A Combination Therapy of C-Dots and Clonidine Hydrochloride Loaded Nanocarriers for Ocular Drug Delivery: Formulation, Characterization and *Ex- Vivo* Evaluation

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Abstract

Purpose: Nanocarrier-mediated ocular drug delivery offers a lot of prospects owing to its better penetration, longer retention, and bioavailability of drugs. Moreover, combination therapy provides several advantages in the treatment of ocular diseases that leads to secondary infections. **Methods:** Herein, a polymeric nanocarrier (NC) has been developed for topical co-administration of clonidine hydrochloride (for the treatment of glaucoma) and paracetamol based fluorescent carbon dots (for anti-bacterial activity). This combination therapy is suitable for simultaneous treatment of glaucoma as well as any secondary bacterial infections. Furthermore, the interaction of the clonidine hydrochloride with the protein α-2a receptor was also studied by molecular docking and MD simulation. To facilitate the ocular delivery as-synthesized carbon dots and clonidine hydrochloride were loaded within Eudragit RL 100 polymeric nanocarriers. The size of nanocarrier was determined by scanning electron microscopy (SEM). The characterization of carbon dots was done by UV-Vis and fluorescence spectroscopy. The ex-*vivo* permeation study and corneal hydration study were performed using goat eye. **Results:** The SEM studies have reported the size of NC as 124 nm. The drug entrapment was reported as 73.3%, while the NC were showing blue emission under UV-light due to presence of the C-dots. The release studies have shown up to 90% of drugs are released in 60 minutes. The percentage corneal hydration was noted as 78%. **Conclusions:** The overall results showed that co-delivery of the carbon dots and clonidine hydrochloride by nanocarriers may be suitable for the treatment of glaucoma and secondary bacterial infection.

Keywords: Nanocarriers, Glaucoma, C-Dot, Clonidine hydrochloride, Ocular Drug Delivery System

Introduction:

Glaucoma is a neurodegenerative ailment which affects the eyes. It is related to increased intraocular pressure (IOP). It may be well-defined as a state that causes advanced neuropathy, characterized by physical changes to the optic nerve head and may lead to significant changes in the patient's visual nature[1]. The eye is an exclusive and interesting organ for therapeutic drug delivery onto the surface as well as in the inner part of the ocular structure. A model drug delivery

for eye must be capable to extend the release of drug and must remain in the eye for long period of time. To offer ocular delivery systems with excellent therapeutic efficacy, the challenge of building a therapeutic system is to achieve an ideal concentration of a drug at the active site for the proper period. The cornea's physiology, anatomy, and barrier function make it difficult for drugs to be absorbed quickly. To keep a therapeutic drug level in the tear film or at the site of action, repeated use of eye drops is required. However, repeated use of extremely concentrated solutions might result in side effects and ocular surface cell damage. The main challenges to ocular surface drug delivery after topical administration are the precorneal loss factors, which include solution drainage, lacrimation, tear dynamics, tear dilution, tear turnover, conjunctival absorption, nonproductive absorption, transient residence time in the cul-de-sac, and the relative impermeability of the corneal epithelial membrane. Only a small amount of the drug (1% or even less of the given dose) is absorbed by the eyes as a result of these physiological and anatomical restrictions. Topical formulations must balance lipophilicity and hydrophilicity with longer contact times in order to be clinically successful[2]. Ophthalmic drug delivery based on nanotechnology is one of the techniques which are used for the delivery of medication for the ocular tissue to overcome the differential, precorneal, dynamic and static ocular restriction. Therefore, a new ocular drug delivery system that uses nanomaterials are appropriate for targeting glaucoma disease[2]. Nanocarriers (NCs) could be such nanomaterial that can potentially target the definite site and has the benefit to load both hydrophilic and lipophilic drugs. Additionally, the NCs have the advantage of rising pre-corneal residence time and lowering the loss of drugs by the formation of tears, which can be achieved by the proper use of polymers[13, 14, 15]. Moreover, NCs also have the advantage of mucoadhesion, which helps to retain the drug for a longer period at the eye[5,6]. Clonidine Hydrochloride is a α-2 agonist that particularly acts on α-2 receptor[3]. It is used to treat open-angle and secondary glaucoma by reducing the IOP of an eye. It helps to lower IOP by reducing aqueous production and moderately increasing uveoscleral outflow.

The fluorescent carbon dots (C-dots) containing graphene quantum and carbon quantum is a new class of carbon nanomaterials which have sizes lower than 10 nm[18,19,29,4,22]. They have attractive properties like chemical inertness, less toxicity, water solubility, biocompatibility, and antibacterial nature. For the combination therapy, these C-dots were synthesized by microwave-assisted one-step synthesis method, using paracetamol as a precursor. Recently, it was found in literature that C-dots were synthesized using aspirin that showed very good anti-inflammatory activity and exhibited very low toxicity[24]. The C-dots synthesized from paracetamol showed bactericidal activity against *E. coli* bacteria. Further, these C-dots were co-delivered along with clonidine hydrochloride inside polymer nanocarriers with the anticipation that the present combination therapy would be suitable for the treatment of glaucoma and secondary bacterial infection. Also, the interaction of the clonidine hydrochloride with the protein α-2a receptor was demonstrated by molecular docking and MD simulation studies.

Materials and Methods

Materials

Clonidine hydrochloride, a gift sample was provided from FDC Limited Mumbai, India. Eudragit RL100 was purchased from Indian fine chemicals, Mumbai. Methanol was purchased

from Merck life science private limited, Mumbai India. Polyvinyl alcohol (PVA) was purchased from SD Fine Chemical LTD Mumbai. Ethanol was purchased from Merck Life Science PVT LTD, Mumbai India. Paracetamol and PEG were purchased from SRL chemicals, Mumbai.

Experimental Methods

a) *Stock Solution preparation*

To prepare 1000 µg/mL stock solution, 100 mg of Clonidine hydrochloride was added in 100 mL of methanol. It was further diluted to make 5, 10, 15, 20, and 25 µg/mL of solutions by serial dilution method.

b) *Preparation of Carbon dots*

The C-dots were prepared by microwave-assisted one-step synthesis method. Precursor (paracetamol) and polyethene glycol (PEG) were mixed in a 3:1 ratio, which was further given pulse heating in a microwave at 800 W until it becomes dark brownish**.** It was further dissolved in distilled water and filtered to obtain the solution of C-dots. Later, dialysis was performed for 24-72 h to remove impurities in the C-dots.

c) *Preparation of drug and polymer suspension*

To prepare the polymer and drug suspension, the polymer (Eudragit RL 100) (30 mg) and drug (Clonidine hydrochloride) (5 mg) were dissolved in a methanol-acetone (1:3 ratio) mixture solution by gentle heating and followed by sonication[7, 8, 16-17]. The pH of the obtained organic phase was adjusted to 4.0 using 0.1 N hydrochloric acid. This phase was further mixed with 1% polyvinyl alcohol (PVA) taking 40 mL as the final volume under magnetic stirring.

d) *Preparation of C-dots and Drug loaded Nanocarriers*

As-prepared drug-polymer suspension was mixed with C-dots **(**240 µL of 10 mg/mL) and chloroform (1 mL) to obtain a clear solution of two immiscible liquids. Further, it was sonicated for 2 min with pulses of 30 sec each at 60 % amplitude forming white cloudy mixture. The obtained solution was kept at -20 ℃ for 12 hours. After 12 hours, the solution was kept for 10 minutes at room temperature to evaporate traces of organic solvent[12]. All the samples were further centrifuged at 8000 rpm for 3 minutes to obtain nanocarriers which were finally dissolved in PBS[9, 10].

The drug entrapment was calculated by the following equation (eq. 1):

$$\%\ \boldsymbol{Drug\ Entrapment} = \frac{\boldsymbol{Amount\ of\ Drug\ Taken - Amount\ of\ Drug\ in\ Spernatent}}{\boldsymbol{Amount\ of\ Drug\ Taken}}\ \boldsymbol{x}\ \mathbf{100}$$

(eq. 1)

Computational Methods

a) *Ligand preparation*

The ligand clonidine hydrochloride (PubChem ID: 20179) was downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and was subjected to LigPrep, Schrodinger, LLC, New

York, NY, 2020-4 to yield stable conformations of ligands for docking under default condition of LigPrep/Epik.

b) *Protein preparation and grid generation*

Crystal structures of the alpha *2a* adrenergic receptor (PDB ID:6KUX) downloaded from protein data bank (RCSB PDB: https://www.rcsb.org/). Protein preparation wizard, default parameters (Glide, Maestro v12.0, Schrodinger, LLC, New York, NY, 2020-4) were used to prepare protein and grid were generated using receptor grid generation. The outer box dimension was X=36 Å, Y=36 Å, Z=36 Å and inner box dimension were kept X=30 Å, Y=30 Å, Z=30 Å. The grid center was kept near the predicted cavity site (PDBsum: http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/) around chain A residue 109,113,114,390,391,408,412,188,189 and 190. The grid center was kept as X= -3.63; Y= -10.12; Z= -32.95 for 6KUX using Glide v8.3, Schrodinger, LLC, New York, NY, 2020-4.

c) *Structure based virtual screening studies*

In the present study structure-based virtual docking was performed using Glide v8.3, Schrodinger, LLC, New York, NY, 2020-4 in order to evaluate the docking score of prepared ligands against the protein (6KUX). Ligands conformation with best docking score was considered for further M D simulation and analysis.

d) *Molecular dynamics simulation study*

Further, for MD simulation, model system was built around the protein-ligand complex using system builder, orthorhombic box with solvent water model (TIP3P) around the complex was performed. Further the box generated was then energy optimized/minimized and the charges of water model were neutralized by adding Na^+ and Cl^- ions with the default concentration of 0.15 M. The standard temperature (300.0 K) and standard pressure (1.01325 bar) were kept. MD simulation was done using the desmond, Schrodinger, LLC, New York, NY, 2019-1 to evaluate the stability of protein-ligand complex, complex RMSD, root mean square fluctuation (RMSF), complex contact bar graph, ligand RMSF and its other properties were analyzed to assess the stability of the complex for 40 ns simulation trajectory using desmond force field (OPLS 2005). The check point interval for the simulation trajectory was kept 240.06 ps.

Result and Discussion

For the combination therapy of the clonidine hydrochloride and fluorescent C-dots which were synthesized from paracetamol that acts as an anti-analgesic and antipyretic agent. As recently our group and others showed that C-dots possess good antibacterial effect while delivered alone or in combination. Therefore, a simple, fast and step microwave-based method was developed for the formulation of C-dots. The as-synthesized C-dots showed strong blue emission while excited by UV light (Trans illuminator). To study the absorbance and corresponding emission spectra of the C-dots UV-Vis and fluorescence spectroscopy studies were performed**.** The spectroscopic studies were completed using Systronic Double Beam Spectrophotometer 2202. The absorbance peak was observed at 242 nm **(Fig.S1a)**, while a blue emission peak was found at 450 nm when excited at 320 nm **(Fig.S1b)** using Analytical, FS 2060 India fluorescence spectrophotometer. The absorbance peak of C-dots at 242 nm was possibly due to π-π* & n-π*

transition as reported previously[23,25, and 26]. Furthermore, the gel electrophoresis technique was used for the measurement of the surface charge of the prepared C-dots. The charge was estimated by measuring the electrophoretic mobility of the C-dots (**Fig.S2**). C-dots were observed to be negatively charged as it was moving towards the positively electrode (**Fig.S2)** in gel electrophoresis. The blue band vividly seen in the gel image under UV conforms the presence of C-dots. The particles move through the cell according to their surface charge and polarity. Therefore, it would be mentioned here that a surface charge is suitable for bactericidal activity as it facilitates interaction with bacterial cell.

Further, to perform an antibacterial test, all the apparatus were autoclaved and the Gram-negative *E.coli* bacteria was cultured and incubated in the presence and absence of C-dot at 37°C with different concentrations [D1 (50 µL, D2 (100 µL), and D3 (200 µL)]. The growth of the bacterial cell in LB media following standard culture conditions. After 12 of the treatments bacterial growth was measured by taking the optical density (OD) of each samples at 595 nm **(Fig.S3)**. The studies were recorded in triplicate copies. The C-dots showed significant bactericidal activity against Gram-negative *E.coli* bacteria which was taken as a model system for probing bactericidal potency of C-dots. Thus, we were interested in the co-delivery of C-dots along with clonidine hydrochloride used for the treatment of glaucoma.

To perform the co-delivery polymeric nanocarriers were synthesized for co-delivery of the clonidine hydrochloride and as-synthesized C-dots. To determine the loading of the drug a calibration curve of drug clonidine hydrochloride prepared using wide range of concentration of drug solution. The prepared diluted solutions were analyzed using UV-Vis spectroscopy by probing the λ_{max} at 210 nm and the calibration curve was prepared **(Fig.S4 a,b)**. Similarly, the loading of the C-dots was also probed by UV-Vis study by taking absorbance at 242 nm. Next, 10 mg/mL of C-dots was co-loaded with 5 mg/mL of drug molecules within this NCs. The percentage loading of C-dots and drug were calculated by taking OD at 242 nm and 210 nm, respectively. The loaded NCs were precipitated and collected by centrifuging at 8000 rpm for 3 min. Scanning electron microscopy (SEM) was used to visualize the shape and size of loaded NCs. SEM images were taken at different magnifications of 2000x and 3500x (**Fig.S5 a,b**). The average size of the NCs was around 124 nm, it was evident from the corresponding particle size distribution (**Fig. S5 c**). The size of the nanocarriers is favorable for ocular delivery [27, 28]. The entrapped drug was calculated by the above-mentioned formula and it was found as 73.3% of drug entrapment. The value of drug entrapment shows better drug availability through the NCs. The nanocarriers were showing blue emission under UV-light due to presence of the C-dots (**Fig. S5 d**).

It was found from the graph that 90% of drugs get a release in 60 minutes from the NCs. The hydration test can be used for measuring ocular damage. Clonidine hydrochloride loaded C-dots NCs show a significant hydration level at the exposed cornea and percentage corneal hydration was noted as 78%. This value shows that nanocarriers have shown good hydration and thus there is better drug bioavailability.

Ex-vivo permeation and corneal hydration study were performed by using goat cornea. The eyes of the goat were selected for the study of *ex-vivo* permeation. A modified Franz diffusion cell was used for the study. Goat eyeballs were collected from a slaughter shop and kept in normal saline (0.9% w/v) solution at 4°C **(Fig.S6 a)**. All the experiments were achieved in aseptic

environment. The eyes were further washed properly with phosphate buffer saline (PBS) and additional tissue was detached. The excised cornea was placed between the donor and acceptor compartments of the diffusion cell taking surface area as 1.5 cm. The lower compartment was full with freshly prepared buffer solution and 1 mL of the samples were taken from lower compartment after permeation from cornea, coming from donor compartment. The study was achieved at 37 ℃ and 50 rpm. Three samples of permeated drugs were taken at different time points and were estimated by observing OD at 210 nm. The permeated samples were collected from the acceptor compartment in the quantity of 1 mL at specific time intervals (10 min, 20 min, 30 min, 45 min & 60 min) and simultaneously it was replaced with the same value of PBS solution. A graph of the average permeation study was plotted between the percentage drug release and time in min (**Fig.S6 b**). Also, corneal hydration study was performed achieved to check the hydration of the cornea after action with C-dots-loaded clonidine hydrochloride nanocarrier formulation. The goat cornea was soaked with NCs and wet weight was noted. The soaked cornea was further kept in a hot air oven at 60℃ up to 3 days. The dry weight was noted after 3 days and corneal hydration was estimated using following equation (eq. 2):

$$\%\ \boldsymbol{Corneal\ Hydration} = \frac{\boldsymbol{Wet\ Wt.of\ Cornea - Dry\ Wt.of\ Cornea}}{\boldsymbol{Dry\ weight\ of\ Cornea}}\boldsymbol{x100} \quad (\boldsymbol{eq.2})$$

Finding mode of action of a drug molecule is very important for any real-life application. Since *in-vivo* studies requires sophisticated laboratory set up and costly reagents, therefore understanding the mode of action of the drug by computation approaches can save time as well as resources. To validate our finding, *in-silico* studies were performed to showcase the interaction of the drug molecules with *α-2* receptor. Previous studies showed that clonidine hydrochloride is a *α-2* agonist, particularly acts on *α-2* receptor that helps in reduction of the IOP by stimulating the same receptors.[3] The interaction of the clonidine hydrochloride (PubChem ID: 20179) with the protein *α-2a* adrenergic receptor (PDB ID:6KUX) was studies by molecular docking and MD simulation.

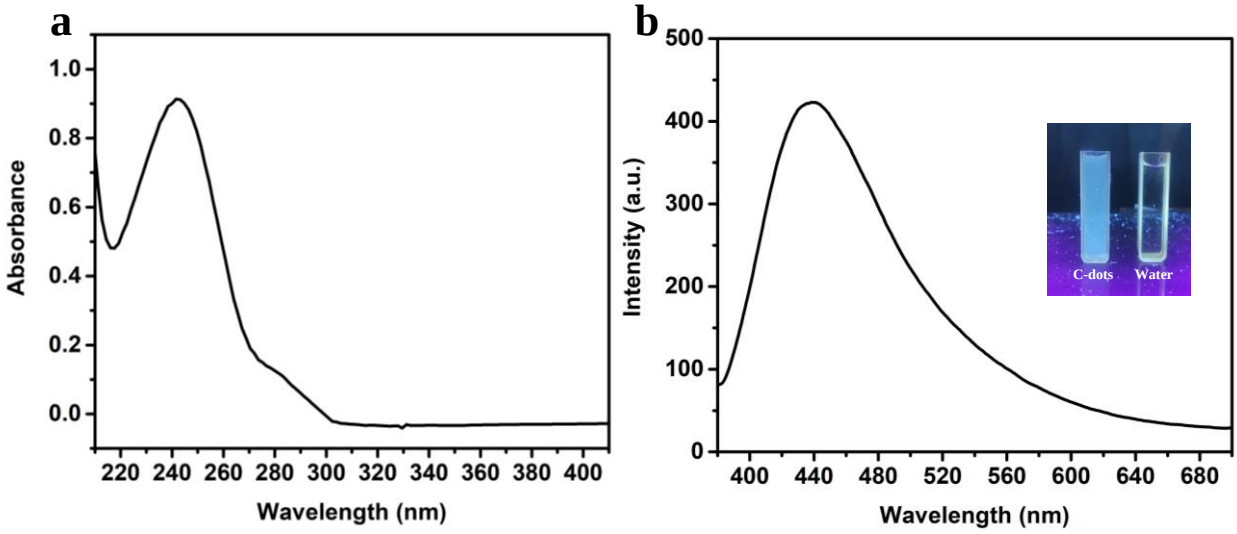

Fig. S1: a) Absorbance and **b)** fluorescence spectra of the C-dots synthesized from paracetamol. Comparative image of fluorescent C-dots with DI water under UV transilluminator (inset picture).

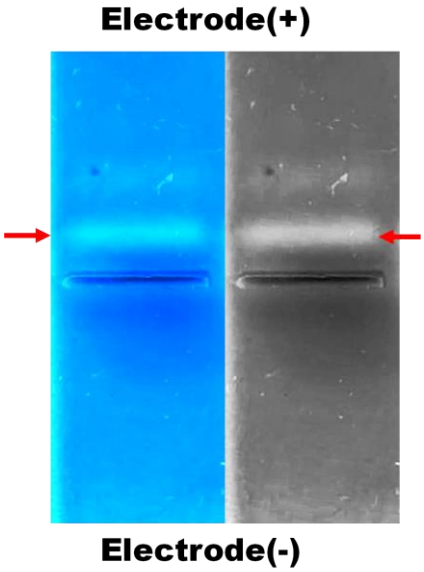

Fig. S2: Gel Electrophoresis study showing the negatively charged C-dots moving towards positive pole (blue emission). Emission band of C-dots was marked with red arrow. Same gel having color as well as black and white images.

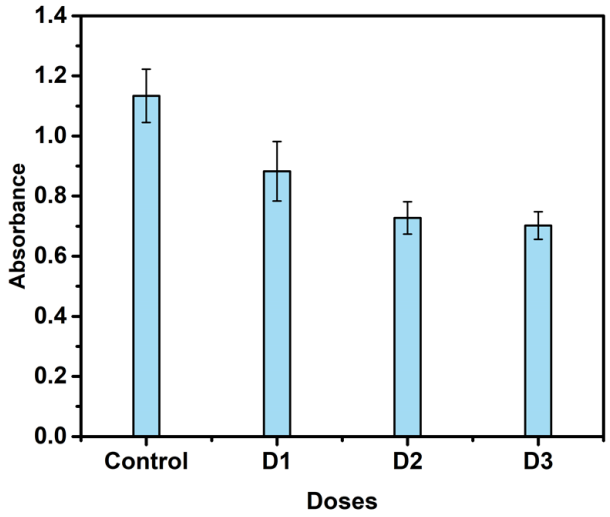

Fig. S3: Dose-dependent antibacterial activity measurement on *E coli* using C-dots under standard bacterial growth conditions. The values are represented as mean ± SD of results from three individual experiments.

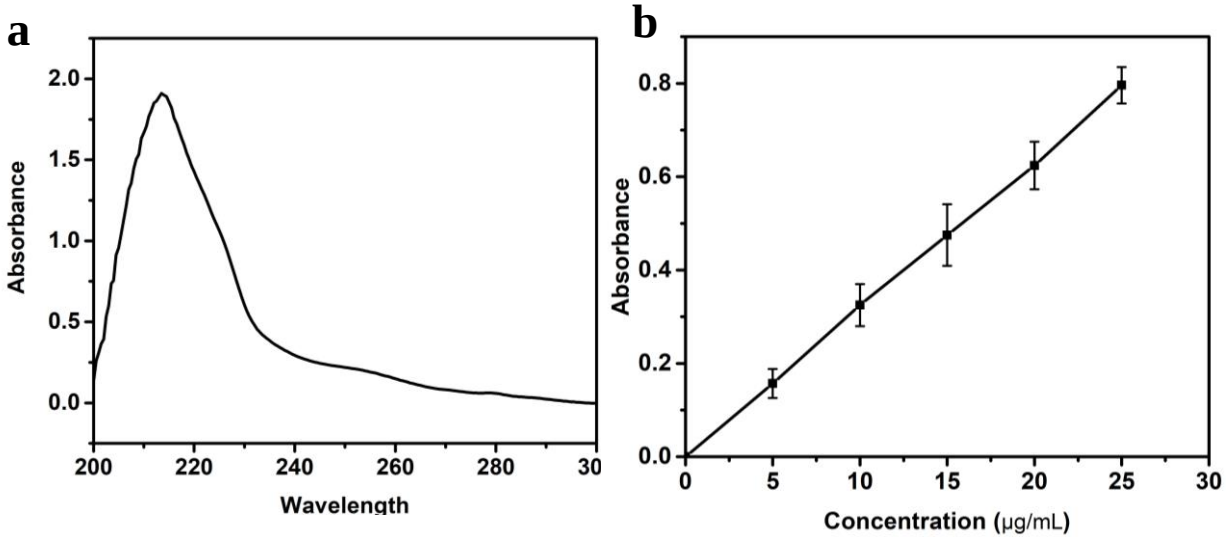

Fig. S4: a) UV-Vis spectrum of drug Clonidine Hydrochloride; **b)** Calibration curve post dilution of drug Clonidine Hydrochloride**.**

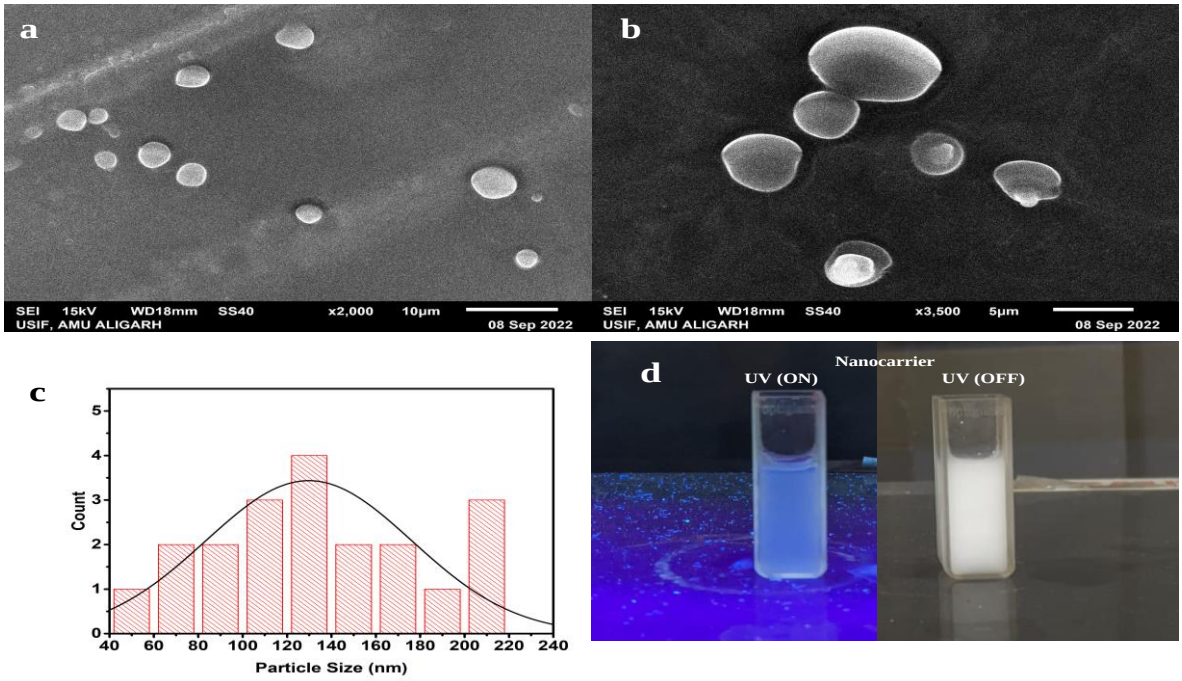

Fig. S5: SEM image of drug and C-dots loaded nanocarrier of magnification **a)** 2000x and **b)** 3500x; **c)** Particle size distribution of the nanocarrier; **d)** Comparative image showing presence of C-dots in the nanocarrier.

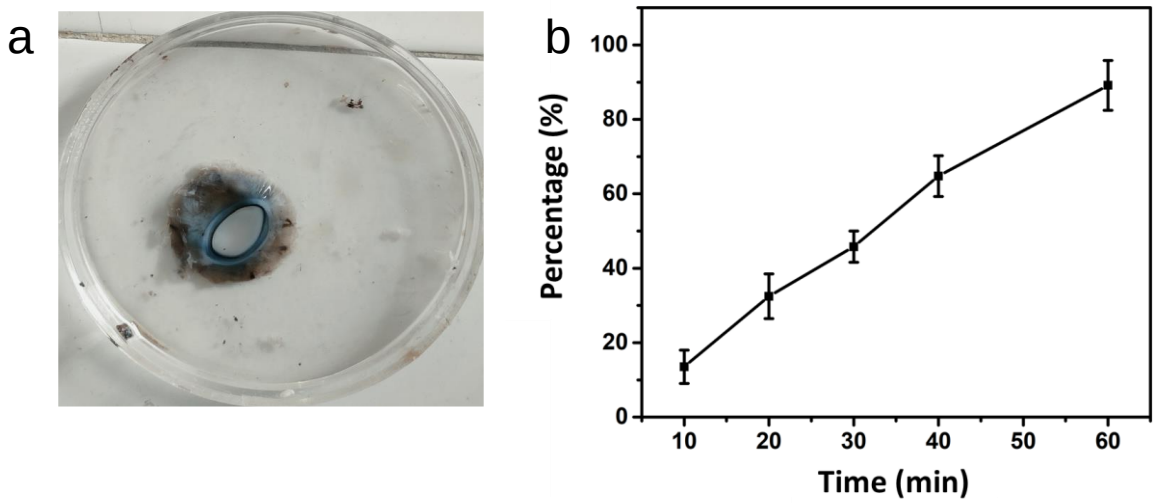

Fig. S6: (a) Goat eyeballs for the study of *ex-vivo* permeation **(b)** Time dependent percentage drug release showing average permeation of drug present in nanocarrier in goat eye. The values are represented as mean ± SD of results from three individual experiments as N=3.

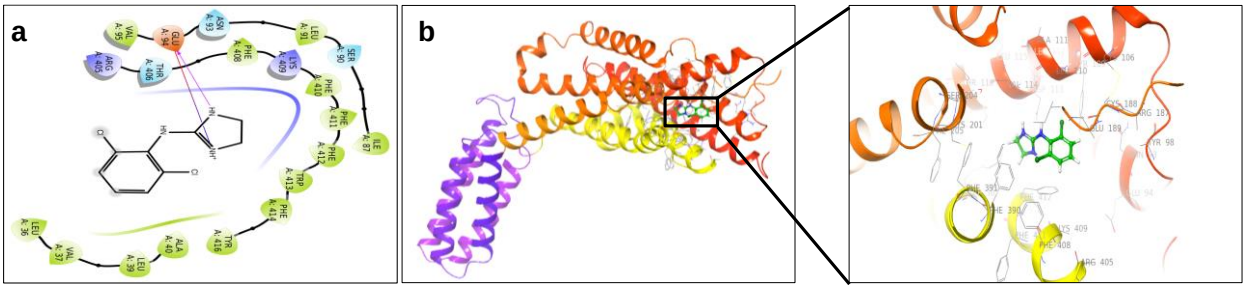

Fig. S7: a) 2D interaction of Clonidine hydrochloride and protein (6KUX); **b)** 3D interaction of Clonidine hydrochloride and protein (6KUX)

The docking score of clonidine hydrochloride against 6KUX are -5.268 Kcal/mol which shows that there is strong binding affinity of the ligand in the cavity with protein (**Fig.S7 a, b and Table S1**). Clonidine hydrochloride forms hydrogen bond with GLU94 (Bond length: 1.62 Å). GLU94 (bond length: 4.64 Å) is also forming salt bridge with ligand. THR460, SER90, ASN93 are involved in polar interaction and the residues like LEU91, PHE408, PHE410, PHE411, PHE412, TRP413, PHE414, TYR416, VAL95, LEU36, VAL37, LEU39 and ALA40 are involved in hydrophobic interaction. Interestingly, we also observe that all these residues are part of active site of the protein. This strongly suggest that clonidine hydrochloride can be effective inhibitor against 6KUX.

Table S1: Molecular Docking scores

Protein PDB ID	**Ligands PubChem ID**	**Ligand Name**	**Docking score (Kcal/mol)**	**Interacting residues**

6KUX	20179	Clonidine hydrochloride	**-5.268**	H bond - GLU94 (1.62 Å) Salt bridge: GLU94 (4.64 Å) Polar interaction – THR460, SER90, ASN93 Hydrophobic interaction – LEU91, PHE408, PHE410, PHE411, PHE412, TRP413, PHE414, TYR416, VAL95, LEU36, VAL37, LEU39 and ALA40

For 6KUX with clonidine hydrochloride, the root mean square deviation (RMSD) graph demonstrated that the protein-ligand complex is stable with increase in simulation time (**Fig.S8 a, b, c, d, e**). The simulation event analysis (SEA) and protein-ligand interaction during the simulation also supports the same. The RMSD mean for Cα chain, side-chain and ligand were 5.003 ± 1.366 Å, 5.651 ± 1.364 Å and 0.452 ± 0.228 Å, respectively. The RMSD of protein was also showing stability after 35 ns whereas in case of ligand there was very little fluctuation observed with protein. Docking and MD simulation results showed that the receptor protein was also showing good stability after formation complex with the drug.

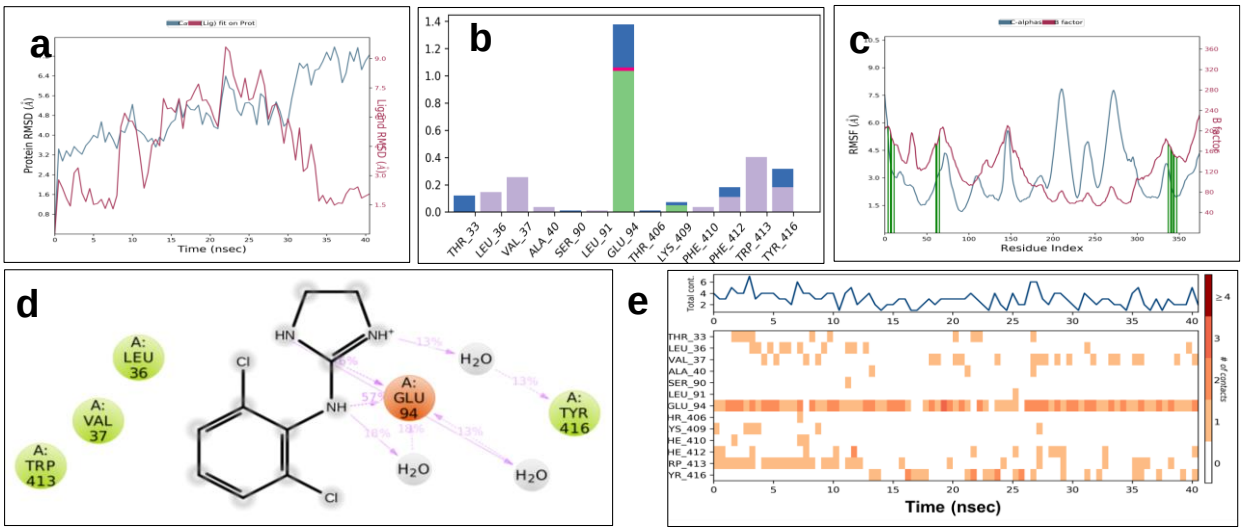

Fig. S8 a) RMSD of protein-ligand complex; **b)** Interaction fraction during simulation; **c)** RMSF /B factor and interacting residues (Green) of protein; **d)** 2D interaction of ligand with protein during simulation; **e)** A representation (timeline) of protein-ligand contacts (H-bonds, Ionic, Water bridges, Hydrophobic).

Conclusion

In brief, we have developed a simple and fast method of synthesis of C-dots from paracetamol that was showing good bactericidal activity. It was co-delivered with clonidine hydrochloride (CH) by developing suitable polymeric nanocarriers. The *in-vitro* results showed that spherical-shaped nanocarriers are having a favorable size for the ocular delivery of the drug. The *ex-vivo* studies also showed that loaded nanocarriers effectively release the drug, thus suitable for ocular

delivery. It is, therefore, deemed that present co-delivery may be suitable for combination therapy of glaucoma and any secondary bacterial infection.

Author Declaration

The authors declare no conflict of interest.

Funding details

This work was not supported by any Funding Agency.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

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