Red-emitting carbon dots and their biological application as antifungal/anti-biofilm agent

Regina Huang

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DATE: May 27, 2020

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THESIS TITLE: Red-emitting Carbon Dots and their Biological Application as Antifungal/Anti-biofilm Agent

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Issued by Graduate School, HKBU
Red-emitting Carbon Dots and their Biological Application as Antifungal/Anti-biofilm Agent

HUANG Regina

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Philosophy

Principal Supervisor: Dr. LEUNG Ken Cham Fai (Hong Kong Baptist University)

May 2020
DECLARATION

I hereby declare that this thesis represents my own work which has been done after registration for the degree of MPhil at Hong Kong Baptist University, and has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications.

I have read the University’s current research ethics guidelines, and accept responsibility for the conduct of the procedures in accordance with the University’s Research Ethics Committee (REC). I have attempted to identify all the risks related to this research that may arise in conducting this research, obtained the relevant ethical and/or safety approval (where applicable), and acknowledged my obligations and the rights of the participants.

Signature: Regine Huang
Date: May 2020
Carbon dots (CD) have emerged as the new eye-catching theranostic nanomaterials due to their distinctive features, including tunable emission, facile surface modification, high biocompatibility and low cytotoxicity. These distinguishing features allow full customizations of CD according to the needs of various studies. Of note, they have been widely employed as nano-vehicles with live-tracking systems in many biological applications to deliver medicine with low bioavailability to targeted sites. *Candida albicans*, a commonly seen commensal fungus accounts for life-threatening infections in humans, is the leading cause of oral candidiasis. Yet, the efficacy of the “gold standard” Amphotericin B (AmB) has been limited due to poor water solubility and dose-dependent cytotoxicity. In addition, the interactions of CD with *Candida* cells/biofilms and human epithelial tissues have not been fully investigated, and very limited studies have been done on CD-based antifungal drugs delivery for topical administration. Herein, AmB-conjugated guanylated CD (CD-Gu⁺-AmB) tackling oral fungal infections were synthesized and possessed potent antifungal/anti-biofilm effects against *C. albicans*. Moreover, CD-Gu⁺-AmB exhibit low cytotoxicity to primary human oral keratinocytes and can selectively accumulate in the cell nuclei. Above all, the first evidence of studying the penetration and exfoliation profiles of CD in a three-dimensional organotypic human oral epithelial tissue model was provided, and the accumulation of CD-Gu⁺-AmB in the epithelial tissue can form a ‘shielding’ layer on oral epithelia against *C. albicans*. This study demonstrates that CD-Gu⁺-AmB may serve as a promising antifungal agent for tackling *C. albicans* and *Candida*-induced oral candidiasis through fast epithelial penetration, extra-/intra-cellular embedding and gradual exfoliation.
Acknowledgements

Time just seems to fly. Two years ago, I quit my job and went back to school. This is never an easy decision—to leave the place I love and set foot on a foreign land—but today I am proud to say: YES! I MADE IT.

The past two years in Hong Kong have been filled with eventful memories and repeating cycles of overcoming self-doubt and gaining self-confidence. However, without the endless love and support from the amazing people in my life, I simply cannot come this far. I would like to first express my sincere gratitude to Prof. Albert Sun-Chi Chan and my supervisor, Dr. Ken Cham Fai Leung, for their unlimited guidance and encouragement throughout the study period. Under the supervision of Dr. Ken Leung, I was able to achieve personal growth and become a better professional. In addition, I would like to deeply thank Dr. Lin Xuan Li for her mentorship. She has always been the sparkles in the sky and inspired me in so many ways. Working with her has been a blessing to my life, and I forever appreciate her guidance. I also want to take the time to thank Mr. Fung Kit Tang for his teaching in organic synthesis techniques. He is always willing to share his ideas and experience with others and encourage me to aim higher. I am also grateful to Ms. Winnie Wu, Mr. Wai Chung Li, Mr. Sing-Ming Chan, Dr. Ruitao Zhou, Mr. Daniel Tritton, Dr. Chak Shing Kwan and Mr. Joshua Lai, for being great traveling companions on this journey. They have lighted up my days in Hong Kong.

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Abbreviations and Acronyms

°C  Degree Celsius  m/z  Mass-to-charge ratio
K   Kelvin         min  Minute(s)
µL  Microliter(s)  h    Hour(s)
mL  Milliliter(s)  d    Day(s)
mg  Milligram(s)  nm   Nanometer(s)
g   Gram(s)       Da   Dalton
ν/ν  Volume per volume eV   Electronvolt
Hz  Hertz         M⁺  Parent molecular ion
MHz Megahertz     J    Coupling constant
3D  Three-dimensional nM  Nanomolar
AmB Amphotericin B MALDI- TOF Matrix-assisted laser
Deborption Ionization- Time of Flight

CA  Candida albicans Me:CO Acetone
CD  Carbon dot(s)  NHS  N'-Hydroxysuccinimide
CDs CD, CD-Gu⁺ and OD  Organic dye(s)
CD-Gu⁺-AmB
CFU Colony-forming unit PBS Phosphate buffered saline
CQD Carbon quantum dot(s) PEI Polyethylenimine
DAPI 4′,6-diamidino-2- DCM Dichloromethane QD  Quantum dot(s)
phenylindole
DLS Dynamic light scattering  QY  Quantum yield(s)
DMF Dimethylformamide  RHGE Reconstituted human
gingival epithelia
DMSO Dimethylsulfoxide  RHOE Reconstituted human oral
epithelia
DOX Doxorubicin  rt  Room temperature
EDC 1-ethyl-3-(3- DOX  Doxorubicin  rt  Room temperature
dimethylaminopropyl) EDC 1-ethyl-3-(3- dimethylaminopropyl) carbodiimide hydrochloride
EtOH Ethanol  SDA  Sabouraud dextrose agar
FA  Formamide  SEM  Scanning electron SEM  Scanning electron
microscope
FL Fluorescence  SGM  SkinEthic growth medium
FT–IR Fourier-transform infrared TEM Transmission electron
spectroscopy  TEM Transmission electron
GQD Graphene quantum dot(s)  UV–vis Ultraviolet-visible
HOKs Primary human oral  XPS X-ray photoelectron
keratinocytes  XPS X-ray photoelectron
IF  Immunofluorescent  XRD X-Ray diffraction
INT Iodonitrotetrazolium  YNB Yeast nitrogen base broth
IR  Infrared
1.1 Introduction

Carbon dots (CD) have attracted numerous interests since their accidental discovery by Xu et al. during the purification of single-walled carbon nanotubes from arc-discharge soot in 2004.\(^1\) They have been termed by many names, including carbon quantum dots (CQD) and graphene quantum dots (GQD);\(^2,3\) however, the “CD” established by Sun et al. is the most universally accepted form.\(^4\) These emerging CD have been defined as photoluminescent carbon-based spherical nanomaterials with an average size of less than 10 nm, and they can be easily obtained from any carbon-containing environmental waste. Apart from having the aforementioned advantages, the amphiphilic CD also exhibit excellent water solubility, surface functionalization availability, photostability, sensitivity, biocompatibility and low cytotoxicity.\(^5,6\) Thus, they have been widely celebrated as the promising alternatives for some biological applications, especially in theranostic technology (Figure 1-1), to overcome the limitations of the environmentally unfriendly quantum dots (QD) and organic dyes (OD).\(^7,9\)

![Figure 1-1](image)

**Figure 1-1.** Schematic illustration of the unique characteristics of CD with tunable fluorescence leading to different applications.
In comparison to QD and OD, CD were often considered as green chemistry. The starting materials of CD are usually inexpensive and easily accessible, and their production only generates little wastes.\(^\text{10}\) For instance, several experiments were carried out by using natural products, such as carbon-containing disposals,\(^\text{11-13}\) fruit wastes,\(^\text{14,15}\) fingernails and hair fiber,\(^\text{16,17}\) just to name a few. In other words, these eco-friendly carbon-based nanomaterials can significantly reduce the cost and minimize possible environmental hazards during the synthetic process, implying the potential of taking them into industrial scales and commercializing them into the new spotlights for green nanotechnology. In addition, their synthetic pathways allow customizations of the CD by introducing different dopants to the system,\(^\text{9,18-20}\) and by modifying the components, CD can be developed into highly sensitive and selective catalysts/detectors.\(^\text{20-22}\) Likewise, owing to the diversity of the elemental composition (mostly carbon, oxygen, hydrogen and nitrogen) of CD’s starting materials, the surface of the particles can be readily abounded with various functional groups, while hydroxyl and amine groups being the most commonly utilized ones. These functional groups passivating on the CD surface allow additional modifications of the particles, which not only contributes to emission tuning but also makes the CD one of the most promising candidates for delivering water-insoluble drugs.\(^\text{23-25}\)

Herein, Chapter 1 wishes to provide a brief overview on CD by first introducing some of the selected approaches of the two synthetic methods (top-down and bottom-up) and evaluate a few factors of determining surface passivation. The evaluation then leads to the discussion of the optical properties of CD, particularly focusing on the ones with red fluorescence. Last but not least, the last two sections will highlight some of the CD applications, including bioimaging.\(^\text{24}\)
fingerprints detection, chemical sensing, biological sensing, drug delivery, photocatalyst and electrocatalysts, followed by the aim of this thesis.

1.2 Synthesis of CD

The synthetic approach of CD plays a crucial role in determining their physical properties, which can guarantee green synthesis by allowing any carbon-containing source to be fully recycled. Although the specific formation mechanism has yet to be confirmed, in general, the synthesis can be classified into two methods—top-down and bottom-up. The top-down method involves downgrading the larger carbon skeleton into a smaller one, such as arc discharge, laser ablation and electrochemical oxidation. Likewise, the bottom-up method converts the carbon-based molecules to form the particles via combustion/thermal treatments and microwave pyrolysis. While the CD synthesized from both methods share similar morphologies, their fluorescence, surface functional groups and quantum yields (QY) can vary from batch to batch. Therefore, to customize the CD, the knowing of the formation process needs to be fully studied and summarized.

Simply put, the top-down method is to degrade the large carbon containing materials into small particles. The model allows the investigators to outline an overview but is unable to provide additional details for the subunits. For instance, the OH and C=O-rich CD synthesized by Tian et al. with size of 4-5 nm were first made by burning the carbon soot inside an upside-down beaker with natural gas then etching with nitric acid. The CD structures containing sp² carbon were confirmed by NMR, and exhibit excitation dependent photoluminescence properties but with a low QY of 0.43%. The study was able to characterize the extrinsic physical properties of CD, but the intrinsic properties such as fluorescence
mechanism still require further investigations. In another study performed by Zhou et al., they successfully converted the multi-walled carbon nanotubes into CD with size of 2-3 nm via electrochemical treatment. However, with the ultraviolet-visible (UV–vis) peak and photoluminescence peak showing at 270 and 410 nm, respectively, the CD only maintained a QY of 0.064% while the fluorescence and formation mechanisms were left unexplained. Despite the convenience of the particle synthesis, top-down method might have failed to be the ideal approach to customize the particles due to many unidentifiable factors. Therefore, the synthetic strategies of the bottom-up method might also be scrutinized.

The bottom-up method utilizes small molecules as building blocks to synthesize the CD via combustion/thermal treatments and microwave pyrolysis, enabling partial control of CD’s physical properties. For instance, Sahu et al. customized highly green-fluorescent CD with size of 3 nm by using orange juice via hydrothermal route. The QY of the CD was determined as 26% and exhibited an excitation-dependent fluorescence. In 2015, Liu et al. developed a new method to synthesize different CD with size of less than 5 nm by mixing the glacial acetic acid into water and treated with P2O5. The CD showed an excitation-independent fluorescence at 480 nm and have a QY of up to 8.12%. Besides the hydrothermal method, microwave-assisted pyrolysis is also one of the most widely employed treatments. Wang et al. demonstrated the photoluminescent CD with multi-color emission in 2011. In comparison to the top-down method, the bottom-up method can afford better control of the starting materials and the resulting fluorescence. Meanwhile, the biological compatible CD do not need severe synthetic condition and can be easily made in home kitchen with carbohydrates (Figure 1-2).
Several strategies were also introduced by employing the combination of both top-down and bottom-up methods during the synthetic process. While maintaining the easiness of obtaining the raw materials, the process provides several modifications of the physical properties. In 2013, Sun et al. synthesized N,S-codoped CD by etching the human hair fiber with concentrated sulfuric acid under 24 h of sonication and dialyzed the products against DI water. The CD synthesized at 150°C has an average size of 5 nm with a QY of 5.1%. This strategy is particularly useful with bulky carbon-based waste. As shown in Table 1-1, all types of CD were summarized according to their synthetic conditions and physical characteristics.
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</table>
1.3 Surface Passivation/Functionalization

Surface passivation and functionalization of CD can be modified at the early stage, making the final product highly selective and sensitive to its environment. Some of the customizations include employing different synthetic methods or introducing various dopants to the system, which is especially beneficial to synthesize the CD with desired characteristics. For example, Liu et al. demonstrated that the CD surface could passivate with highly branched polyethylenimine (PEI) by simply mixing the component with glycerol in a one-pot microwave pyrolysis.\(^{39}\) Oxidative treatment with strong acids is another simple yet effective way to introduce carbonyl and carboxyl groups on the surface of CD. The functional groups passivated on the CD surface can greatly increase the water solubility without any further modification.\(^{41}\)

Apart from utilizing different synthetic methods, the elemental compositions of the starting materials can also change the functionalities of CD. In 2011, Wang et al. used organosilanes carrying long-chain amines to form CD.\(^{37}\) The fabrication of carboxyl groups allows additional conjugation with silica particles, which can contribute to the development of theranostic applications. Yang et al. synthesized amine-functionalized CD by hydrothermal carbonization of chitosan.\(^{42}\) The chitosan with readily available amino groups can form the fluorescent CD without needing to modify the CD surface. Besides the carbonyl- and amine-rich molecules, other elements, such as sulfur, phosphorus and boron, can also be doped into the system.\(^{20,43,44}\) For instance, Dong et al. synthesized N,S-codoped CD by reacting the carbon (citric acid) and sulfur (L-cysteine) sources \textit{via} the hydrothermal method.\(^{18}\) The exterior of CD was effortlessly passivated with sulfur-containing functional groups that change the fluorescence. Moreover,
additional N,S-codoped study suggests that these CD have high binding affinity to Hg^{2+}. Phosphorus is another common dopant that has been introduced to the CD system. Chandra et al. prepared N,P-codoped CD via hydrothermal route for Fe^{3+} detection. Besides nonmetallic elements, metals can also be invited into the CD skeleton usually in the presence of acid and base. In comparison to other nonmetallic dopants, metal dopants seem to have greater impacts on the charge density and charge transition formed between the graphitic matrix and the metal ions.

Apart from controlling the surface passivation/functionalization in a one-pot synthesis, functional groups can also be invited to the system through direct conjugation. Many studies related to biological applications have employed both direct and indirect pathways to achieve their demanded outcome.

### 1.4 Optical Properties

Another selling point of CD is perhaps greatly attributed to their inborn tunable fluorescence. Although the fluorescence mechanism is still an ongoing debate, several studies have indicated that the fluorescence feature could be due to a combination of many factors. The most frequently seen explanation, however, is possibly induced by the bandgap transitions of the π-conjugated domains. Each individual π-conjugated domain is uniquely distant from one another, allowing the formation of various electronic energy bandgap. When the domains bridged, self-quenching might occur, which could be caused by the lack of transition radiations to the ground state (Figure 1-3).
Figure 1-3. Two types of CD with weak/bright emission. Strong UV absorbance CD (left) show weak or no emission, whereas low UV absorbance CD (right) demonstrate bright multi-color emission.

The other widely accepted factor could be associated with surface defects from the imperfect $sp^2$ and $sp^3$ hybrid carbons. These carbons create energy traps that lead to different energy gaps, and hence generate strong multi-color emissions. As suggested by Sun et al., these energy traps can be described by the quantum confinement effect. In brief, the movement of electrons is confined in nano-sized particles, thereby, the energy levels are discrete, which widens the energy gap.

To tune the emission, some studies have been performed to investigate the fluorescence of CD. As shown in Figure 1-4, Ding et al. applied silica column chromatography on the CD to confirm that the emission might be controlled by the surface oxidation state instead of the size. The purification process via silica column
enables the findings of different surfaces states of the CD samples. When the oxidation state increases, CD emission will shift towards the NIR region.\textsuperscript{54} Additionally, the emission can also be influenced by the amount of graphitic nitrogen embedded in the CD skeleton.\textsuperscript{55} This type of nitrogen, securely installed in the CD structure with no neighboring space, can promote the generation of midgap state within the HOMO-LUMO gap of the undoped system. Recent studies have also shown that fluorescence emissions can be pH-dependent.\textsuperscript{6,56,57} Liu et al. discovered that the fluorescence intensity of CD decreases when the pH of the solution is not neutral.\textsuperscript{57} Interestingly, some found out that the fluorescence intensity decreases when the solution is slightly basic or within a certain range.\textsuperscript{6,58} In the slightly basic environment, deprotonation of the carboxyls on CD might occur, thereby, changing the electrostatic interaction between the particles and solvent system. Yet, these findings were performed with many variations, and the pH-dependent fluorescence emissions were often found to be controversial to one another. Therefore, additional work must be performed for further investigations.

\textbf{Figure 1-4.} (A) One-pot synthesis and purification for CDs with (B) eight distinct samples of fluorescence characteristics under 365 nm UV light. (C) Corresponding PL emission spectra of the eight samples.\textsuperscript{54}
1.5 Applications

CD have been widely applied in several applications, including biolabeling, bioimaging, metal ion sensing, fingerprint detection, photocatalysis and drug delivery due to their excellent water solubility, photostability, sensitivity, biocompatibility and low cytotoxicity. Specifically, metal ion sensing has been the most commonly exploited application to detect Hg$^{2+}$ and other heavy metals in aqueous medium. Wang et al. introduced the N,S-codoped CD can detect Hg$^{2+}$ with low detection limit of 10 nM. Yuan et al. synthesized Germanium(III)-doped CD that also enables the detection of Hg$^{2+}$. Recently, Gao et al. have successfully prepared a red-emitting CD via a one-pot synthesis to detect Pt$^{2+}$, Au$^{3+}$ and Pd$^{2+}$. The sensitivity/selectivity of CD can be due to the availability of the surface functionalities. Apart from the detection of metal ions, CD can also be detected anions and some small molecules. For example, the Gd(III)-doped CD with dual fluorescence could successfully perform MRI probing by showing great $T_1$-weighted MRI contrast. B,N,S-codoped CD with red fluorescence can also be utilized to detect Ag$^+$ and $L$-cysteine with detection limits as low as 0.35 µM and 0.045 µM, respectively. However, unlike the fluorescence mechanism of metal ion sensing, the fluorescence could be unquenched in the detection of anions. Regardless of the metal ions sensing, CD have also been modified for pathogenic fungal detection. A recent study has modified the CD with amphotericin B for Candida albicans detection. Nevertheless, the binding mechanism and the utilization of the applications still require additional investigation.

A large part of the reasons that make CD popular among the currently applicable nanomaterials is because of their excellent bioavailability and low cytotoxicity. Therefore, they have been considered as promising candidates for
bioimaging agents. Zuo et al. synthesized highly efficient and selective F-doped CD with red fluorescence to detect Ag\(^+\) in both normal and cancer cells at the optimal concentration of 60 µg/mL. In comparison to QD, the optical properties can be easily achieved by CD, and at the same time, these CD can maintain good chemical and photochemical stabilities. Furthermore, the cell viability has increased in most of the CD, which also indicates that CD is suitable for biological application. For instance, mice injected with 8-40 mg/kg of PEGylated-CD can survive up to 28 days, which is comparable to the NaCl control group.

Aside from being bioimaging agents, CD can also be advanced into drug delivering agents with real-time tracking. Wang et al. developed a type of dual emissive doxorubicin (DOX)-conjugated CD with low cytotoxicity that can fast penetrate into cancer cells. DOX, an anticancer drug, can be pH-controlled and released on the tumor sites. In another system designed by Zheng et al., CD show great promises for delivering anticancer drugs by having minimal side effects and toxicity. The findings suggest that the employment of CD as drug delivering vehicles for anticancer treatment is feasible. In addition to being anticancer carriers, CD can also become a platform to transport genes into the cell. The PEI-passivated CD carrying DNA can diffuse and be found in the cytosol of the cells. The CD, other than benefiting from strong photoluminescence alongside excellent water solubility and photostability, can mediate transfection into the cells. CD offer a compact all-in-one package that enables live tracking and delivering of the medicine at the same time, and thereby, assuring the possibilities of more efficacious drug delivering systems.

Despite the aforementioned applications, photocatalysis has also drawn tremendous attention. Owing to their adjustable emission wavelength (aka. tunable
band gap), CD have been seen as the alternative to overcome the shortcomings of easily damaged organic compounds.\textsuperscript{50} The alkali-assisted CD via electrochemical fabrication with TiO\textsubscript{2} and SiO\textsubscript{2} demonstrated the process of photodegradation is efficient, whereas TiO\textsubscript{2} and SiO\textsubscript{2} by themselves show no/little sign of photocatalytic activity.\textsuperscript{40} In another study performed by Christensen \textit{et al.}, they discovered that CD can generate reactive oxygen species when mixed in solutions containing free radicals.\textsuperscript{70} The oligomeric aminopolymers/PEG-passivated CD allow the particles to produce singlet oxygen upon UV light radiation, implying the potential usage of antioxidant applications. Although the findings of CD as photocatalysts lack significant improvements, additional investigations could be done to provide more valuable insights to the field.

\textbf{1.6 Aim of the Thesis}

As discussed in the previous sections, CD can afford several advantages including excellent biocompatibility, water solubility, photostability and low cytotoxicity. In this thesis, CD with red fluorescence were synthesized to increase the length of penetration depth to tissues. And, to achieve the emission wavelength, different solvent systems, temperature and time were being fully investigated. The as-synthesized CD were then conjugated with a hydrophobic drug, i.e., amphotericin B, and served as trackable medicine vehicles to eliminate \textit{Candida albicans}, a commensal fungus, and its biofilms. Subsequently, the system was applied on a well-established, 3D organotypic human oral epithelial tissue model to study the penetration and gradual exfoliation profiles of CD, which interestingly, can form a “shielding layer” against the invasion of \textit{C. albicans}. 

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Chapter 2: Oral Epithelia Embedded with Red-Emitting Amphotericin B-Conjugated Carbon Dots Prevent the Adherence and Invasion of Candida albicans

2.1 General Consideration

Oral candidiasis, the most commonly appeared fungal infection of oral mucosa in humans, is often found in immunocompromised/chronically ill individuals such as HIV/AIDS, cancer and diabetic patients.\(^{71,72}\) The invasive candidiasis can elicit a chain of immune responses and advance to lethal systemic infections.\(^{73}\) The outset of this serious infection is mainly due to a ubiquitous commensal microorganism, Candida albicans, colonizing in the human body since infancy. This dynamically mercurial fungus can switch to pathogenic form in response to various local/systemic factors such as denture wearing, dry mouth, age, immunodeficiency, endocrine disorders and malnutrition.\(^{72,74}\) Therefore, the management of oral candidiasis is highly recommended, including maintaining good oral hygiene and applying antifungal drugs topically or systemically.

The most-used families of antifungal drugs—the azoles, the allylamines and the polyenes—have been prescribed in clinic to deal with the infections.\(^{75}\) In particular, amphotericin B (AmB), one of the polyenes, has long been considered as the “gold standard” for treating systemic fungal infection.\(^{76}\) It has been reported that fluconazole-resisted oral thrush in HIV\(^+\) patients could be eliminated by oral AmB,\(^{77}\) and its oral suspension is the major choice for the refractory cases of oral candidiasis. However, its clinical application is seriously restricted by the dose-dependent toxicity, and its clinical bioavailability is limited because of the poor water solubility. As a consequence, several lipid-based AmB formulations were
introduced to the market, in hopes of decreasing toxicity with the reference of conventional AmB while providing a comparable clinical effect.\textsuperscript{78} In addition, the ultradeformable liposomes containing AmB showed potent antifungal effect and a profound penetration and accumulation in human skin.\textsuperscript{79} Meanwhile, nanotechnology is extensively employed on developing oral formulation of AmB. Carbon nanotube, polymeric nanocarriers or other solid lipids were functionalized alongside AmB to enhance the drug efficiency, improve oral bioavailability or reduce adverse reactions.\textsuperscript{60,80-85} Nevertheless, the penetration profile of AmB or its nano-formulations in oral epithelia were barely addressed in the reports. Therefore, urgent attention is required before advancing to the next stage of research.

Carbon dots (CD), an emerging amphiphilic carbon-based nanomaterial with an average diameter less than 10 nm, have attracted great attention since their discovery in 2004. Owning to their inexpensiveness, facile surface modification, tunable emission, excellent bioavailability and low cytotoxicity, they have been widely employed in chemical/biological sensing, bioimaging and especially, drug delivering.\textsuperscript{45,50} As fluorescent trackable drug vehicles, CD have been broadly developed as targeting delivery system with tailed functions for anti-cancer treatment.\textsuperscript{62,86,87} However, the reports of delivering antifungal drugs using CD are still very limited, and most of them are still applying as fluorescence probes for detecting fungal cells.\textsuperscript{88-91} Moreover, the interaction of CD with fungal cells, together with the penetration and exfoliation patterns of CD in the epithelial tissues \textit{via} topical application, has not yet been fully exploited. Concurrently, the increase of local concentration of antifungal drug may inhibit the growth of the fungal cells without affecting the symbiosis of the oral environment.
Herein, we developed a water soluble red-emitting CD-based AmB delivery system that possess potent antifungal and anti-biofilm effect on *C. albicans* (Scheme 2-1 A). These CD with low cytotoxicity could accumulate in the cell nuclei of the host cells. In order to study the interaction between CD and the oral epithelium, a well-established three-dimensional (3D) organotypic human oral epithelial model was utilized in this study. Notably, the as-synthesized CD could fast penetrate the epithelia and then exfoliate from the tissue along with the tissue growth. And most importantly, the pretreatment of the CD-based AmB delivery system given via topical application, could form an antifungal barrier in the epithelia and have a long-term resistance to *C. albicans* invasion (Scheme 2-1 B).

![Scheme 2-1](image_url)

**Scheme 2-1.** (A) The as-synthesized red-emitting guanylated carbon dots with amphotericin B-conjugation (CD-Gu⁺-AmB) present potent anti-biofilm effect on two-day-old biofilms of *C. albicans* and could accumulate in *Candida* cells by disrupting their membrane integrity under confocal microscope. (B) The pretreatment of CD-Gu⁺-AmB on reconstituted human oral epithelia (RHOE) could maintain the integrity of the tissue and protect it from the invasion of *C. albicans*. 
2.2 Results and Discussion

2.2.1 Synthesis of CD, CD-Gu+ and CD-Gu+-AmB.

Scheme 2-2. The scheme of synthesizing CD, CD-Gu+ and CD-Gu+-AmB.

The AmB-conjugated CD (CD-Gu+-AmB) was synthesized as shown in Scheme 2-2. First, the red-emitting CD was initiated by citric acid, ethylenediamine and formamide via hydrothermal method according to previous reports. The aforementioned chemicals could enrich the functional groups on the surface and graphitic nitrogen embedded in the skeleton of CD, granting the particles with blue and red fluorescence, respectively. The amine groups on the CD’s surface were then guanylated (CD-Gu+) to specify the conjugation between the carboxyls from CD-Gu+ and amine groups from AmB in the subsequent coupling using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride/N-hydroxysuccinimide (EDC/NHS). The guanylation may amplify the electrostatic interactions of the AmB-conjugated CD with the negatively charged polysaccharides of C. albicans cells/biofilms. After the guanylation, the carboxyl groups on CD-Gu+ were
activated by EDC coupling and stabilized by NHS. Before the actual AmB conjugation, the CD-Gu⁺-NHS-esters intermediate was first isolated and conjugated with an amine-modified bodipy (NH₂-bodipy) with green fluorescence ($\lambda_{ex} = 500$ nm and $\lambda_{em} = 525$ nm) to preliminarily assess the feasibility of the methodology. As presented in Scheme 2-3, an extra emission peak at 525 nm of CD-Gu⁺-bodipy was observed on the fluorescence spectra, implying the successful conjugation of NH₂-bodipy and the intermediate. Based on these pilot testing and verification, the amine groups from AmB were successfully conjugated with CD-Gu⁺-NHS-esters intermediate to obtain CD-Gu⁺-AmB for subsequent experiments.

**Scheme 2-3.** The verification of the linking of NH₂-bodipy with the isolated NHS-esters intermediate. Emission spectra of CD-Gu⁺-bodipy having an extra emission peak at 525 nm.
2.2.2 Morphology of CDs

The transmission electron microscopy (TEM) images of the as-prepared CD, CD-Gu⁺ and CD-Gu⁺-AmB were shown in Figures 2-1 A, B and C, respectively. The synthesized CD have a measured size distribution of 4.72 ± 1.25 nm (Figure 2-2 A) with lattice spacing of 0.25 nm (Figure 2-1 A inset), corresponding to the typical (100) in-plane lattice;⁵⁴ and a broad peak of X-ray diffraction (XRD) pattern with wide angle at approximately 25° (Figure 2-2 B), corresponding to the (002) plane of graphene, both simultaneously confirmed the partial graphitic structure of CD.

![Figure 2-1](image)

**Figure 2-1.** TEM images of (A) CD with (inset) lattice spacing of 0.25 nm, (B) CD-Gu⁺ and (C) CD-Gu⁺-AmB.
Figure 2-2. (A) Estimated particle size of the CD (n = 108). (B) The XRD patterns with wide angle at approximately 25°.

Figure 2-3. The zeta-potentials of CD, CD-Gu⁺ and CD-Gu⁺-AmB had a net electrical charge of −12.67, −8.68 and −19.16 mV, respectively.

The zeta-potentials (Figure 2-3) indicated that the well-dispersed CD, CD-Gu⁺ and CD-Gu⁺-AmB had a net electrical charge of −12.67, −8.68 and −19.16 mV, respectively. The negatively charged CD could be due to the carboxyl groups outnumbering the amine groups on the particle surfaces. After the guanylation, CD-Gu⁺ became less negative, owing to the conversion of amine groups to more positively charged guanidiniums. CD-Gu⁺-AmB were the most negatively charged ones among these CDs, due to the hydroxyl groups from conjugated AmB.
2.2.3 Optical Properties of CDs

The optical properties of different CDs were evaluated by fluorescence (FL) and ultraviolet-visible (UV–vis) spectroscopy. All types of CDs typically exhibited dual excitation-independent peaks at 480 (first) and 630 nm (second) (Figure 2-4 A, B and C). The fluorescence of the first and the significantly dominated second peaks could be attributed by the functional groups on the surface and embedded elements in the graphitic skeleton of CDs. Figure 2-4 D summarized the shift of the normalized emission bands of CDs, namely the first emission band with a 20 nm red-shift after each subsequent modification and the second one with the same wavelength. The phenomenon could be due to the conversion of amines to positively charged guanidiniums (Gu\(^+\)) and the introduction of additional \(sp^2\) carbon to the system, leading to surface defects and the increase in oxidation state of the CDs.\(^{54,55,66,92}\)

![Figure 2-4. FL spectra of (A) CD, (B) CD-Gu\(^+\), (C) CD-Gu\(^+\)-AmB and (D) summarized CDs.](image-url)
Meanwhile, the UV–vis spectra (Figure 2-5) displayed two major absorption bands that were commonly observed in red-emitting CD: a typical sharp $\pi-\pi^*$ transition band of the aromatic C=C bonds from 200 to 250 nm and a broad $n-\pi^*$ transition one of the C=N/C=O bonding at approximately 560 nm. With reference to CD, the confinement of CD-Gu$^+$’s C=N/C=O band was attributed to the increasing $n-\pi^*$ transitions. In addition, CD-Gu$^+$-AmB demonstrated three distinctive peaks from AmB molecule at approximately 372, 392 and 417 nm as shown in Figure 2-6, and the conjugation efficiency of 2.5% by mass was found.

**Figure 2-5.** UV–vis spectra of the CDs.

**Figure 2-6.** UV–vis of 10, 5, 4, 3, 2 and 1 µg/mL AmB.
2.2.4 Quantification of AmB on CD-Gu⁺-AmB

To estimate the conjugated AmB quantity, the Beer-Lambert Law equations of CD-Gu⁺ and free AmB were first obtained and validated by the calibration curves at 372, 392, 409 and 535 nm from concentration of 2 to 50 µg/mL:

\[ A = \varepsilon cl \]

Where \( A \) is the absorbance,
\( \varepsilon \) is molar absorption coefficient,
\( c \) is the concentration,
\( l \) is the optical path length.

![Figure 2-7](image)

**Figure 2-7.** The calibration curves of CD-Gu⁺ at (A) 534 and (B) 417 nm, and free AmB at (C) 417 nm. It was assumed that the AmB was fully conjugated on the CD-Gu⁺-AmB, and the AmB was not released into the testing solution during the measurement.

After obtaining the equations (Figure 2-7), the absorbance of CD-Gu⁺-AmB was matched with the absorbance of CD-Gu⁺ at 535 nm to determine the concentration of CD-Gu⁺ in CD-Gu⁺-AmB, which was used to calculate the baseline of each distinctive peak of AmB afterwards. Then, the calculated AmB baseline was subtracted from the measured AmB peak of CD-Gu⁺-AmB, and the concentration of conjugated AmB was computed by plugging the AmB absorbance into the free AmB equation. For instance, the as-obtained equation for CD-Gu⁺ at
417 nm is equal to \( y = 0.01352x + 0.001475 \), and the absorbance value for CD-Gu\(^+\) in CD-Gu\(^+\)-AmB at 534 nm was found to be 1.322. The value was then calculated the concentration CD-Gu\(^+\) in CD-Gu\(^+\)-AmB, which is approximately 26.91 µg/mL. After finding the concentration, the as-calculated concentration can be used to determine the absorbance of CD-Gu\(^+\) baseline in CD-Gu\(^+\)-AmB at 417 nm. The known value of CD-Gu\(^+\)-AmB at 417 nm was then subtracted by the baseline number to find the absorbance of AmB from equation \( y = 0.1185x - 0.02973 \). Finally, concentration of AmB was divided by the concentration of CD-Gu\(^+\)-AmB to determine the wt%.

2.2.5 Functionalities of CDs

The Fourier-transform infrared spectroscopy (FT–IR) and X-ray photoelectron spectroscopy (XPS) were conducted to investigate the functional groups of different CDs. The \( \nu N–H \) band of CD-Gu\(^+\) had slightly intensified in comparison to CD due to the covalent bonding of guanidinium resonance structure (Figure 2-8). Likewise, the slightly intensified \( \nu O–H \) band and reduced \( \nu C=O \) band of CD-Gu\(^+\)-AmB indicated the successful conjugation of AmB to CD through EDC/NHS coupling.

![Figure 2-8. FT–IR of CD, CD-Gu\(^+\) and CD-Gu\(^+\)-AmB.](image)
Figure 2-9. XPS analysis of CDs.
XPS analysis was performed to obtain more detailed profiles of the elemental composition. **Figure 2-9** further reveals that the CDs were composed of three fundamental elements: C, N and O. In CD, the C1s were differentiated into four bands, including C=C/C–C (284.7 eV), C–N (285.5 eV), C–O (287.0 eV) and COOH (288.5 eV). The N1s was divided into two bands, representing the pyridinic-N and pyrrolic-N bands located at 399.0 and 400.5 eV, respectively. Meanwhile, the O1s peak presented with two bands: C=O (530.6 eV) and C–O (532.3 eV). The elemental composition of CD-Gu+ was identical to CD, except for the C1s. The C–N band had merged and intensified with the C–O band, accounting for the higher electron density from the guanidinium groups. For the CD-Gu+-AmB, the vanishing COOH band from C1s was due to the successful AmB conjugation of the EDC/NHS coupling, whereas the appearing –OH band from O1s was from the OH groups on sidechain of the AmB molecules.

**2.2.6 Interactions of CDs with *C. albicans* and the antifungal effect of CD-Gu+-AmB.**

The diffusion patterns of different CDs in the biofilms were assessed by two-day-old ATCC 90028 (standard-type) and SC5314 (wild-type) *Candida* biofilms. Typically, the *Candida* cells were stained by SYTO9 (λex = 488 nm) with green fluorescence, while the interacted CDs with red fluorescence were excited by 543 nm HeNe laser. Interestingly, different interactive profiles of the CDs with *C. albicans* were observed. As delineated by circled bubbles in **Figure 2-10 & 2-11**, majority of the red-emitting CD and CD-Gu+ was adhered to the cell walls of *C. albicans*, and sparsely accumulated within the *Candida* cells as vivid pixels. The adherence to the cell walls may be caused by the integrity of the cell membrane,
thereby only allowing a few small amphiphilic CD and CD-Gu⁺ to penetrate into the cells. After the conjugation of AmB to CD-Gu⁺, CD-Gu⁺-AmB were mainly found accumulating within the Candida cells with a drastic decrease in cell numbers after 24 h treatment. It was speculated that the destruction of the cell membranes could be induced by the AmB conjugated on CD-Gu⁺. It has been documented that AmB, as a polyene antifungal drug, can bind to the ergosterol within the fungal membrane to form ion channels and change the membrane permeability. This may lead to a leakage of the cytoplasm of Candida cells and eventually result in cell death. Moreover, it has been reported that the pore size of the ion channels varies from 1.6 to 16 nm depending on the concentration of AmB. These findings suggested that the AmB-formed pores were in nano-scale, allowing the penetration and accumulation of CD-Gu⁺-AmB in the Candida cells.
Figure 2-10. Confocal images showing diffusion patterns of CD, CD-Gu⁺ and CD-Gu⁺-AmB (100 µg/mL) treated two-day-old *C. albicans* (SC5314) biofilms.
**Figure 2-11.** Confocal images showing diffusion patterns of CD, CD-Gu⁺ and CD-Gu⁺-AmB (100 µg/mL) treated two-day-old *C. albicans* (ATCC 90028) biofilms.
The antifungal properties of different CDs were assessed in both planktonic mode and two-day-old biofilms of SC5314 and ATCC 90028 Candida cells. As shown in Table 2-1 & 2-2, both minimum inhibitory concentrations (MIC) of CD and CD-Gu+ did not show discernable inhibitory effects on the planktonic cells of SC5314 at the concentration of 1000 µg/mL. Meanwhile, the conjugation of AmB on CD-Gu+ could maintain its antifungal effect at the concentration of 15.63 and 31.25 µg/mL (equivalent to 0.39 and 0.78 µg/mL of AmB concentration, respectively) in accordance with after 24 and 48 h treatments. Likewise, the MIC of ATCC 90028 of CD and CD-Gu+ also shows similar inhibitory effect, whereas that of CD-Gu+-AmB were 7.81 and 31.25 µg/mL (equivalent to 0.20 and 0.78 µg/mL of AmB concentration, respectively). The numbers indicate that there was a 3-fold enhancement of the AmB efficiency.

Table 2-1. The MIC of CD, CD-Gu+, CD-Gu+-AmB and AmB against the planktonic mode of C. albicans (SC5314) at 24 and 48 h.

<table>
<thead>
<tr>
<th>Antifungal agents</th>
<th>Minimum inhibitory concentration (µg/mL)</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>&gt;1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD-Gu+</td>
<td>&gt;1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD-Gu+-AmB</td>
<td>15.63</td>
<td></td>
<td>31.25</td>
</tr>
<tr>
<td>AmB</td>
<td>1.25</td>
<td></td>
<td>2.50</td>
</tr>
</tbody>
</table>

Table 2-2. The MIC of CD, CD-Gu+, CD-Gu+-AmB and AmB against the planktonic mode of C. albicans (ATCC 90028) at 24 and 48 h.

<table>
<thead>
<tr>
<th>Antifungal agents</th>
<th>Minimum inhibitory concentration (µg/mL)</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>&gt;1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD-Gu+</td>
<td>&gt;1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD-Gu+-AmB</td>
<td>7.81</td>
<td></td>
<td>31.25</td>
</tr>
<tr>
<td>AmB</td>
<td>0.625</td>
<td></td>
<td>2.50</td>
</tr>
</tbody>
</table>
The \( \text{Log}_{10} \) of the colony-forming units (CFU) were presented to determine the concentrations of the survived \( \text{C. albicans} \) in the two-day-old biofilms after 24 h treatment of CD, CD-Gu\(^+\), CD-Gu\(^+\)-AmB with a series of concentrations (250, 100, 50 and 10 \( \mu \)g/mL) and AmB (6.25, 2.50, 1.25 and 0.25 \( \mu \)g/mL, equivalent to conjugated AmB of tested CD-Gu\(^+\)-AmB concentration). After 24 h treatment, CD-Gu\(^+\)-AmB displayed a comparable anti-biofilm effect with free AmB at the testing concentrations, demonstrating a significant decrease of the \( \text{Log}_{10} \) (CFU) even at 10 \( \mu \)g/mL as compared with the control group (Figure 2-12).

![Graph 1](image1)

![Graph 2](image2)

**Figure 2-12.** The concentrations of live \( \text{C. albicans} \) (SC5314 & ATCC 90028) in the two-day-old biofilms. The assay was performed in duplicate on three different occasions, and the data are presented as mean ± SD with the asterisk indicates the significant differences between the treatment and control groups \((p < 0.05)\).
In addition, FEG–SEM images show that the CD-Gu⁺-AmB- and AmB-treated *Candida* cells displayed a deflated appearance with reference to the ones with smooth surfaces from control, CD- and CD-Gu⁺-treated groups for both *C. albicans* SC5314 and ATCC 90028 (Figure 2-13 & 2-14). Similar observation and results were obtained, indicating the promising adhesion capacity of CDs on *C. albicans* and their antifungal/anti-biofilm effects.
Figure 2-13. The FEG–SEM images showing the morphology of SC5314 *C. albicans* treated with CD, CD-Gu⁺, CD-Gu⁺-AmB (100 µg/mL) and AmB (2.50 µg/mL).
Figure 2-14. The FEG–SEM images showing the morphology of ATCC 90028 C. albicans treated with CD, CD-Gu⁺, CD-Gu⁺-AmB (100 µg/mL) and AmB (2.50 µg/mL).
2.2.7 Internalization and cytotoxicity of CDs in primary human oral keratinocytes (HOKs).

To assay the cellular internalization and potential cytotoxicity after 24 h treatment, the interactions of different CDs with primary human oral keratinocytes (HOKs) were analyzed by staining the cell lysosomes and nucleus with Lysotracker DND-26 (green) and Hoechst 33342 (blue), respectively, and the red channel represents the treatment of different CDs. As shown in Figure 2-15, CDs can be internalized in the human oral cells within 2 h, and the red fluorescence can be identified in the cells after 24 h treatment with some of the CDs can be found accumulating in the nucleoli. Surprisingly, the cells with red signals in the nuclei did not show obvious staining of Lysotracker DND-26. Since the staining of Lysotracker DND-26 is based on the acidity of the environment in the cellular compartments, it may suggest that the CDs could affect the acidic condition of the lysosomes, which consequently disturb their staining. However, further studies are required to find out whether and to what extent CDs could influence intracellular microenvironmental conditions.

In addition, the viability of HOKs was assessed by using CCK-8 assay. Although CDs could be found in the nuclei, the different concentrations of CDs tested did not show any significant cytotoxicity to the HOKs after 24 h treatment (Figure 2-16).
Figure 2-15. Fluorescence images of CD, CD-Gu⁺ and CD-Gu⁺-AmB (100 µg/mL)-treated HOKs for 2 and 24 h. The white arrows indicate the accumulation of CD-Gu⁺-AmB in the cell nuclei. (Scale bar: 20 µm)
Figure 2-16. After 24 h treatment of CD, CD-Gu⁺, CD-Gu⁺-AmB (250, 100, 50 and 10 µg/mL) and AmB (6.25, 2.50, 1.25 and 0.25 µg/mL).

As promising fluorescent agents, CD can offer several advantages, such as low cytotoxicity, excellent biocompatibility and photostability, making them the ideal nanomaterials for in vitro and in vivo bioimaging.\(^{101}\) It has been documented that CD can be internalized in C6 glioma cells and confined at endosomes and mitochondria with some evenly distributed in endoplasmic reticulum and Golgi complexes, and the fluorescence intensity at such locations diminished after extending the incubation time.\(^{102,103}\) In this study, since the as-synthesized CD were prepared from citric acid and ethylenediamine, their surface was enriched with amine groups. Even after the guanylation and AmB conjugation, CD-Gu⁺ and CD-Gu⁺-AmB still uphold a positively charged guanidine groups. As such, it is believed that the amine-rich and positively charged guanidine groups granted their featured affinity to nucleoli. As the ribosome factory, the negatively charged ribosomal RNAs are synthesized, processed and assembled with ribosomal proteins in nucleoli.\(^ {104}\) Thus, CDs with amine-rich or positively charged groups presented the featured accumulation in nucleoli.
2.2.8 Penetration and exfoliation profiles of different CDs in oral epithelium tissues. The penetration and exfoliation profiles of CDs were evaluated in the reconstituted human oral epithelia (RHOE), followed by assessment of their anti-invasion properties. The immunofluorescence staining of E-cadherin (green) was performed on the slices, and the cell nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI, blue fluorescent DNA stain). After treating the RHOE with different CDs for 2, 6 and 12 h, the red-emitting CDs were subsequently detected in tissue slices (Figures 2-17 A, 2-18 A & 2-19 A). It is noteworthy that the intensity of red fluorescence increased along with the treatment duration, suggesting the in situ accumulation of CDs in the tissue. Particularly, the red signal can be readily observed at 2 h in the entire tissue layers, indicating rapid, constant and effective penetration of CDs in RHOE.

Apart from penetration and accumulation, CDs could also exfoliate from the tissue. After treatment with different CDs for 6 h, the RHOE were cultured in SkinEthic growth medium (SGM) up to 8 d. The tissues were collected at 6 h (immediately after CD-Gu⁴⁺-AmB treatment), 2, 4 and 8 d for the cryosection, and stained with E-cadherin for fluorescent microscopy. The images show a slightly positional shifting of CD-Gu⁴⁺-AmB (red) in RHOE in the experimental period. Along with a prolonged culture period in the SGM, the intensity of red fluorescence gradually decreased, together with the shortened red band (86.8 to 51.3 µm) in the tissue slides. Additionally, the accumulation of CD-Gu⁺-AmB in the cell nuclei was also identified. Of note, the red signal greatly declined in the RHOE at 8 d. Notably, no red fluorescence was found in the newly grown layers at the bottom of RHOE (Figures 2-17 B, 2-18 B & 2-19 B).
Figure 2-17. (A) Penetration profile of CD-Gu⁺-AmB. (B) Exfoliation profile of CD-Gu⁺-AmB. The white arrows indicate the accumulation of CD-Gu⁺-AmB in the cell nuclei. (Scale bar: 50 µm)
Figure 2-18. (A) Penetration profile of CD. (B) Exfoliation profile of CD. (Scale bar: 50 µm)
Figure 2-19. (A) Penetration profile of CD-Gu⁺. (B) Exfoliation profile of CD-Gu⁺.
(Scale bar: 50 µm)
The RHOE used in this study represent the non-keratinized stratified squamous epithelia in oral cavity. The permeability of these tissues is greater than the keratinized ones with stratum corneum.\textsuperscript{105,106} We also examined the penetration profiles of different CDs in reconstituted human gingival epithelia (RHGE) with keratinized stratum corneum. Interestingly, different penetration patterns were observed: all CDs were accumulated in the stratum corneum without further penetration in the underlying cellular layers during the 12 h experimental period (Figure 2-20). This may be due to the absence of stratum corneum that acts as physical and biological barrier, which all forms of CDs were able to fully penetrate the RHOE at 2 h and appeared in both inter- and intra-cellularly. Moreover, the natural mucosa can be self-renewable with the turnover time ranging from 14 to 20 d due to the cell proliferation.\textsuperscript{106} RHOE used in this study were maintained for proliferation in SkinEthic growth media up to 8 d. The red fluorescence reflecting the penetrated CDs in the upper layer decreased along with the culture time period, indicating the gradual exfoliation of the CDs, while the lower layer without red fluorescence represented the newly grown tissue. These exciting findings reveal that CDs can effectively penetrate the oral epithelial tissue via topical administration and yet exfoliate from the tissue through cell proliferation and/or potential detachment from the tissue surface.
Figure 2-20. The fluorescent images of cryosections from RHGE treated with CD, CD-Gu⁺ and CD-Gu⁺-AmB (200 µg/mL) for 0, 2, 6 and 12 h. (Scale bar: 50 µm)
2.2.9 CD-Gu⁺-AmB-embedded RHOE against the invasion by C. albicans.

C. albicans is the most common commensal fungus, and it may become a pathogen under various predisposing local and systemic factors. Oral candidiasis as a superficial fungal invasion is often initiated by the colonization of yeast cells on oral epithelial layer, and it subsequently progresses through the induced endocytosis and active penetration of the yeast-generated hyphae and pseudohyphae. The fluorescence results in Figure 2-17 A revel that CDs per se after 6 h-treatment enabled penetration and accumulation within all layers of RHOE. To study the anti-invasion activity of CD-Gu⁺-AmB, the RHOE were pretreated with PBS, different CDs or AmB for 6 h, then challenged by C. albicans SC5314 (4 × 10⁴ CFU/mL) for 24 and 48 h. Both RHOE and their culture media were collected for staining and biological analysis (Figure 2-21).

Figure 2-21. Schematic diagram of the experimental design for 24 and 48 h. At each time point, the tissue and culture media were harvested for the following biological analysis.

After the periodic acid-Schiff staining (PAS), the polysaccharides-rich Candida yeasts, hyphae and pseudohyphae were identified with bright red signals in the tissue slices. Both CD and CD-Gu⁺ were unable to prevent Candida invasion. It is noted that C. albicans SC5314 as a highly invasive pathogenic strain causes significant pro-inflammatory responses. In the C. albicans (CA) control, CD and CD-Gu⁺ groups, the hyphae and pseudohyphae aggressively grew on the tissue...
surface and further extended into underlying cell layers. This induces notable histopathologic alternations in RHOE (e.g. vacuolization, spongiosis, and cell layer detachment) and eventually lead to tissue destruction at 24 and 48 h (Figure 2-22 A). Whereas, the pretreatment of AmB to some extent exhibited anti-invasive effect on *C. albicans*. Some yeast cells could be observed on the tissue surfaces at 24 h, and the yeast-transformed hyphae and pseudohyphae appeared and yet invaded into the tissue at 48 h, suggesting the insufficient protection of AmB against *Candida*. Notably, no yeasts, hyphae and pseudohyphae were detected in the CD-Gu⁺-AmB-treated tissues that presented with the structure similar to the ones from the control group.

Moreover, the immunofluorescent staining of E-cadherin highlighted that the pretreatment of CD and CD-Gu⁺ could not prevent the *C. albicans*-induced tissue destruction. As shown in Figures 2-22 B and 2-24, the green fluorescence of E-cadherin in the tissue slices from the CA control, CD and CD-Gu⁺ groups dramatically decreased at 24 and 48 h with reference to the control group, implying the degradation of E-cadherin by *C. albicans* and self-released enzymes. Remarkably, the CD-Gu⁺-AmB group presented clearly with green signals at 24 and 48 h, demonstrating the overall integrity of E-cadherin. AmB group showed a similar profile with slight invasion by *Candida*. E-cadherin is a calcium-dependent cell-cell adhesion molecule, and it plays an important role in mediating epithelial behavior and maintaining tissue integrity. One of the invasive approaches of *C. albicans* is to degrade E-cadherin by secreting lytic enzymes and/or manipulating the activity of epithelial cell calpain.
**Figure 2-22.** The cryosection of the collected tissue samples was performed with (A) periodic acid-Schiff (PAS) and (B) immunofluorescent (IF) staining (green representing E-cadherin), respectively (Scale bar: 50 µm). The red arrows indicate the yeast, hyphae or pseudohyphae located on/in RHOE.
Figure 2-23. The amount of released LDH in the culture media at 24 and 48 h was assessed by measuring the absorbance of reduced tetrazolium salt (INT) via a coupled enzymatic reaction at 490 and 680 nm (A). The concentration of IL-8 (B) and IL-1β (C) in the media was measured using ELISA. The asterisk indicates the significant differences between the other groups and CA control groups ($p < 0.05$).

Furthermore, lactate dehydrogenase (LDH) is one of the intracellular enzymes, and it can be released to the culture media when the cell membranes are damaged. Thus, the increased concentration or activity of LDH in the tissue culture media could estimate C. albicans-initiated destruction.$^{114,115}$ The relative concentration of LDH from different groups at 24 and 48 h was assessed using a colorimetric assay (Figure 2-23 A). The results showed that both CD-Gu⁺-AmB and AmB groups exhibited relatively lower levels of LDH than the CA control, in consistence with the immunofluorescent findings. Additionally, the invasion of C. albicans stimulates innate host response with production of pro-inflammatory cytokines such as IL-1β and IL-8 for tackling the infection.$^{116}$ As the pretreatments of CD and CD-Gu⁺ failed to stop the invasion of C. albicans, both IL-1β and IL-8 levels markedly increased at 48 h. In contrast, the expression of these cytokines in
both CD-Gu⁺-AmB and AmB groups remained at far lower levels at 48 h than the CA control group ($p < 0.05$). No significant difference was found between the CD-Gu⁺-AmB and AmB groups (Figure 2-23 B & C). Taken together, CD-Gu⁺-AmB embedding may effectively prevent the adherence and invasion of *C. albicans* in the RHoe.

AmB with a relatively high molecular weight (924 Da) displays poor solubility in water and most polar organic solvents (*e.g.* ethanol and acetone), and its tissue absorption is mainly depended on the passive diffusion via the intestinal epithelia, thereby these intrinsic properties greatly limit its bioavailability and utilization for oral healthcare. In recent years, novel approaches have been increasingly developed to address this critical issue. Indeed, nanotechnology-based drug delivery system has been proposed for effective oral healthcare. Regarding the lipid-associated formulations of AmB, the size (> 100 nm) and the surface charge of the lipid complex are the major drawbacks for limiting their penetration in epithelia via topical administration. It is apparent that carbon dots with low costs, flexible functional surfaces, nano-scaled size and traceable fluorescence show great advantages in refining the drug delivery system for treatments of various oral diseases. The present study demonstrates that the favorable properties of CD-Gu⁺-AmB including instant epithelial penetration, extra- and intra-cellular embedding as well as gradual exfoliation are highly promising for generating shielding layers as an effective approach against noxious microbial infections like *Candida*-induced oral candidiasis and related systemic complications.
Figure 2-24. Different CDs-embedded RHOE against the invasion by *C. albicans* SC5314 (4 × 10⁴ CFU/mL) for 24 and 48 h. The tissues at each time point were collected and cryosectioned for the fluorescent microscopy. (Scale bar: 50 µm)
2.3 Experimental Procedures

2.3.1 Chemicals

Citric acid, ethylenediamine, 1-ethyl-3-(3-dimethylaminopropyl) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and amphotericin B (AmB) were purchased from Sigma-Aldrich (St. Louis, USA). Formamide (FA) and 1H-pyrazole-1-carboxamidine hydrochloride (PCH) were ordered from Meryer (Shanghai, China). Ethanol (EtOH) was acquired from VWR International (Fontenay-sous-Bois, France). Acetone (Me₂CO), while dichloromethane (DCM), dimethyl sulfoxide (DMSO), ethyl acetate (EtOAc), and methanol (MeOH) were acquired from RCI Labscan Ltd. (Bangkok, Thailand). Deionized (DI) water (18.2 MΩ.cm) was obtained from Milli-Q ICW3000 water system.

2.3.2 Preparation of nitrogen-doped CD

The CD were prepared via hydrothermal method as reported in the literature with minor modifications.⁶⁶,⁹² In a typical procedure, 6.0 g of citric acid and 2.1 mL of ethylenediamine were dissolved in 80 mL of formamide under vigorous stirring at room temperature to obtain a transparent solution. The solution was transferred into a tightly sealed 100 mL Teflon-lined autoclave afterward and placed in a preheated oven at 180°C for 4 h. After cooling to ambient temperature, the crimson mixture was precipitated in 10 mL of Me₂CO and then separated at 8000 rpm for 5 min using centrifuge (Centrifuge 5804, Eppendorf, Hamburg, Germany). The precipitates were washed with Me₂CO/EtOH for five times to remove the unwanted precursors, and the remaining residue was dispersed in 10 mL of DI water and dialyzed against 1000 mL of distilled water for 3 d (500 Da, Bomei, Viskase,
Illinois, United States). Finally, the nitrogen-doped CD were collected through freeze-drying and stored at 4°C.

2.3.3 Synthesis of bodipy

**Compound 1.** 4-formylbenzoic acid (1.0 g, 6.6 mmol), EDC-HCl (2.0 g, 10.4 mmol) and NHS (2.2 g, 19.1 mmol) were dissolved in DCM (25 mL). The mixture was stirred for 24 h at room temperature. The solvent was removed by rotary evaporator and the residue was re-dissolved in EtOAc (50 mL). The organic layer was washed with water (3 × 25 mL) and then brine (25 mL). The organic layer was dried with anhydrous MgSO₄. The product was then purified by flash silica gel column chromatography using EtOAc/n-hexane (1:1 = v/v) as eluent to afford compound 1 as white solid. Yield: 1.02 g, 62%. \(^1\)H NMR (400 MHz, CDCl₃, 298 K) δ 10.14 (s, 1H), 8.31 (d, \(J = 8.5\) Hz, 2H), 8.03 (d, \(J = 8.6\) Hz, 2H), 2.94 (s, 4H) (Appendix Figure S2). \(^1\)C NMR (101 MHz, CDCl₃, 298 K) δ 191.30, 169.06, 161.19, 140.47, 131.34, 130.13, 129.87, 25.83 (Appendix Figure S3).

**Compound 2.** Compound 1 (0.71 g, 2.87 mmol) and 2,4-dimethylpyrrole (0.56 g, 5.88 mmol) were dissolved in degassed DCM (450 mL). Ten drops of trifluoroacetic acid were added slowly, and the mixture was allowed to stir at room temperature for 24 h. DDQ (0.75 g, 3.3 mmol) was added to the mixture and allowed to react for 4 h at room temperature. Then triethylamine (NEt₃) (4.8 mL) was added followed by BF₃·OEt₂ (4.8 mL). The mixture was further stirred for 24 h at room temperature. The organic layer was washed with water (3 × 300 mL). The organic layer was concentrated, and the product was purified by silica gel column chromatography using EtOAc/n-hexane (1:1 = v/v) as eluent to afford Compound 2 as orange solid. Yield: 0.26 g, 19.5%. \(^1\)H NMR (400 MHz, CDCl₃, 298 K) δ 8.27
(d, J = 8.3 Hz, 2H), 7.50 (d, J = 8.3 Hz, 2H), 6.00 (s, 2H), 2.93 (s, 4H), 2.56 (s, 6H), 1.38 (s, 6H) (Appendix Figure S4). \(^1^3\)C NMR (101 MHz, CDCl\(_3\), 298 K) \(\delta\) 169.35, 161.44, 156.50, 142.99, 142.23, 139.39, 131.45, 130.86, 129.21, 125.93, 121.83, 25.86, 14.96, 14.79 (Appendix Figure S5). HRMS calculated for C\(_{24}\)H\(_{22}\)N\(_3\)O\(_4\)BF\(_2\) [M]\(^+\): 465.1670, found: 465.1658 (Appendix Figure S6).

**Compound 3.** Compound 2 (48.3 mg, 0.10 mmol) in DCM (10 mL) was added dropwise to a DCM solution (14 mL) containing 4 mL of ethylenediamine. The mixture was stirred for 12 h. The mixture was washed with water (30 mL) and then brine (30 mL). The organic layer was collected and dried with anhydrous Na\(_2\)SO\(_4\). The product was then purified by silica gel chromatography using DCM/MeOH (10:1 = v/v) as eluent to afford compound 3 as orange solid. Yield: 42 mg, 98.6%.

\(^1^H\) NMR (400 MHz, CDCl\(_3\), 298 K) \(\delta\) 7.96 (d, J = 8.3 Hz, 2H), 7.38 (d, J = 8.3 Hz, 2H), 5.98 (s, 2H), 3.55 (q, J = 5.7 Hz, 2H), 3.00 (t, J = 5.7 Hz, 2H), 2.55 (s, 6H), 1.35 (s, 6H) (Appendix Figure S7). \(^1^3\)C NMR (101 MHz, CDCl\(_3\), 298 K) \(\delta\) 166.83, 156.04, 143.07, 140.46, 138.44, 135.17, 131.18, 128.58, 127.98, 121.58, 42.43, 41.28, 14.78, 14.75 (Appendix Figure S8). HRMS calculated for C\(_{22}\)H\(_{25}\)BF\(_2\)N\(_4\)O [M+H]\(^+\): 410.2088, found 410.2085 (Appendix Figure S9).

**2.3.4 Guanylation and conjugation of amphotericin B on CD**

For electropositivity enhancement, the primary and secondary amines on the as-prepared CD were converted to guanidiniums. In short, 0.2 g of the nitrogen-doped CD in 10 mL of MeOH was sonicated for 10 min and stirred for half an hour at room temperature. Then, 0.2 g of PCH was added to the well-dispersed CD solution with continuous stirring for an additional 4 h. Next, the solution was centrifuged at
10000 rpm for 10 min and washed with MeOH for five times. The guanylated CD (CD-Gu⁺) were vacuum dried and kept at 4°C.

For the upcoming conjugation, the carboxyl groups on CD-Gu⁺ were first activated by EDC coupling and stabilized by NHS. It was achieved by stirring 0.1 g of CD-Gu⁺, EDC and NHS in a DCM/DMSO (1:4, v/v) co-solvent system for 2 h at room temperature. The CD-Gu⁺-NHS-esters intermediate were harvested via centrifugation at 10000 rpm for 10 min and then washed with DCM for three times followed by vacuum drying. The successful activation of the carboxyl groups on CD-Gu⁺ was first validated in a preliminary study by adding an amine modified bodipy (NH₂-bodipy, λ_ex = 500 nm and λ_em = 525 nm) to the as-obtained CD-Gu⁺-NHS-esters intermediate. In brief, 4.0 mg of NH₂-bodipy were reacted with 20.0 mg of CD-Gu⁺-NHS-esters intermediate in MeOH overnight. After centrifugation and rinsing out with MeOH for five times, the particles (CD-Gu⁺-bodipy) were vacuum dried and re-dispersed in an ethanol/water mixture (1:1, v/v). Their optical properties were measured using LS 50B fluorescence spectrometer (PerkinElmer Inc., Waltham, United States).

Referring to AmB conjugation, 4.0 mg of AmB were first dissolved in 10 mL of MeOH to obtain a clear solution. Then, 20.0 mg of CD-Gu⁺-NHS-esters intermediate were dispersed in the solution for overnight stirring. The AmB-conjugated CD (CD-Gu⁺-AmB) were gathered through centrifugation and washed with 10 mL of MeOH for three times. Finally, the final products were collected via vacuum drying and stored in the freezer for additional characterizations.
2.3.5 Characterization of CD, CD-Gu⁺ and CD-Gu⁺-AmB.

The morphology of CD, CD-Gu⁺ and CD-Gu⁺-AmB was assessed using transmission electron microscopy (TEM, Tecnai G2 20 S-TWIN, Thermo Fisher Scientific, Massachusetts, United States), and the average size of CD was determined by using ImageJ (Version 1.51s, National Institutes of Health, USA) from more than 100 particles randomly selected from multiple TEM images. The X-ray diffraction (XRD) patterns of the prepared samples were recorded with Bruker D8 Advance X-ray Diffractometer, and its full text was obtained from X Pert-Philips X-ray diffractometer with monochromatized CuKα radiation of wavelength 1.5406 Å at 55 kV and 40 mA. The zeta potentials of different CDs were measured by DelsaMax Pro (Beckman Coulter, Indianapolis, US), and each sample was measured for six times to obtain the average value. The optical properties of the CDs were evaluated by fluorescence spectrophotometer (FL, Perkin Elmer LS 50B) and UV–visible spectrophotometer (UV–Vis, Cary 8454 UV–Vis, Agilent Technologies, California, United States). The functional groups of each CD type were determined by Nicolet Magna 550 Series II Fourier transform infrared spectroscopy (FT–IR, Nicolet Instrument, Madison, United States) and X-ray photoelectron spectroscopy (XPS) by SKL-12 spectrometer modified with VG CLAM 4 multi-channel hemispherical analyzer.

2.3.6 Microbes and culture conditions

The wild-type of Candida albicans, SC5314, obtained from the archival collection of Faculty of Dentistry, The University of Hong Kong, was used in this study. The fungi were cultured on Sabouraud dextrose agar (SDA) and incubated in the aerobic
chamber at 37°C. The strains were inoculated in yeast nitrogen base broth (YNB) with 50 mM of glucose (YNB50) overnight before using.

2.3.7 Antifungal properties in planktonic mode and mono-species biofilms

The antifungal properties of CD, CD-Gu+ and CD-Gu+-AmB were assessed and then compared with free AmB in both planktonic mode and two-day-old mono-species biofilms. The stock solution of AmB was prepare in DMSO and then diluted with yeast nitrogen base broth until further use.

In the planktonic mode, broth microdilution assay was performed to measure the MIC of different CDs and AmB. In brief, the Candida strain was first inoculated in YNB50 overnight, and then harvested by centrifugation at 1000 rpm for 5 min at 4°C using HITACHI CR22N high-speed refrigerated centrifuge (Hitachi Ltd., Tokyo, Japan). The cell pallets were washed with phosphate buffered saline (PBS, pH 7.2) twice and then re-suspended in PBS at the concentration of 10^7 cells/mL. Two-fold serial dilutions of CD, CD-Gu+, CD-Gu+-AmB and free AmB were prepared with YNB50 in 96-well plate. Then, the cell suspension was added into each well with final concentration of 10^6 cells/mL, and the growth of fungi in each well was observed for 48 h. The lowest concentration without visual fungal growth was recorded as the MIC.

The two-day-old biofilms of Candida were cultured as described by Chandra et al. with some modifications. The cell suspensions of C. albicans in PBS at the concentration of 10^7 cells/mL were first prepared respectively as described previously. Then, 200 µL of Candida suspension was added in each well of 96-well plate. Two plates were inoculated for the study. After the inoculation, the plates were placed in the aerobic chamber at 37°C for the two-hour adhesion of
the yeast cells. Afterwards, the cell suspensions were aspirated, and the plates were washed with PBS once to remove the non-adherent yeast cells. Fresh YNB with 100 mM of glucose (YNB100) was added in each well for the maturation of the *Candida* biofilms. The *Candida* biofilms were incubated at same condition for 2 d and then treated with CD (10, 50, 100, 250 µg/mL), CD-Gu⁺ (10, 50, 100, 250 µg/mL), CD-Gu⁺-AmB (10, 50, 100, 250 µg/mL) and free AmB (0.25, 1.25, 2.50, 6.25 µg/mL, equivalent drug amount compared with CD-Gu⁺-AmB at each concentration) for 24 h. Then, the yeast cells of the biofilms from the treated and control groups in the other plate were collected by pipette and diluted by PBS to the proper concentrations. 50 µL of each diluted yeast cell suspension was inoculated on SDA and incubated at 37°C. After 24 h, the colonies growing on the plates were counted and analyzed. All of the assays were performed on three different occasions in duplicate.

**2.3.8 Confocal scanning laser microscopy and field emission scanning microscopy.**

The diffusion and interaction of different CDs in the two-day-old biofilms were assessed using Olympus FLUOVIEW FV 1000 confocal scanning laser microscope with FV-10 ASW system equipped with 543 nm HeNe laser and 488 nm Argon laser (Olympus Corporation, Tokyo, Japan). The biofilms were cultured and matured for 2 d in µ-Slide 8 well chambers with polymer coverslip bottom (ibidi GmbH, Munich, Germany) as described previously. CD, CD-Gu⁺ and CD-Gu⁺-AmB at concentration of 100 µg/mL were applied to the biofilms for 24 h, respectively. Then, the treatments were removed, and the biofilms were stained with SYTO9 (Live/Dead BacLight™ viability kit; Thermo Fisher Scientific) for
30 min at room temperature. The fluorescence images were acquired and analyzed by FV10-ASW 4.0 Viewer (Olympus Corporation) and ImageJ (Version 1.51, National Institutes of Health, USA).

At the same time, the strain was also inoculated on Thermanox plastic coverslips (NUNC™, Rochester, USA) with diameter of 15 mm in 12-well plate to form biofilm as described. After 2 d, CD, CD-Gu⁺, CD-Gu⁺-AmB at concentration of 100 µg/mL and AmB (2.50 µg/mL) were applied on the biofilms for 24 h. Then, the treatments were removed, and the biofilms were gently washed with PBS twice and subsequently fixed with 4% paraformaldehyde (PFA) overnight. The fixed biofilms were dehydrated sequentially using 70%, 80%, 90% and absolute EtOH. The well-prepared biofilm samples were mounted on aluminum stands and coated with palladium using an ion sputter coater (JEOL JFC1 100; JEOL, Tokyo, Japan). The morphology of the Candida cells after different CDs and AmB treatment were assessed using Hitachi S4800 FEG–SEM (Tokyo, Japan) or Leo 1530 FEG–SEM (Carl Zeiss Meditec AG, Jena, Germany).

2.3.9 Cell culture, imaging and viability

Primary human oral keratinocytes (HOKs) were cultured accordingly and prepared for the following imaging assay. In brief, HOKs and their culture media (oral keratinocyte medium, OKM) were ordered from ScienCell (San Diego, USA). The cells at passaged 3 were seeded in poly-L-lysine pre-coated µ-Slide 8 well chambers with glass bottom (ibidi GmbH, Munich, Germany) at the density of $2 \times 10^4$ cells/well, and then cultured in OKM containing 100 units/mL of penicillin and 100 mg/mL of streptomycin. All the cells were incubated at 37°C with 5% CO₂ in a humidified incubator until reaching 80% confluence. Then, different CDs were
dispersed in the cell culture media (OKM) at concentration of 100 µg/mL. HOKs were treated with CDs media for 2 and 24 h, respectively. After the treatment, the cells were washed with blank media three times to remove the extra CDs. Afterwards, fresh media containing 75 nM of Lysotracker DND-26 and 1 µg/mL of Hoechst 33342 were added to the cells for staining lysosomes and nuclei. The cellular fluorescent images were recorded by Olympus 1 × 2-UCB fluorescent microscope (Tokyo, Japan). The cells without CDs treatment were considered as control group.

The cell viability of HOKs was assessed using Cell Counting Kit-8 (CCK-8, Sigma-Aldrich, St Louis, USA). In brief, HOKs at passage 3 were first seeded in poly-L-lysine pre-coated 96-well plate at density of 2 × 10^4 cells/well overnight for attachment, respectively. Then, different CDs (CD, CD-Gu⁺, CD-Gu⁺-AmB) with various concentrations (250, 100, 50 and 10 µg/mL) and AmB (6.25, 2.50, 1.25 and 0.25 µg/mL, equivalent to conjugated AmB of tested CD-Gu⁺-AmB concentration) were applied to the cells for 24 h. After the treatment, the culture media in each well containing CDs or AmB were discarded, and then replaced with fresh culture media (100 µL) and CCK-8 reagent (10 µL). The cells were cultured with the media containing CCK-8 reagent for 2 h. The absorbance of the supernatant was measured at 450 nm using SpectraMax M2 Microplate Reader (Molecular Devices, California, USA).

2.3.10 Penetration and exfoliation profiles of different CDs in engineered oral epithelium tissues

The engineered commercialized oral epithelium tissues, i.e. reconstituted human oral epithelia (RHOE), were selected for testing in this study. The tissues and their
culture media, including SkinEthic maintenance medium (SMM) and SkinEthic growth medium (SGM), were ordered from EPISKIN (Lyon, France). Upon arrival, the tissues were taken out immediately from the agarose gel and placed carefully onto 6-well plates containing 1 mL of SMM for incubation overnight. Then, CD, CD-Gu⁺ and CD-Gu⁺-AmB were dispersed in PBS at concentration of 200 µg/mL, respectively. 0.5 mL of different CD dispersions were applied topically to the tissues for 0, 2, 6, 12 h. After the treatment, the CD dispersions were removed, and the tissue surface was gently rinsed with PBS for three times to remove the loosely attached CDs. The tissues were subsequently fixed using 4% PFA at 4°C overnight, followed by the dehydration in a serial of sucrose solution (10%, 20% and 30%). Regarding the exfoliation, the tissues were first treated with different CDs dispersion for 6 h and then washed with blank PBS three times. After the treatment, the tissues were continuously cultured in SGM for 2, 4 and 8 d. During the tissue culture, the media were changed every day, and the tissue surface was gently rinsed with artificial saliva once per day. At each time point, the tissues were fixed and dehydrated as described previously.

The dehydrated tissues were then embedded in Tissue-Tek O.C.T. compound (Sakura Finetek USA, Torrance, USA), and then sectioned in cryostat chamber (Cryocut 1800, Reichert-Jung, Depaw, USA). The tissue sections with a thickness of 10 µm were mounted on polysine slides (Thermo Fisher Scientific, Waltham, USA) and prepared for immunofluorescent staining of E-cadherin. The tissue sections on the slides were firstly washed with PBS to remove the O.C.T. compound and then blocked by 10% normal goat serum (Thermo Fisher Scientific, Waltham, USA) for 60 min at the room temperature. Subsequently, sections were incubated with mouse monoclonal primary antibody against E-cadherin (67A4,
Santa Cruz biotechnology, Dallas, USA) at 4°C overnight in a dilution ratio of 1:50. After removal of primary antibody, the sections were incubated with secondary antibody, *i.e.* Alexa Fluor 488 goat anti-mouse IgG (1:400, A11029, Thermo Fisher Scientific, Waltham, USA) for 90 min at room temperature in the dark. Then, the samples were mounted with a Fluoro-Gel II mounting medium containing DAPI (Electron Microscopy Sciences, Hatfield, USA) and observed under an Olympus 1 × 2-UCB fluorescent microscope (Tokyo, Japan).

**2.3.11 Challenge of *Candida* on different CDs or AmB pretreated engineered oral epithelium tissues for 48 h**

The challenge of *C. albicans* on RHOE was performed as described by Schaller *et al.* with some modifications. First, *C. albicans* was inoculated on SDA agar plate 2 d prior to RHOE arrival and then incubated for 24 h. On the second day, some *Candida* cells were taken from the agar plate and suspended in saline solution. The cells were washed with saline twice and collected using centrifugation at 500 g for 10 min. Then, *Candida* cells were suspended in YND50 at concentration of $2 \times 10^4$ cells/mL and cultured for 16 h at 37°C with orbital shaking (80 rpm). The washing procedure was repeated on the third day, and the *Candida* cells was re-suspended in YND50 at concentration of $4 \times 10^5$ cells/mL and incubated at 37°C with orbital shaking for another 24 h. At the same day, the newly arrived tissues were immediately transferred in 6-well plates containing 1 mL of SMM per well to activate the tissues. After the overnight incubation, the tissues were pretreated with PBS, different CDs (CD, CD-Gu⁺, CD-Gu⁺-AmB) PBS solution at concentration of 200 µg/mL and AmB PBS solution (5 µg/mL) for 6 h. After the pretreatment, RHOE were washed with PBS three times to remove the loosely attached CDs or
drug molecules on the tissue surface. Meanwhile, the *Candida* cells were centrifuged, washed with saline three times and suspended in PBS with final concentration of $4 \times 10^7$ cells/mL. Then, 50 µL of *Candida* suspension was inoculated to the upper layer of RHOE. The tissues pretreated and inoculated with PBS were considered as control, while the ones pretreated with PBS and inoculated with *Candida* suspension were denoted as CA control. After the inoculation, RHOE were continuously incubated for 48 h with SMM changing every 24 h. At each time point (24 and 48 h), the culture media were collected, and the tissues were fixed in 4% PFA at 4°C overnight and then harvested for cryosection. All the treatments were performed on three different occasions in triplicate. The E-cadherin in the tissue slices from different groups were stained as described previously. For the histological changes, periodic acid-Schiff (PAS) staining was performed on the tissue slides. The tissue samples of different groups obtained from three occasions displayed a similar fluorescence intensity changes of E-cadherin and histopathologic alternations.

The tissue destruction caused by the *Candida* cells was measured by using Pierce Lactate Dehydrogenase (LDH) Cytotoxicity Assay Kit (Thermo Fisher Scientific, Waltham, USA). 50 µL of culture media from different groups collected at two time points were added to the 96-well plate and then reacted with 50 µL of reaction mixture for 30 min at room temperature. Afterwards, the reaction was stopped by adding 50 µL of stop solution per well. The absorbance of the final solution was measured at 490 nm and 680 nm using a SpectraMax M2 Microplate Reader (Molecular Devices, California, USA). Meanwhile, the cytokines (IL-8 and IL-1β) in the culture media were assessed using enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems, Minneapolis, USA).
2.3.12 Statistical analysis

Data are displayed as mean and standard deviations (SD). The significance difference between the different CDs treated and control groups, or different CDs-treated and control groups compared with CA control group was determined by one-way analysis of variance with multi-comparisons by Tukey’s test using GraphPad Prism 7.

2.4 Conclusion

In summary, the AmB conjugation favorably modulated the interactions of CDs with the *Candida* cells, and yet the guanylated red-emissive CDs with AmB conjugation possessed potent effects against *C. albicans* in its planktonic and biofilm forms. The as-synthesized CDs with low cytotoxicity were internalized in HOKs and further accumulated in the cell nuclei. Importantly, the present study provided the first evidence on the penetration and exfoliation profiles of CDs in a well-established 3D organotypic human oral epithelial model *via* topical administration. Notably, the penetration and accumulation of CD-Gu⁺-AmB could form a constant and sustainable shielding layer on oral epithelia against *C. albicans*. This study highlights that CD-Gu⁺-AmB may serve as a promising antifungal agent for tackling *Candida*-induced oral candidiasis, through instant epithelial penetration, extra- and intra-cellular embedding as well as gradual exfoliation.
Chapter 3: Concluding Remarks and Future Work

Carbon dots (CD) have emerged as the new eye-catching nanomaterials within the last decade. Herein, Chapter 1 gives a brief overview of the CD, including their synthetic approaches, fluorescence mechanisms, and applications. Notably, owning to tunable emission, high biocompatibility, facile surface modification and low cytotoxicity, CD have been widely celebrated as the promising candidates for drug delivery with traceable fluorescence properties.

*Candida albicans*, a commensal fungus that have been colonizing in human body since infancy, can transform into life-threatening pathogen in immunocompromised individuals. Meanwhile, the efficacy of Amphotericin B (AmB), a common antifungal agent in clinic, is limited due to poor water solubility and dose-dependent toxicity. In Chapter 2, the first application of CD eliminating *Candida albicans* in both planktonic and biofilm forms is introduced by conjugating red-emissive CD with AmB. The conjugation of AmB on guanylated CD (CD-Gu+) can improve the bioavailability and maintain the antifungal activity of AmB at the same time. The AmB-conjugated CD nanotheranostic platform acts as an all-in-one package that shows potent antifungal and anti-biofilm effects with traceable fluorescence properties. Meanwhile, the findings suggest that AmB-conjugated, guanylated CD (CD-Gu+-AmB) with low cytotoxicity can be internalized and accumulate in the nuclei of the primary human oral keratinocytes (HOKs). The study was continued to investigate the penetration and exfoliation profiles of CD in reconstituted human oral epithelia (RHOE), which demonstrates the first evidence that CD-Gu+-AmB can form a shielding layer, arming on the RHOE tissues, to prevent the invasion of *C. albicans.*
For extension studies, synthesis of CD with different dopants and modification is under development. Of note, the loading efficiency of AmB on CD can be enhanced by the introduction of additional functional groups on the surface of CD. Meanwhile, the reconstituted human gingival epithelia (RHGE) with keratinized stratum corneum were inspected, and interestingly, different penetration patterns were observed. Unlike the CDs in RHOE (which CDs can fully penetrate the RHOE within 2 h and appeared in both inter- and intra-cellularly), the CDs in RHGE were found accumulating in the stratum corneum during the first 12 h. This might be due to the stratum corneum of RHGE that could act as a physical and biological barrier, which hinders the entering of CDs. Cell-penetrating peptides (CPP) are polycationic peptides that are used by the viruses to deliver their genetic codes into cells. Particularly, CPP containing disulfide bonds (CPD) are being utilized to facilitate the penetrating activity in cells by having dynamic thiol-disulfide exchange. It is noteworthy that the stratum corneum is mainly made up of lipids and keratin, a polypeptide chain bonded together with disulfide bonds. Thus, it is hypothesized that the modified CDs might be able to cross the stratum corneum by entangling the CPD on the CDs.

Furthermore, recent studies have shown that *C. albicans* could be one of the causes of Alzheimer’s disease, the most common cause of dementia. However, the bioavailability of the drugs is restricted by the blood-brain barrier (BBB), and only allows 2% of the small molecules to pass through the highly functional structure uniting the brain and spinal cord capillaries. Therefore, by utilizing CPD with AmB-conjugated CD, it is hoped that the cell intake of the drugs can be increased and the penetration to corneum layers can be enhanced, leading to promising applications in the future.
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Appendix

Figure S1. Synthetic scheme of BODIPY dye (compound 3). (a) EDC, NHS, rt, 24 h; (b) (i) 2,4-dimethylpyrrole, TFA, DCM, rt, 24 h; (ii) DDQ, NEt3, BF3·OEt2, rt, 24 h; (c) ethylenediamine, DCM, rt, 12 h.

Figure S2. $^1$H NMR spectrum (400 MHz, CDCl3, 298 K) of compound 1.
Figure S3. $^{13}$C($^1$H) NMR spectrum (101 MHz, CDCl$_3$, 298 K) of compound 1.

Figure S4. $^1$H NMR spectrum (400 MHz, CDCl$_3$, 298 K) of compound 2.
Figure S5. $^{13}$C-$^{1}$H NMR spectrum (101 MHz, CDCl$_3$, 298 K) of compound 2.

Figure S6. HRMS (MALDI–TOF) analysis of compound 2: calculated for C$_{24}$H$_{22}$N$_{3}$O$_{4}$BF$_{2}$ [M]$^{+}$ $m/z$ 465.1670, found 465.1658.
Figure S7. $^1$H NMR spectrum (400 MHz, CDCl$_3$, 298 K) of compound 3.

Figure S8. $^{13}$C($^1$H) NMR spectrum (101 MHz, CDCl$_3$, 298 K) of compound 3.
Figure S9. HRMS (MALDI–TOF) analysis of compound 3: calculated for C\textsubscript{22}H\textsubscript{25}BF\textsubscript{2}N\textsubscript{4}O \([\text{M+H}]^+\) \(m/z\) 410.2088, found 410.2085.
CURRICULUM VITAE

Academic qualification of the thesis author, Ms. HUANG Regina:

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