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Ultra- sustained-release multi-particulate dosage form of Doxycycline as a platform of repurposing therapeutics in the fight against COVID 19: in-vitro and in-silico study

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Abstract

Purpose: Many recent studies highlighted Doxycycline beneficial multiple effects against SARS-COV2 either as a monotherapy or combination therapy. The creation of novel drug delivery systems is an attractive approach for the repurposing of drugs in a trial to fight COVID 19. In this study, ultra-sustained-release dosage forms were designed.
Methods: A simple and cost-effective method was followed. Eudragit L100 (EL100), a pH sensitive water insoluble polymer, was combined with Doxycycline (DOX) in different ratios to form multi-particulate systems (MPs) with different drug release rates.
Results: All the prepared MPs significantly sustained DOX release to reach a complete drug release after about 23.9, 142.57 and 165.58 h in case of MP1, MP2 and MP3, respectively compared to the pure drug which attained 100 % drug release within 30 min. The drug release was diffusion-dependent. Also, the prepared MPs showed satisfactory flow properties. The formula attaining the most extended drug release was subjected to characterization. In-silico molecular modeling study proved the high binding affinity of DOX/EL100 to S1-RBD of SARS-CoV-2. This high interaction may interfere with virus attachment to the host receptors and hence inhibit virus infection.
Conclusion: This study shed the light on a possible platform to prepare ultra-long-acting dosage forms having different drug release rates by just simple, yet attractive, modifications which can offer promising therapeutic approaches.

Keywords: Doxycycline, Eudragit L100, sustained-release, Covid 19 and in-silico

1. Introduction

A severe acute respiratory pandemic virus known as SARS-CoV2 emerged the world and representing as an unexpected threat to human life. Coronaviruses are spherical in shape surrounded with spikes giving them a crown appearance to electron microscopy, with a tiny size (65–125 nm in diameter) and contain a single-stranded RNA as a nucleic material[1].The attachment and the entry of the viruses to the host cells were established through the glycoprotein spikes on their outer surface which bind to a cellular receptor [1]. A coronavirus' entry mechanism relies on cellular proteases including human airway trypsin-like protease (HAT), cathepsins and transmembrane protease serine 2 (TMPRSS2) that split the spike protein and establish further penetration changes [2] which mean that the spike glycoprotein recognizes the host cell receptor and fuses its membrane with it [3]. Fever, dry cough, and tiredness are the most commonly recorded symptoms. Breathing problems, sputum development, runny nose, pneumonia and diarrhea are other symptoms.

There is a must to try to stop the disease progression which may bring about organ failure leading to death[4]. Since there is neither any vaccine nor any specific antiviral drug available against SARS-COV2, extensive efforts have been made to explore effective therapeutic options against the virus all over the world[5]. Repurposing of existing drugs can present a solution. The development of novel drug delivery technologies is an attractive solution for the repurposing of drugs. One of the drugs that have been used recently in combination with hydroxychloroquine against coronavirus as a better alternative to azithromycin was doxycycline hydrochloride (DOX) [6]. Doxycycline hydrochloride, is a tetracycline antibiotic with a relatively broad spectrum activity that is widely used in human and

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veterinary medicine[7]. According to the Biopharmaceutics Classification System (BCS), DOX hydrochloride is classified as BCS Class I as is the most soluble form among the other DOX forms[8]. It is more lipophilic than other tetracyclines, such as methacycline and oxytetracycline, and as a result has greater tissue penetration and antimicrobial properties[9]. Also, DOX exhibits an anti-inflammatory effect in combination with its antiviral activity against some infections caused by several RNA viruses, such as dengue fever [10]. An in vitro study done by Rothan et al., showed that DOX suppress dengue virus infection in monkeys cell line (Vero cells) through the inhibition of dengue serine protease enzymes and consequently viral host entry [11]. Another study showed that DOX can chelate zinc compounds on matrix metalloproteinases (MMPs) of mammalian cells [12], which contribute with cell fusion and viral replication[13]. Recently, it was reported that safe tolerability profile for DOX, potential alleviation of lung sequelae as well as providing coverage against atypical bacterial pneumonia such as Mycoplasma pneumoniae and Legionella
pneumophilia favor its use as an attractive medication for the treatment of COVID-19[14]. Another recent study declared that DOX is widely investigated in clinical trials, either as a monotherapy or combination therapy, and that it was repurposed for COVID-19 treatment [15]. Also, many recent publications highlighted its multiple effects against SARS-COV2 and/or attenuation of disease complications and rapid recovery [16-19]. However, administration of DOX may be accompanied by different side effects like digestive disturbances, dental discoloration [20] and development of antibiotic resistance [21]. Therefore, design of a controlled-release dosage form presents a great advantage by maintaining the drug concentration for a prolonged time after a single administration. This would decrease doses and total drug intake, resulting in fewer side effects. This can also have an additional advantage in the treatment of Covid-19 where the patient needs to take multiple medicines which can result in different drug-drug interaction and decreased patient compliance besides the economic burden.

Methacrylic acid co-polymers (Eudragits®) are widely used to prepare various controlled release dosage forms [22]. Eudragit® L 100 (EL100) is an anionic hydrophobic pH-sensitive polymer which is soluble in intestinal pH, therefore it was extensively investigated for the preparation of controlled-release oral delivery systems [23], however, only few studies have been focused on the complexation between drugs and Eudragits of opposite charges [24, 25]. Based on the possible electrostatic complexation between EL100 and DOX, it was expected to extend drug release by the designed simple formulation.

Multi-particulate systems could be an alternative to the modified-release single-unit tablets and capsules[26]. These multiple unit dosage forms are composed of numerous independent subunits (microparticles), each of which serves as an autonomous reservoir of the drug and can release it in a desirable manner independent of the other subunits. Multi-particulate delivery systems are particularly well-suited for the preparation of modified-release solid oral dosage forms (delayed- or sustained-release), which provide advantages such as less variable gastrointestinal transit and a lower risk of dose dumping[27]. Also, Multi-particulate drug delivery systems are advantageous because they can allow controlled drug release profiles and organ-targeted release [28], in addition to decreasing the risk of localized mucosal damage and "all-or- none" success [29, 30]. Generally, the development of oral multi-particulate drug delivery systems is based on the formation of a matrix or coated drug particles using one or more carrier polymers to control drug release[31-33] using some sophisticated and expensive process. For this reason, our goal was to develop a sustained release multi-particulate dosage form composed of DOX/EL100 complexes using a simple and cost-effective solid dispersion technique.

As mentioned before that SARS-CoV2 must bind to specific receptors on the surface of host cells to allow their entry into cells and start their replication and infection. SARS-CoV2 attached to ACE2 receptors allocated at the surface of host cells by anchoring S-proteins ligands, S1 subunit. S1 subunit has the receptor binding domain (RBD), responsible for the high affinity binding to ACE2 receptor [34, 35] . Based on the mechanism of viral infection and its binding with the host cell through its protein surface, any compound interacting with S1-RBD could interfere with virus attachment to host receptors and hence inhibit infection. A molecular docking analysis was therefore conducted to identify and understand the SARS-CoV2 interaction and binding affinity of DOX with S1-RBD and the developed formulation.

2. Materials and methods

2.1. Materials

Doxycycline hydrochloride (DOX) was kindly donated by Memphis Pharmaceutical Company, Cairo, Egypt. Eudragit L100 (EL100) was kindly offered by Evonik Röhm GmbH, PharmaPolymers, Germany. All other reagents were of analytical grade supplied by El-Nasr Company for Pharmaceutical Chemicals, Cairo, Egypt.

2.2. Methods

2.2.1. Preparation of DOX/Eudragit multi-particulate formulae using evaporation method

Doxycycline solid dispersion was prepared by solvent evaporation method using Eudragit L100 as a carrier at different ratios; 1:1, 1:2 and 1:3 (MP1, MP2 and MP3, respectively). EL100 was dissolved in ethanol with continuous stirring. DOX was than dissolved into the polymeric solution. The solvent was allowed to evaporate at 70 °C and finally the coherent mass was scrapped, pulverized, sieved and kept in a dissector till use.

2.2.2. Determination of Encapsulation efficiency (E.E.)

It presents the quantity of the drug entrapped in the formula in comparison to the total quantity of the drug added during the preparation process. The actual amount of the drug in the multiparticulate formula was calculated following a direct method. A known weight (100 mg) of the formulae was dispersed in phosphate buffer pH 6.8 and shacked for 12 h in a shaker incubator (G.F.L. type 3032). The solution was then filtered and appropriate dilution was done before measuring the absorbance at 274 nm using UV spectrophotometer (.Shimadzu UV spectrophotometer 2401/PC, Japan).

$$EE\% = (\text{Actual drug amount})/(\text{Theoritical amount of drug})\text{x}100$$

2.2.3. In vitro dissolution studies

The dissolution of DOX was assessed using the USP Dissolution Tester Apparatus 1 (rotating basket) following the pH shifting method. Samples of the prepared formulae containing 100 mg of DOX were filled in transparent hard gelatin capsules (number 00) and put in the dissolution media composed of 750 mL of 0.1 N HCl (pH 1.2 simulating gastric pH). After 2 h, 250 ml of 0.26 M trisodium phosphate was added to each vessel to adjust the pH to 6.8[36] and the experiment was then run for 24 h. The speed of the paddle was set at 50 rpm and the temperature was maintained at 37± 0.5 °C. At time intervals of 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6 and 24 h, samples were withdrawn from the dissolution medium and replaced by fresh equal volumes. The samples were filtered through Millipore filters (0.45 µm) and analyzed for DOX content by measuring its absorbance at 297 nm for pH 1.2 and 274 nm for pH 6.8. Experiments were carried out in triplicate. Dissolution profiles were plotted and drug release kinetics was determined by finding the best fitting model (zero-order, Higuchi and first-order). Also, T100 (time to achieve 100 % drug release) was calculated using linear regression analysis.

2.2.4. Evaluation of the flow properties

The flow properties of the selected formula were evaluated and compared to the crude drug. The following parameters have been recorded: angle of repose, Carr's index and Hausner's ratio. The angle of repose (θ) was measured based on the conventional fixed height cone method [37].The powder was poured through a funnel positioned at a fixed height (H) until the apex of the powder conical pile formed just reaches the tip of the funnel, and the angle of repose (θ) was calculated:

$\tan\theta = H/r$, where r is radius of the pile of powder.

The Carr's (compressibility) index and the Hausner's ratio were determined on the basis of the bulk and tapped powder density [37]. The powder was poured into a graduated cylinder of 25ml. The powder was tapped until no additional volume change was detected.

Percentage compressibility was calculated based on the following equation:

Carr's Index = $\rho_p - \rho_b / \rho_p$ x 100

The density of bulk and tapped powder (g/cm3) were measured as the weight of the powder divided by its volume before and after tapping.

The Hausner's ratio is determined by the following equation:

Hausner's Ratio = volume before tapping / volume after tapping.

All measurements were tripled and the average was determined for each powder.

2.2.5. Characterization for the selected formula

2.2.5.1. X-ray powder diffraction (XRPD)

DOX, EL100, and the selected formula (MP3) x-ray powder diffraction patterns have been reported using X-ray diffractometer (Scintag Inc., USA). Samples were irradiated using Ni filtered, CuKa radiation at a voltage of 45 kV, and a 9 mA current at a scanning rate of 1° min−1 with 2θ diffraction angle over a range of 0°–90°.

2.2.5.2. Fourier transformation infrared (FTIR) spectroscopy

Infrared spectra of DOX, EL100, and the formula (MP3) were determined as KBr discs using a Shimadzu 435 U-04 IR spectrophotometer.

2.2.5.3. Scanning electron microscopy

A scanning electron microscope was used to investigate the surface morphology of the DOX, EL100, and the formula (MP3) . Using double-sided adhesive tape, the powders were fixed onto a brass stub and then made electrically conductive by vaccum coating with a thin layer of gold (approximately 150 A°) for 30 seconds. The photos were taken at an excitation voltage of 20 KV.

2.2.6. Molecular docking studies

Molecular docking study was conducted using C-Docker 2.5 software in the interface of Accelry's discovery studio 4.1. The envelope glycoprotein structure of Chimeric S- receptor-binding domain

(RBD) of SARS-COV-2(Covid19) (PDB ID: 6vw1) [38] were obtained from Protein Data Bank (www.rcsb.org). The protein structure was designed using Protonate 3D Protocol in MOE with default options, and this was accomplished by adding hydrogen atoms to the amino acid residues, completing the missing residues. The protein structure was minimized using SMART minimizer algorithm.
DOX (guest) and the host Eudragit L100 were constructed and prepared using Ligand preparation protocol. The molecular docking approach was carried out using C-Docker algorithm for DOX (guest) and the host was used to support the rational design of this study. Interaction of the guest with the target shell of the SARS-CoV-2 and host was studied[39] .

2.2.7. Data analysis

All the studies were conducted three times and the data were expressed as mean ± standard deviation (S.D.). Using SPSS® software, the statistical significance of differences was determined by ANOVA. A value of $p < 0.05$ was considered to be significant.

3. Results

The prepared formulations, consisted of DOX/EL100 complexes, were prepared using a simple and cost-effective method based the solid dispersion technique. The produced systems had a white-colored powder form appearance which was characterized and evaluated as follows:

3.1. Encapsulation efficiency (E.E.)

The encapsulation efficiency in all the prepared formulae was above 95 % (95.6%, 96.1% and 96.2% for MP1, MP2 and MP3, resp.) showing the suitability and reproducibility of the method of preparation and the uniform distribution of the drug within the polymeric matrix.

3.2. In vitro dissolution studies

The dissolution behavior of the free drug and the prepared formulae are shown in figure 1 and the release parameters are listed in table 1. It is observed that all the formulae exhibited a sustained drug release profile while the crude drug showed an abrupt and complete release during the first 30 min., this was expected because the drug is soluble in the gastric pH but the EL100 is not and hence it hindered the fast drug release. All the prepared DOX/EL100 multi-particulate formulae showed a bimodal release pattern depending on the pH of the dissolution media (table 1). At both tested pH, the Higuchian model was the most dominant in all cases proving a diffusion-dependent drug release from the polymeric matrix. Previous literature listed that drug release from different Eudragit® types matrices followed the square root of time law where the predominating mechanism of drug release is the Fickian diffusion, which is generally the mechanism followed in case of water insoluble polymeric systems [40].
The release rate was affected by the DOX/EL100 ratio, increasing of EL100 amount decreased the release rate. This is clear in the calculated expected time for complete drug release (T100) which was in the following order MP1 < MP2 < MP3. The T100 in the case of MP1 was 23.9 h and increased 5.96 folds to reach 142.57 h (5.94 days) in case of MP2, while it increased by 6.62 folds to reach 165.58 h (6.89 days) in case of MP3. These results prove the possibility of preparation of ultra-long- acting preparations having different drug release rates by performing just small modifications in the designed formulae. The suggested formulae can be administered once-daily or once-weekly according to the patient status and need.

Multiple units' dosage forms of small-size particles have proved to have a long GIT residence time [28-30]. A previous study has declared a long GIT residence time reaching 48 hrs for ibuprofen-loaded microspheres and a higher bioavailability compared to the conventional drug suspension [41]. Another study reported the advantages of oral dosage forms with ultra-long-term GIT residence time beyond 24 h and up to a week or even longer [42]. In the same context, it was proved that the weekly administration of long-acting anti-retrovirals using a novel oral delivery system composed of drug-polymer matrices is a promising intervention helping to control the HIV epidemic worldwide [43].

3.3. Flow properties studies

The classification of flow properties of a certain powder are based on different parameters, namely: angle of repose (Θ), Carr's index (CI) and Hausner ratio(HR) [44]. It was found that Θ <30, HR<1.25 and CI<15 indicate excellent flow properties while, Θ = 30-40, HR=1.25-1.5 and CI=15-25 are an indication of good flow properties. However, Θ >40, HR>1.5 and CI>25 predict poor flow properties. Good flow property of a powder is a crucial factor for handling and processing. As listed in table 2, it is clear that all the prepared multi-particulate formulae have satisfactory flow properties, while the crude drug has poor properties (explained below). This prove that the suggested preparation process based on solid dispersion technique seems to be a suitable one for preparing free-flowing powders
Considering the obtained results, MP3 was the formula showing the most prolonged release profile

(T100=6.89 days), hence, was submitted to additional characterization.

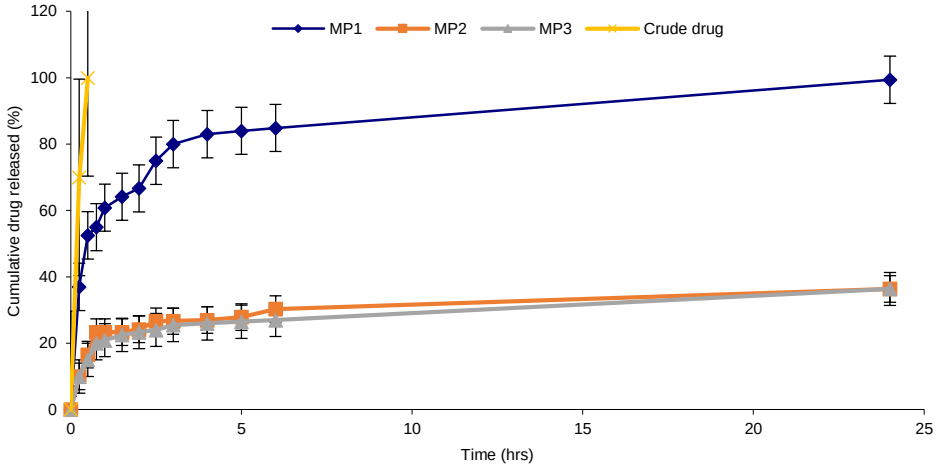

Figure 1. *In vitro* DOX release profiles of different prepared formulations in comparison with the crude drug form.

Table 1. Release data of the prepared formulae

Formula	pH	Release kinetics						T100 (h)
		Zero order		Higuchi order		1st order		
		Rate (mg/h)	R2	Rate (mg/h1/2)	R2	Rate (mg/h)	R2	
MP1	**1.2**	**14.88**	**0.799**	**30.49**	**0.887**	**0.12**	**0.733**	**23.90**
	6.8	**0.94**	**0.900**	**6.493**	**0.945**	**0.004**	**0.870**	
MP2	**1.2**	**7.078**	**0.709**	**14.86**	**0.826**	**0.179**	**0.634**	**142.57**
	6.8	**0.448**	**0.944**	**3.042**	**0.966**	**0.006**	**0.927**	
MP3	**1.2**	**6.862**	**0.759**	**14.25**	**0.866**	**0.179**	**0.683**	**165.58**
	6.8	**0.536**	**0.988**	**3.603**	**0.992**	**0.007**	**0.977**	
Crude drug	**1.2**	**NA**						**0.5**

Table 2. Flow properties of the prepared formulae

Formula	Angle of repose (Ɵ)	Hausner ratio (HR)	Carr's index (CI)
MP1	37.56 ± 0.26	1.45 ± 0.02	22.05 ± 0.31
MP2	36.86 ± 0.33	1.42 ± 0.01	21.10 ± 0.20
MP3	36.19 ± 0.29	1.41 ± 0.02	20.09 ± 0.19
Crude drug	43.15 ± 0.35	1.62 ± 0.03	25.65 ± 0.18

3.4. **Characterization of the selected formula (MP3)**

3.4.1. **X- ray powder diffraction**

Figure 2 shows X- ray diffraction patterns of DOX, Eudragit L100, and the formulae MP3. The intense peaks of DOX indicate its crystalline nature (Fig. 2a), while the broad peaks in EL100 diffractogram may prove its amorphousness (Fig. 2b). The diffraction pattern of the formula (MP3) shows the disappearance of the sharp peaks of DOX which demonstrates the loss of its crystallinity and may prove the formation of a new solid phase (Fig.2c).

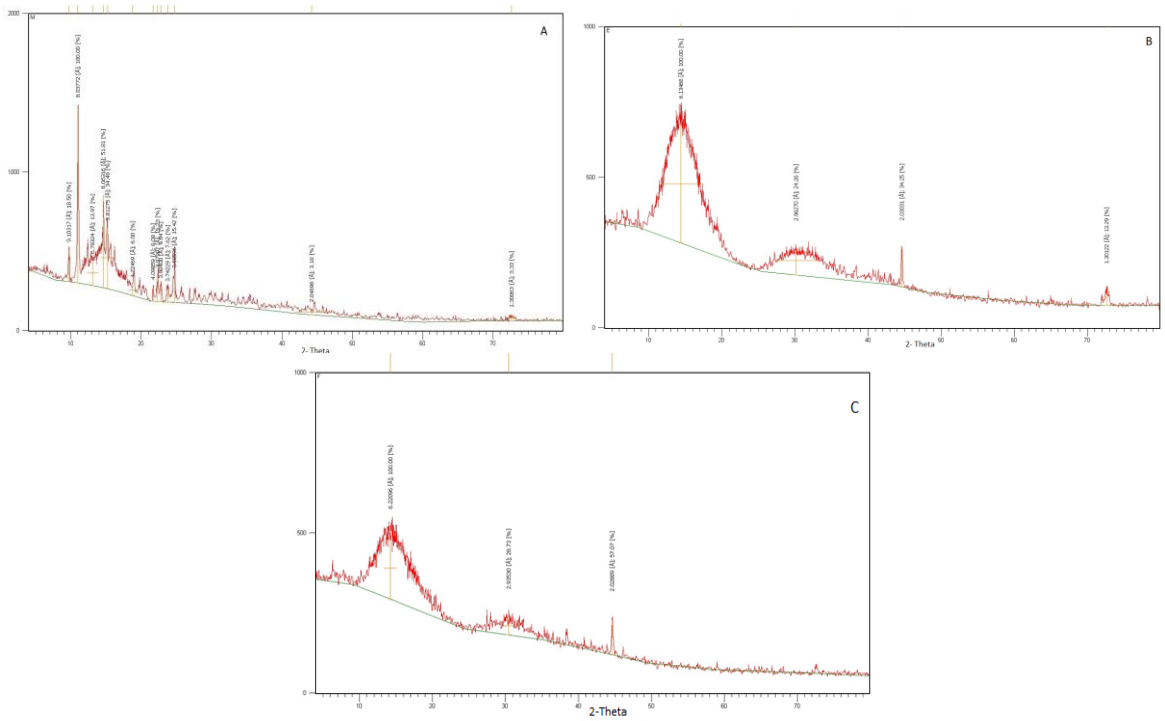

Figure 2. X-rays diffractgrams of DOX (A), EL100 (B) and MP3 (C)

3.4.2. FTIR

As illustrated in Fig.3A, the distinct peaks belonging to the drug can be seen at 3300-3550 cm^{-1}(O–H, N–H and C–H stretching), 1689 cm^{-1}(carbonyl group), 1045 cm^{-1}(C–O–C stretching)[45], and 1596 cm^{-1}(amino groups related to amide II)[46] in addition to the fingerprint region. Fig.3B elucidates the characteristics peaks of EL100 at 3500, 3000, 1736, 1195 and 1165 cm^{-1} which are attributed to the free form of carboxylic acid, CH stretching vibration, the dimer of carboxylic ester, and C-O bonds of carboxylic ester and acid, respectively [47, 48]. In the IR spectrum of MP3 (Fig.3C), the characteristic peaks of DOX at 3300-3550 cm^{-1} are not detected indicating the existence of interaction between DOX and EL100 possibly by hydrogen bonding. Also, the fingerprint region shows obvious changes compared to the drug spectrum.

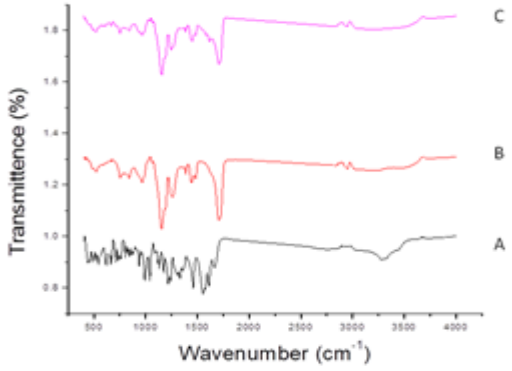

Fig.3: The FTIR spectra of DOX, EL100 and MP3

3.5. SEM

The scanning electron micrographs of DOX, EL100 and MP3 are presented in Fig.4. The micrograph of DOX demonstrates irregular rectangular-shaped crystals (Fig.4A), while that of EL100 shows nearly smooth spherical particles (Fig.4B). On the other hand, the micrograph of MP3 (Fig.4C) clearly elucidates perfectly spherical microparticles (about 5 µm diameter) with a rough surface which may be due to the deposition of drug particles on the polymeric surface during the preparation. This photograph may be an indication of the formation of a new solid phase [49]. On another hand, this can explain the better flow properties reported above for the prepared formula compared to the crude drug. As the drug has the form of irregular rectangular-shaped crystals which can cause great inter-particulate friction that decreases the flowability, while the perfectly spherical shape of MP3 can decrease the resistance to flow and increase particle velocity. This also can be an explanation for the best flowing properties of MP3 compared to the other prepared formulae, as, when the proportion of EL100 having a smooth spherical surface increases in the formula the net forces inhibiting powder flow decrease.

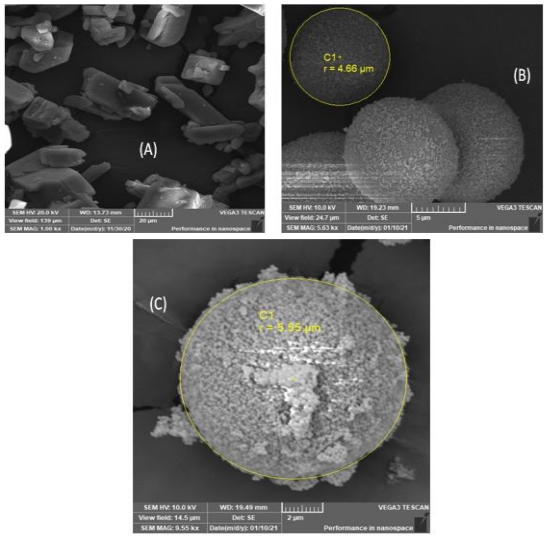

Figure 4. SEM photographs of DOX (A), EL100 (B) and MP3 (C)

3.6. Docking study

SARS-CoV2 must bind to specific receptors on the surface of host cells to allow their entry followed by their replication and infection. SARS-CoV-2 is attached to ACE2 receptors allocated at the surface of host cells by anchoring S-proteins ligands, S1 subunit. S1 subunit has areceptor binding domain (RBD) which is responsible for the high affinity binding to ACE2 receptor [34, 35, 50]. A compound which interacts with S1-RBD could interfere with virus attachment to host receptors and hence inhibit virus infection. Therefore, molecular docking study was performed to identify the interaction and binding affinity of DOX and DOX/EL100 with S1-RBD of SARS-CoV-2.

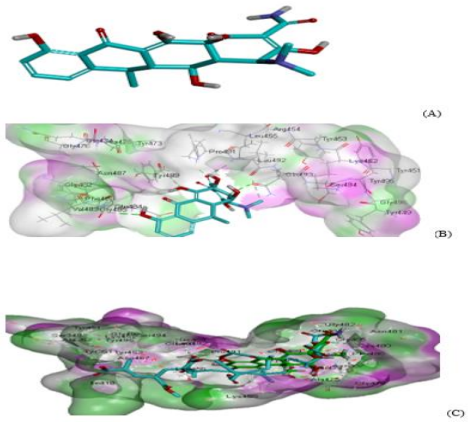

Fig.5. (A) 3D structure of the Doxycycline (guest), (B) 3D interaction diagram of Doxycycline with the key amino acids in the S-RBD of SARS-COV-2 and (C) Doxycycline-Eudragitl100 showing the interactions with the key amino acids in the S-RBD of SARS-COV-2.

The outcome of docking studies of DOX and DOX/EL100 with S1-RBD are presented in figure 5. The interaction of the free DOX as well as DOX/EL100 complex at the binding site showed high stability with binding energy of -46.83 and -51.21 Kcal/mol, respectively. The interaction of DOX with S1-RBD involved the formation of 4 hydrogen bonds, three hydrogen bonds acceptor with Phe486, Leu 455and Tyr489 and one hydrogen bond donor with Gln 493 in addition to the hydrophobic interactions detected with Leu455, Ser494 and Phe 497 (Fig.1B). While the complex DOX/EL100 revealed the formation of 9 hydrogen bonds, five hydrogen bonds acceptor with Ser 469, Cys480 and Gly482, Asp481 and four hydrogen bond donor with Gly 476, Ser469 and Gly482 (Fig.1C). This finding can indicate the higher degree of binding between DOX/EL100 and S1-RDB which may enhance the higher anti-infective efficacy of the DOX/EL100 complex compared to that of the free drug.

Conclusion

Seeing that many medicines were used, including anticoagulants, bronchodilator, antibiotics and antiviral, in an attempt to combat of Covid-19; patients may find difficulties or incompliance accompanying the treatment period which might last for several weeks until complete recovery. For this reason, reduction of the frequency of drug administration is a must. Novel drug delivery systems can be a great tool for repurposing of drug use. The development of oral drug delivery systems providing prolonged release of medications over days or weeks could make a great transform in patient life by significantly decreasing burden in disease management, ameliorating outcomes and helping to control the pandemic. In this study, ultra-long-acting multi-particulate formulae loaded with Doxycycline were suggested. The rate of drug release was changed by simple, yet important, formulation modifications to provide a once-daily to once-weekly administered solid dosage form. The prepared formulae powder had good flow properties. Promising in-vitro as well as in-silico results were obtained. Further biological investigations are required to confirm these results.

Conflict of Interest: The authors declare no conflict of interest.

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