *S. aureus* bacteria. Figures 5a and 5b shows inhibition of *E. coli* and *S. aureus*, respectively, for the polymers and monomers coated sutures placed in the agar plates. The antibacterial activity results of the coated sutures were obtained as the area of the zone of inhibition (ZOI, mm^2) and the corresponding data are summarized in Table 3. Interestingly, the results from the ZOI studies were different from the solution-based MIC determination trend. Figure 5a shows the *E.coli* growth inhibition profile on the coated sutures for polymers and monomers after incubation for 24 h and their corresponding normalized ZOI values are 468.7

mm^2 (**C_0P_1**), 321.9 mm^2 (**C_2P_2**), and 216.1 mm^2 (**$C_{10}P_3$**) and monomers 300.2 mm^2 (**1**), 0 mm^2 (**2**), and 166.5 mm^2 (**3**). Similarly, Figure 5b shows the *S. aureus* growth inhibition profiles on the polymers and monomers after incubation for 24 h and their corresponding normalized ZOI values are 185.2 mm^2 (**C_0P_1**), 113.7 mm^2 (**C_2P_2**), and 2.1 mm^2 (**$C_{10}P_3$**) and monomers 92.4 mm^2 (**1**), 0 mm^2 (**2**), and 42.3 mm^2 (**3**). It was found that the area of the ZOI for **C_0P_1** polymer having no spacer was the most significant 469 and 185 mm^2 for *E. coli* and *S. aureus*, respectively, whereas for **$C_{10}P_3$** (216.1 and 2.1 mm^2 for *E. coli* and *S. aureus*, respectively) was the lowest for both types of bacteria. At the same time, **2** did not show any significant activity even when twice the amount was added than other compounds. The trend for ZOI determination for *E.coli* and *S. aureus* was observed as **C_0P_1**>**C_2P_2**>**1**>**3**>**$C_{10}P_3$**>**2**. Notably, a couple of interesting facts were observed from this study, i) **C_0P_1** exhibited remarkable activity over both bacteria compared to all PBs as well as the monomers including ciprofloxacin itself, ii) Spacer polymer **$C_{10}P_3$** exhibited significant activity for *E.coli*, but almost no activity was observed for *S.aureus*, iii) There is a clear and distinct activity trend was observed among the polymers, *i.e.,* increasing the spacer content led to reduction of inhibiting activity. These observations support the hypothesis that drug-polymers without the spacer can greatly alter the drug activity. More importantly, in this case, it is possible to obtain better antibacterial activity than the parent ciprofloxacin itself. Thus, this study may open up a new window in drug-polymers and may provide the opportunity to discover a new generation of antibiotics that the world is currently looking for, especially in this pandemic time.

Table 3. Zone of inhibition (ZOI in area mm^2) of compounds (**C_0P_1, C_2P_2, $C_{10}P_3$, 1, 2, and 3**) against panels of gram-negative (*E. coli*) and gram-positive (*S. aureus*) bacterial strains.[a]

S.No.	Samples	Spacer content (%)	Normalization factor (*NF*)[b]	*E. coli*		*S.aureus*	
				ZOI (mm^2)	Normalized[c] ZOI (mm^2)	ZOI (mm^2)	Normalized[c] ZOI (mm^2)
1	**C_0P_1**	0	1.0	468.7	468.7	185.2	185.2
2	**C_2P_2**	29	1.4	229.9	321.9	81.2	113.7
3	**$C_{10}P_3$**	53	2.1	102.9	216.1	0	<2.1[d]
4	**1**	-	1.0	300.2	300.2	92.4	92.4
5	**2**	-	1.0	0	0	0	0
6	**3**	-	1.0	166.5	166.5	42.3	42.3

[a]All samples were coated (4 mg/mL); [b]Normalization Factor (*NF*) = molecular weight of monomer of **C_2P_2** or **$C_{10}P_3$**/molecular weight of monomer of **C_0P_1**; [c]Normalized ZOI = ZOI×NF; [d]If activity considered to be 1.

2.3. Hydrolysis study of Polymers. The hydrolysis study for PBs such as **C_0P_1**, **C_2P_2**, and **$C_{10}P_3$** was performed by using the HPLC–LCMS technique employing CH_3CN/H_2O 1:1 with a flow rate of 1 mL per minute (min) associated with other parameters such as column temperature (25 °C), sample temperature (37 °C), injection volume (20 µL) and monitored with photodiode array (PDA) detector (for details see Section D, Supporting Information). Since **C_0P_1** non-spacer polymer does not hydrolyze at neutral pH significantly due to stronger tertiary amide bonds, the release of the drug has been observed by altering the pH into acidic (pH = 3) using 1% (*v/v*) formic acid addition into all polymer solutions (100 µg/mL for **C_0P_1**, 1 mg/mL for **C_2P_2** and **$C_{10}P_3$**) and the release profiles of all polymers are shown in Figure 6.

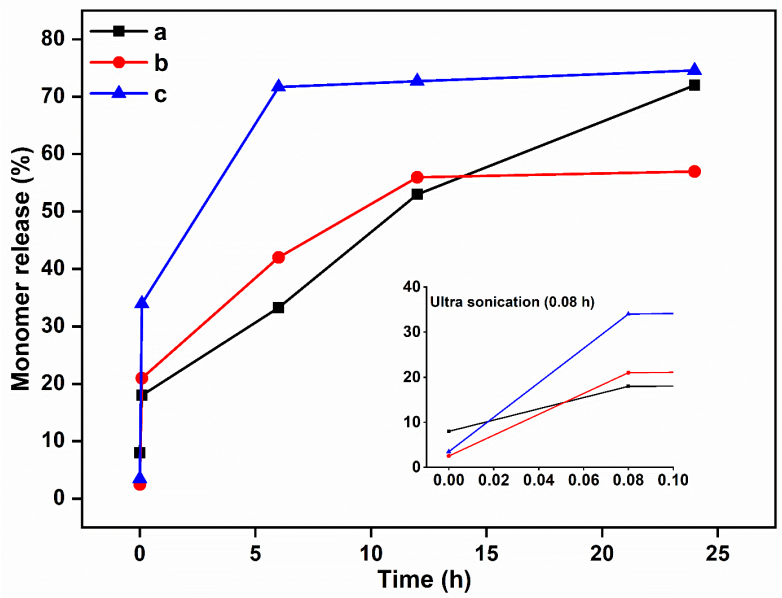


Figure 6. Acid-catalysed hydrolysis of a) **C_0P_1**, b) **C_2P_2**, and c) **$C_{10}P_3$** to release the percentage of drug ciprofloxacin **1** and **3**, respectively, *via* under the condition of acidic buffer solution of formic acid (0.024 M, pH 3), at 37 °C with sampling intervals 0.08, 6,12, and 24 h. Note: the inset shows the initial 0–0.08 h was under sonication and the rest of the time with stirring only.

The LC-MS profiles (Figure S24–S31, Supporting Information) indicated the release of ciprofloxacin (RT = 3.70 min) and monomer **3** (RT = 5.93 min) from **C_0P_1** and spacer polymers (**C_2P_2** and **$C_{10}P_3$**), respectively, has been observed over time. However, the same rate of release has not been observed with **C_2P_2**, and the appearance of some new peaks has been observed,

which indicates that some degradation or fragmentation occurred due to fast hydrolyzing imine bonds (Figure S26, Supporting Information).

The spacer polymers **C_2P_2** and **$C_{10}P_3$** were studied by ^{1}H NMR to further quantify the hydrolysis reaction (Figures S32 and S33, Supporting Information). The ^{1}H NMR data of **C_2P_2** and **$C_{10}P_3$** indicated sharp decrease of the amide peaks at 9.90 – 10.05 ppm, i.e., up to 96 and 98%, respectively within 24 h, though 95% of acid-catalysed hydrolysis occurred within 2 h. Whereas, only partial imine hydrolysis was observed as indicated by the appearance of aldehyde proton at 8.35 ppm which showed 10 and 8% for imine hydrolysis after 24 h for **C_2P_2** and **$C_{10}P_3$**, respectively. We infer that the hydrolysed amine is in equilibrium with imine due to the presence of acid.[59]

According to our hypothesis, since homo-polymerisation of **1** by a condensation reaction to synthesize **C_0P_1** does not involve functional group modification on ciprofloxacin, the released monomer would act similar to the established mechanism of ciprofloxacin. The hydrolysis study results do suggest that monomer ciprofloxacin is being released from the polymer **C_0P_1**. The mechanism of biocidal activity of ciprofloxacin is well-studied and it is believed to inhibit DNA replication by promoting cleavage of bacterial DNA in the DNA–enzyme complex such as DNA–gyrase and DNA–type IV topoisomerase.[60] This further corroborates the lower activity observed in the solution study for **C_0P_1** compared to **1** presumably due to slow-release behaviour/sustained drug release, *i.e.,* even at pH = 3 only 69% release was achieved after 24 h (Figure 6).

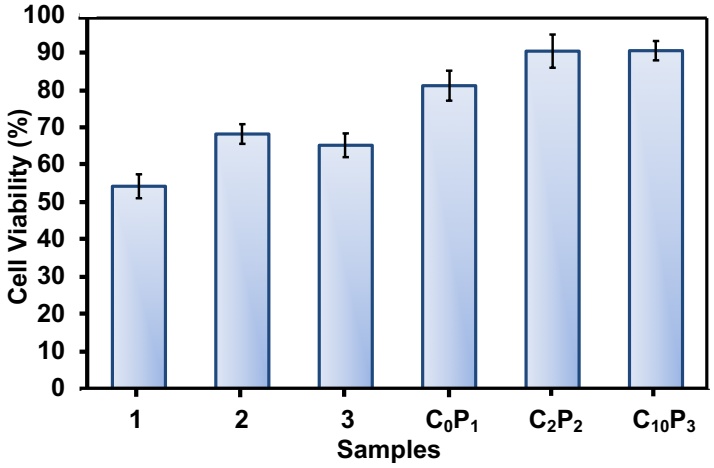


Figure 7. Biocompatibility study (MTT assay) of monomers (**1**, **2**, and **3**) and polymers (**C_0P_1**, **C_2P_2**, and **$C_{10}P_3$**) on mouse fibroblast cell line (L929). The concentration of all compounds was 1 μg/mL.

2.4. Biocompatibility Assay. Ciprofloxacin is not toxic to eukaryotic cells at low concentrations.[61] However, to rule out the possibility of cytotoxicity of the synthesised biocides, biocompatibility (MTT) assay was carried out using a mouse fibroblast cell line (L929). The anti-proliferative effects were assessed employing EZcount™ MTT cell Assay Kit by treating monomers (**1**, **2**, and **3**) and polymers (**C_0P_1**, **C_2P_2**, and **$C_{10}P_3$**). The assay was performed in triplicate with various test compounds (1µg/mL). Figure 7 displays the cell viability (%) after 48 h exposure to the newly synthesised polymers of ciprofloxacin derivatives. The data revealed that the polymers were more biocompatible than its monomers. It was further found that spacer polymers showed significantly higher biocompatibility than **C_0P_1**, presumably due to the weaker hydrolysable imine and primary amide bonds than relatively stronger tertiary amide bonds in **C_0P_1**.

3. SUMMARY AND CONCLUSION

To unravel the role of the spacer effect, we have designed and synthesised a new type of polymeric biocides (PB) **C_0P_1**, which we refer to as a true drug-polymer because upon hydrolysis/degradation, only the monomer drug units are released with no other extra mass. This not only enables more drug release in lesser amounts but also obviates the toxicity of non-drug parts. As a proof of concept, a well-known antibiotic molecule having AB-type monomer, *i.e.,* ciprofloxacin, was polymerised for the first time to obtain a large molecular weight (973.27 kDa) PB having 0% non-drug content. This true drug-polymer was compared with two other new polymers **C_2P_2** and **$C_{10}P_3$**, with increased non-drug spacer content of 29 and 53%, respectively. Antibacterial inhibition studies by both solution and suture coating revealed a clear trend, *i.e.,* improved activity when the non-drug spacer content decreased even when the activity was normalized with the spacer content ratio. Spacer polymers exhibited comparatively lower activity even with fast releasing capability due to imine and primary amide bonds.

Interestingly, though the non-spacer polymer **C_0P_1** showed decreased activity in solution against monomer **1** probably due to the slow hydrolysis nature, whereas increased activities of 1.5 and 2.2 times for E.coli and 1.6 and 88 times for S. aureus against spacer polymers **C_2P_2** and **$C_{10}P_3$**, respectively, were observed in suture coating application. The uniform coating ability for **C_0P_1** to adhere nylon thread was achieved effectively using polymerisation, without which monomer **1** suffers with poor solubility and coating ability. The hydrolysis study suggests that the **C_0P_1** polymer shows sustainable monomer release over the other two spacer

PBs due to stronger tertiary amide bonds. As evidenced by the higher melting point (409 °C), better biocompatibility and excellent coating ability on nylon thread, we expect $\mathbf{C_0P_1}$ is a promising candidate for advanced biomaterial and medical applications such as coating of suture and other surgical devices. As we have checked only one application for the true drug-polymer ($\mathbf{C_0P_1}$), *i.e.,* coating on surgical nylon sutures, there is no doubt that the true-drug polymer of antimicrobials can open a wide range of new applications, including activity against drug-resistant bacteria. Our lab is currently exploring this direction of research.

ASSOCIATED CONTENT

Supporting Information

Electronic supplementary information (ESI) available: Synthetic procedures and characterisation data of the monomers and polymers, SEC, MIC, and agar disc diffusion assay, cytocompatibility, hydrolysis study data of polymers.

AUTHOR INFORMATION

Corresponding Author

Govindasamy Jayamurugan – *Institute of Nano Science and Technology, Knowledge City, Sector 81, SAS Nagar, Manauli PO, Mohali, Punjab 140306, India;* orcid.org/0000-0001-9870-5209; E-mail: jayamurugan@inst.ac.in

Authors

Shaifali Sartaliya – *Institute of Nano Science and Technology, Knowledge City, Sector 81, SAS Nagar, Manauli PO, Mohali, Punjab 140306, India*

Vijayendran Gowri – *Institute of Nano Science and Technology, Knowledge City, Sector 81, SAS Nagar, Manauli PO, Mohali, Punjab 140306, India*

Vainni Chopra – *Institute of Nano Science and Technology, Knowledge City, Sector 81, SAS Nagar, Manauli PO, Mohali, Punjab 140306, India*

Himadri Shekhar Roy – *Institute of Nano Science and Technology, Knowledge City, Sector 81, SAS Nagar, Manauli PO, Mohali, Punjab 140306, India*

Deepa Ghosh – *Institute of Nano Science and Technology, Knowledge City, Sector 81, SAS Nagar, Manauli PO, Mohali, Punjab 140306, India*

Notes

The authors declare no competing financial interest.

ACKNOWLEDGEMENTS

The SERB supported this work, Department of Science and Technology (DST), grant no. SB/S2/RJN-047/2015. GJ thank the DST-SERB for the Ramanujan Fellowship. The authors thank Panjab University (SAIF facility) for NMR measurements.

REFERENCES

1 World Health Organization. (2016c). Health care without avoidable infections: The critical role of infection prevention and control. World Health Organization. World Health Organization. Available at: http://apps.who.int/iris/ bitstream/10665/246235/1/WHO-HIS-SDS-2016.10-eng.pdf [Accessed: 2nd December 2016].

2 WHO publishes list of bacteria for which new antibiotics are urgently needed. World Health Organization. Available at http://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which- new-antibiotics-are-urgently-needed. [Accessed 15 November 2018].

3 D. M. Morens and A. S. Fauci, *Cell,* 2020, **182**, 1077−1092.

4 C. Hobson, A. N. Chan and G. D. Wright, *Chem. Rev.,* 2021, **121**, 3464−3494.

5 W. Li, F. Separovic, N. M. O'Brien-Simpson and J. D. Wade, *Chem. Soc. Rev.,* 2021, **50**, 4932–4973.

6 T. M. Rawson, D. Ming, R. Ahmad, L. S. P. Moore and A. H. Holmes, *Nat. Rev. Microbiol.,* 2020, **18**, 409−410.

7 World Health Organization, Regional Office for Europe & International Federation of Red Cross Red Crescent Societies. Infections and infectious diseases: a manual for nurses and midwives in the WHO European Region. Copenhagen (Denmark): WHO Regional Office for Europe; 2001.

8 Centers for Disease Control and Prevention, National Healthcare Safety Network. Surgical Site Infection (SSI) Event. January 2021. Available at http://www.cdc.gov/nhsn/PDFs/pscManual/9pscSSIcurrent.pdf.

9 J. D. Siegel, E. Rhinehart, M. Jackson, L. Chiarello, Healthcare Infection Control Practices Advisory Committee. *Management of multidrug-resistant organisms in health-care settings*, 2006. "www.cdc.gov/infectioncontrol/guidelines/mdro/index.html/MDROGuideline2006.pdf". [Accessed July 24, 2021].

10 H. C. Neu, *Science,* 1992, **257**, 1064–1073.

11 R. Namivandi-Zangeneh, E. H. H. Wong and C. Boyer, *ACS Infect. Dis.,* 2021, *7*, 215−253.

12 D. J. Payne and M. N. Gwynn, *Nat. Rev. Drug Discovery,* 2007, **6**, 29−40.

13 W. Chin, G. Zhong, Q. Pu, C. Yang, W Lou, P. F. De Sessions, B. Periaswamy, A. Lee, Z. C. Liang, X. Ding, S. Gao, C. W. Chu, S. Bianco, C. Bao, Y. W. Tong, W. Fan, M. Wu, J. L. Hedrick and Y. Y. Yang, *Nat. Commun.,* 2018, **9**, 917(1)−917(14).

14 E. D. Brown and G. D. Wright, *Nature,* 2016, **529**, 336−343.

15 N. F. Kamaruzzaman, L. P. Tan, R. H. Hamdan, S. S. Choong, W. K. Wong, A. J. Gibson, A. Chivu, and M. d. F. Pina, *Int. J. Mol. Sci.,* 2019, **20**, 2747(1)–2747 (31).

16 D. Kumar, S. Gihar, M. K. Shrivash, P. Kumar and P. P. Kundu, *Int. J. Biol. Macromol.,* 2020, **163***,* 2097–2112.

17 W. Zheng, M. Anzaldua, A. Arora, Y. Jiang, K. McIntyre, M. Doerfert, T. Winter, A. Mishra, H. Ma and H. Liang, *Biomacromolecules,* 2020, **21**, 2187−2198.

18 J. L. Hedrick, Y. Y. Yang and C. Yang, U. S. Patent US10995177B2, May 4, 2021.

19 C. Sanchez, B. Julián, P. Belleville and M. Popall, *J. Mater. Chem.,* 2005, **15**, 3559–3592.

20 E. Rusen, A. Mocanu, L. C. Nistor, A. Dinescu, I. Calinescu, G. Mustatea, S. I. Voicu, C. Andronescu and A. Diacon, *ACS Appl. Mater. Interfaces,* 2014, **6**, 17384−17393.

21 X. Chen, D. Hou, L. Wang, Q. Zhang, J. Zou and G. Sun, *ACS Appl. Mater. Interfaces,* 2015, **7**, 22394−22403.

22 J. Zhao, L. Ma, W. Millians, T. Wu and W. Ming, *ACS Appl. Mater. Interfaces,* 2016, **8**, 8737−8742.

23 Q. Liu, P. Singha, H. Handa and J. Locklin, *Langmuir,* 2017, **33**, 13105−13113.

24 A. Chen, H. Peng, I. Blakey and A. K. Whittaker, *Polym. Rev.,* 2016, **57**, 276−310.

25 A. Muñoz-Bonilla and M. Fernández-García, *Prog. Polym. Sci.,* 2012**, 37,** 281–339.

26 C. Ergene, K. Yasuhara and E. F. Palermo, *Polym. Chem*., 2018, **9**, 2407–2427.

27 M. M. Konai, B. Bhattacharjee, S. Ghosh and J. Haldar, *Biomacromolecules,* 2018, **19**, 1888–1917.

28 P. Li, X. Li, R. Saravanan, C. M. Li and S. S. J. Leong, *RSC Adv.,* 2012, **2**, 4031–4044.

29 A. Peschel and H. G. Sahl, *Nat. Rev. Microbiol.,* 2006, **4**, 529–536.

30 A. Sasireka, R. Rajendran, P. Palanisamy and R. Vairamuthu, *ChemistrySelect,* 2019, **4**, 1166–1175.

31 J. S. Wolfson and D. C. Hooper, *Clin. Microbiol. ReV.,* 1989, **2**, 378–424.

32 Drug Information online. See, www.drugs.com

33 G. L. Y. Woo, M. W. Mittelman and J. P. Santerre, *Biomaterials,* 2000, **21**, 1235–1246.

34 S. P. Parwe, P. N. Chaudhari, K. K. Mohite, B. S. Selukar, S. S. Nande and B. Garnaik, *Int. J. Nanomed.,* 2014, **9**, 1463–1477.

35 B. Dizman, M. O. Elasri and L. J. Mathias, *Biomacromolecules,* 2005, **6**, 514–520.

36 C. A. G. N. Montalbetti and V. Falque, *Tetrahedron,* 2005, **61**, 10827–10852.

37 C. L. Allen, A. R. Chhatwal and J. M. J. Williams, *Chem. Commun.,* 2012, **48**, 666−668.

38 E. Turos, B. E. Anderson, R. S. Cormier, J. C. Thomas, R. J. Kapolka and G. Roma, US Patent US8143398B1, March 27, 2012.

39 K. A. Gould, X. S. Pan, R. J. Kerns and L. M. Fisher, *Antimicrob. Agents Chemother.,* 2004, **48**, 2108–2115.

40 H. Mesallati and L. Tajber, *Pharm. Res*., 2017, **34**, 2425–2439.

41 M. C. Lawson, R. Shoemaker, K. B. Hoth, C. N. Bowman and K. S. Anseth, *Biomacromolecules,* 2009, **10**, 2221−2234.

42 R. Zhang, M. M. Jones, H. Moussa, M. Keskar, N. Huo, Z. Zhang, M. B. Visser, C. Sabatini, M. T. Swihart and C. Cheng, *Biomater. Sci.,* 2019, **7**, 287−295.

43 P. R. Judzewitsch, T.-K. Nguyen, S. Shanmugam, E. H. H. Wong and C. Boyer, *Angew. Chem. Int. Ed.,* 2018, **130**, 4559−4654.

44 L. Zhang, D. Addla, J. Ponmani, A. Wang, D. Xie, Y.-N. Wang, S.-L. Zhang, R.-X. Geng, G.-X. Cai, S. Li and C.-H Zhou, *Eur. J. Med. Chem.,* 2016, **111**, 160−182.

45 C. W. Tabor and H. Tabor, *Annu. Rev. Biochem*., 1976, **45**, 285–306.

46 A. Porcheddu, G. Giacomelli and I. Piredda, *J. Comb. Chem.,* 2009, **11**, 126–130.

47 H. Kim, R. Rajamanickam and J.-W. Park, *Int. J. Greenh. Gas Con.,* 2017, **59**, 91–98.

48 T. Golub, G.-U. Dou, C.-C. Zeng and J. Y. Becker, *Org. Lett.,* 2019, **19**, 7961–7964.

49 S. Chandrasekhar, G. P. K. Reddy, M. U. Kiran, C. Nagesh and B. Jagadeesh, *Tetrahedron Lett*., 2008, **49**, 2969–2973.

50 B. Ramalingam, T. Parandhaman and S. Das, *ACS Appl. Mater. Interfaces,* 2016, **8**, 4963−4976.

51 E. Y. Lee, D. Y. Choi, D. K. Kim, J. W. Kim, J. O. Park, S. Kim, S. H. Kim, D. M. Desiderio, Y. K. Kim and K. P. Kim et al, *Proteomics,* 2009, **9**, 5425−5436.

52 A. B. Gogra, J. Yao, E. H. Sandy, S. Zheng, G. Zaray, Z. Hui, *J. Am. Sci.,* 2010, **6**, 78–88.

53 D. Zhang, D. Addla, J. Ponmani, A. Wang and D. Xie, *et al. Eur. J. Med. Chem.,* 2016, **111**, 160−182.

54 E. Vavříková, S. Polanc, M. Kočevar, K. Horváti, S. Bősze, J. Stolaríkova, K. Vavrová and J. Vinsova, *Eur. J. Med. Chem.,* 2011, **46**, 4937–4945.

55 S. Ilyas, R. R. Srivastava and H. Kim, *Sci. Total Environ.,* 2020, **749**, 141652.

56 F. Vilchez, P, J. Martínez, R. S. García, G. Bori, F. Maculé, J. Sierra, L. Font, J. Mensa and A. Soriano, *Clin. Microbiol. Infect.,* 2011, **17**, 439−444.

57 H. Kownhar, E. M. Shankar, R. Vignesh, R. Sekar, V. Velu and U. A. Rao, *J. Antimicrob. Chemother.,* 2008, **61***,* 758–760.

58 J. T. Poolman and A. S. Anderson, *Expert Rev. Vaccines,* 2018, **17**, 607−618.

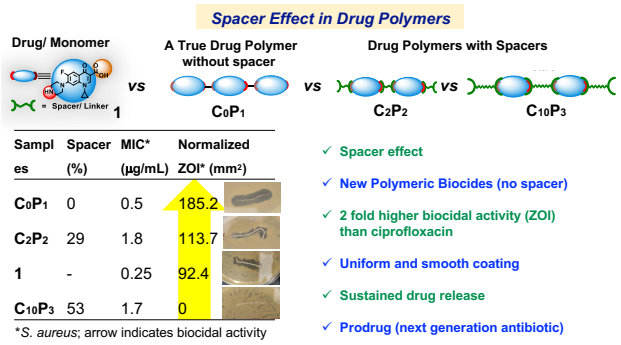
59 M. Ciaccia and S. D. Stefano, *Org. Biomol. Chem.,* 2015, **13**, 646−654.

60 D. M. Campoli-Richards, J. P. Monk, A. Price, P. Benfield, P. A. Todd and A. Ward, *Drugs,* 1988, **35**, 373−447.

61 J. S. Wolfson and D. C. Hooper, *Clin. Microbiol. Rev*., 1989**, 2**, 378−424.

TOC

Unravelling the effect of non-drug spacers on a true drug-polymer and a comparative study of their antimicrobial activity



A true drug-polymer comprising of 100% drug units was found to show enhanced biocidal activity and the polymerization helped to achieve better physical property, which is friendly for suture coating application.