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Carbon Dots as Bioactive Antifungal Nanomaterials

Elisa Sturabotti,*[a] Alessandro Camilli,[b] Francesca Leonelli,[b] and Fabrizio Vetica*[b]

Nowadays, the widespread diffusion of infections caused by opportunistic fungi represents a demanding threat for global health security. This phenomenon has also worsened by the emergence of contagious events in hospitalisation environments and by the fact that many fungi have developed harsh and serious resistance mechanisms to the traditional antimycotic drugs. Hence, the design of novel antifungal agents is a key factor to counteract mycotic infections and resistance. Within this context, nanomaterials are gaining increasing attention thanks to their biocidal character. Among these, carbon dots (CDs) represent a class of zero-dimensional, photoluminescent and quasi-spherical nanoparticles which, for their great and tuneable features, have found applications in catalysis, sensing and biomedicine. Nevertheless, only a few works define and recapitulate their antifungal properties. Therefore, we aim to give an overview about the recent advances in the synthesis of CDs active against infective fungi. We described the general features of CDs and fungal cells, by highlighting some of the most common antimycotic mechanisms. Then, we evaluated the effects of CDs, antimicrobial drugs-loaded CDs and CDs-incorporated packaging systems on different fungi and analysed the use of CDs as fluorescent nano-trackers for bioimaging, showing, to all effects, their promising application as antifungal agents.

Introduction

Infections caused by opportunistic fungi are becoming a major problem for public health security and, over time, they have significantly contributed to nosocomial infectious conditions.[1] The wide diffusion of those disorders represents a severe therapeutic challenge especially for patients that experience situation of immunosuppression and hypersensitivity phenomena that overlaps with prior clinical conditions, such as human immunodeficiency virus (HIV) and AIDS patients, that have undergone organ and bone transplant or under cancer treatments. Nowadays, *Candida* and *Aspergilli* families have developed the most severe drug-resistance response, that can lead to mucosal or systemic invasive infections.[2]

For instance, resistance phenomena to fluconazole have occurred when *Candida albicans* infections were treated in HIV-infected patients.[3]

The increase in the use of antimycotic drugs goes along with the growth of resistance phenomena making it necessary the improvement of novel therapies able to counteract the microorganism inertia. The scientific community is actively working on the discovery and development of therapies able to implement the spectra of active treatments for microbial infections. To support the research, nanoscience has been widely exploited and several types of nanomaterials have exhibited excellent biological features and biocidal character. Besides the most known nanoparticles based on metals or metal oxides,[4] carbonaceous nanomaterials find application in the antimicrobial field. Among the others, carbon dots (CDs) are a class of fluorescent amphiphilic nanomaterials, with average dimensions less than 10 nm and quasi-spherical shape. Since their discovery in 2004, CDs have gained the attention of the scientific community due to their peculiar properties, such as the low cost production, water solubility, abundance of superficial functional groups, intrinsic tuneable fluorescence and good biocompatibility, finding applications in catalysis, nanomedicine, sensing, and biomedicine.[5] CDs can be prepared from reaction of small or big organic compounds (bottom-up approach) or exfoliating larger carbon sources (top-down approach), following solvothermal, microwave or electrochemical treatments. The choice of the synthetic conditions and of the starting material is certainly critical to afford nanoparticles with the desired properties. It is not straightforward to predict CDs antifungal activity due to disparate parameters involved in their production and to the necessity of a deep characterization to asset the nanomaterials properties. Moreover, to the authors knowledge, the majority of the works on CDs as antimicrobial agents discuss their role against a broad spectrum of bacteria[6] while only scant papers investigate their antimycotic character. Therefore, this concept paper is proposed as a tool to compare and outline the current strategies to synthesise or modify CDs thus obtaining nanomaterials with enhanced antifungal activity. The features that concur in the expression of the antimycotic character are rigorously collected and evaluated for CDs obtained by bottom-up approach, considering the type of precursors employed or the post-synthesis functionalization carried out. Furthermore, the exertion of CDs as fluorescent nano-trackers to image fungi or to engineer packaging for food protection are discussed, shining a light onto these recent trends (Figure 1).

[a] *E. Sturabotti*
Center for Cooperative Research in Biomaterials (CIC biomaGUNE), Paseo de Miramón 194, Donostia-San Sebastián 20014, Spain
E-mail: esturabotti@cicbiomagune.es

[b] *A. Camilli, F. Leonelli, F. Vetica*
Department of Chemistry, Sapienza University of Rome, Piazzale Aldo Moro 5, 00185 Rome, Italy
E-mail: fabrizio.vetica@uniroma1.it

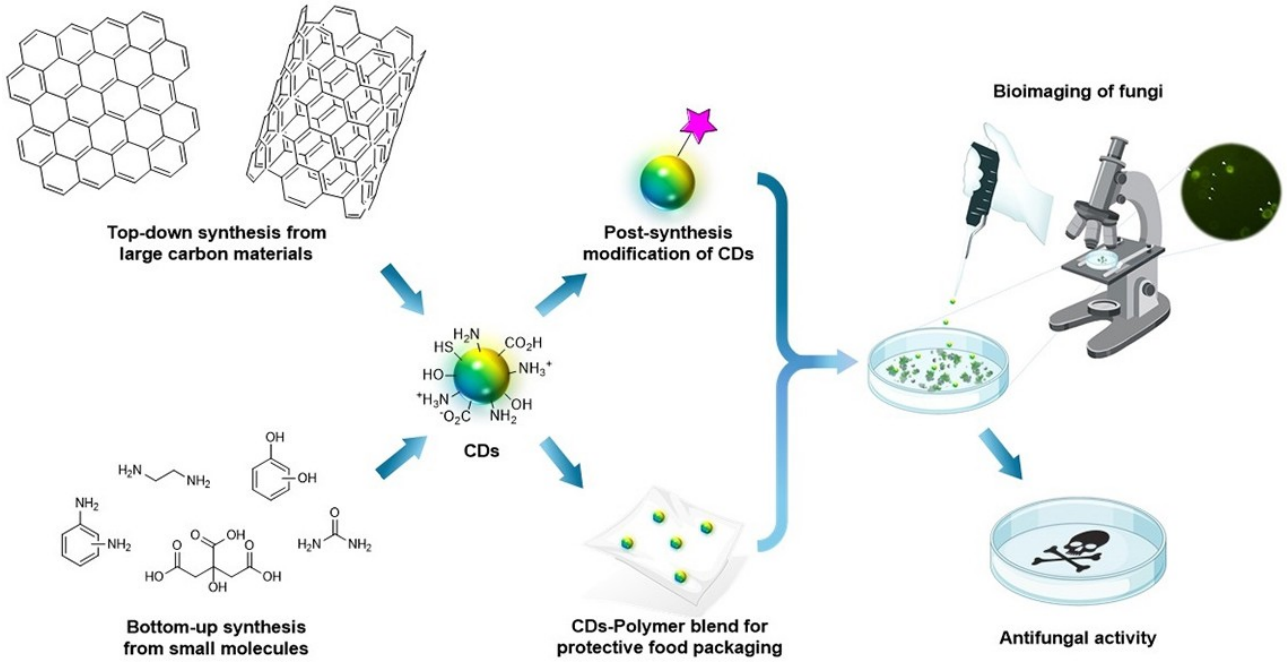
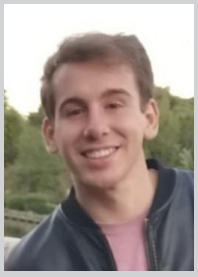


Figure 1. Schematic illustration of top-down and bottom-up approaches for CDs synthesis (left side of the illustration). Furthermore, a representation of the strategies used for the preparation of antimycotic CDs, drug-loaded CDs, and of CDs-blended polymeric films is reported, together with an example of their application as imaging bio-probe for fungal visualization (right-side of the illustration).

Elisa Sturabotti was born in Tivoli in 1992. She received her bachelor's and master's degrees with honours in Industrial Chemistry at University of Rome "La Sapienza" in 2014 and 2016 and, in the same University, she defended her PhD in 2021. After one year of postdoctoral fellowship at "La Sapienza", in 2023 she joined the CICbiomaGUNE research centre in San Sebastian, as postdoctoral researcher, where she continues to work. Her area of interest deals with the synthesis, functionalization and characterization of polymers, biopolymers and fluorescent nanoparticles for biological and medical application.



Alessandro Camilli was born in Rome in 1998. He graduated cum laude in Chemistry at the University of Rome "La Sapienza" in 2023, with a thesis about the synthesis and the study of bioactivity of polymers and nanomaterials functionalized with thymol. In 2024 he received the award as an excellent graduate for academic merits from the same university. He is currently pursuing his PhD under the supervision of Prof. Francesca Leonelli. His research concerns the tailored design, characterization and study of antimicrobial properties of Carbon NanoDots with applications in drug delivery and in the synthesis of blended polymeric materials.

Francesca Leonelli was born in Rome in 1974. She graduated with honours in Chemistry at the University of Rome "La Sapienza" in 1999 and she received her Ph.D. in Chemistry in 2003 at the same university. She continued to work there as a postdoctoral fellow until 2008. From 2008 to 2022, she was a researcher in the Chemistry Department, and in 2022, she became an associate professor at the same university. Francesca Leonelli's research primarily focuses on the synthesis of organic compounds, particularly molecules of biological interest.

Fabrizio Vetica received his Master Degree in Organic Chemistry at "La Sapienza" University of Rome. Afterwards, he obtained his Ph.D. in Organic Chemistry in 2018 under the supervision of Prof. Dieter Enders at RWTH Aachen University. Subsequently, he worked as Lab-team leader at BASF SE working on heterogeneous catalysis. In 2019 he joined ISOF-CNR in Bologna as Postdoc. Since 2020 he is Assistant Professor of Organic Chemistry in the Department of Chemistry at Sapienza University of Rome. His research interests are: asymmetric organocatalysis, natural products synthesis, electroorganic synthesis and synthesis of carbon dots for catalytic and biomedical applications.

Carbon Dots: Synthetic Strategies and General Features

Before going into the details of the CDs antimycotic activity, it is imperative to make a thorough and clear description of the most representative features that distinguish this class of nanomaterial. For the purposes of this concept paper, the following information could be helpful to delineate the origin of any possible structural peculiarity of CDs, that may result in different antifungal properties. Still, many efforts need to be made in this direction, thus properly predicting or clarifying the structure-activity relationship. The comparison of many scientific articles may generate confusion about the definition of carbon dots. In fact, the term "CDs" (that we have chosen to follow for this paper) is a generic expression used to designate a class of photoluminescent and zero-dimensional carbon-based nanomaterials. This definition is not representative of the great variability that distinguishes the nanoparticles in terms of both physicochemical and photophysical properties. The most accredited classifications divide CDs in four families: Graphene Quantum Dots (GQDs), Carbon Quantum Dots (CQDs), Carbon NanoDots (CNDs) and Carbon Polymer Dots (CPDs).[7] GQDs are mainly made up of graphene lattices, that give to the nanoparticle a discoidal shape, while the CQDs possess a quasi-spherical shape and a crystalline structure due to the interplane stacking between graphene sheets. In both cases, photoluminescence arises from quantum confinement effects. On the other hand, CNDs have a quasi-spherical shape, possess a carbonised and amorphous core (depending on the ratio between the sp^3/sp^2 hybridised carbon), in which the fluorescence reasonably comes from defect states or molecular fluorophores linked to the main structure. Lastly, the CPDs have a quasi-spherical shape, a carbonaceous core and exhibit both functional groups and polymers on the surface. In the latter case, fluorescence is due to crosslinked-enhanced emission states and to the presence of fluorophores. All the aforementioned properties can be finely tuned in the final materials, since they strictly depend on the reaction conditions and the nature of the precursors. As mentioned before, CDs can be synthesised *via* two different approaches, known as top-down and bottom up. The former provides for the oxidative cleavage of large carbon materials, such as graphene, graphene oxide and graphite, through electrochemical or chemical oxidation. These methods require harsh conditions and produce nanoparticles with many surface defects that are usually associated with unattractive photoluminescence properties. Also, CDs deriving from top-down approaches are scarcely reproducible and give often unsatisfactory yields.

In light of this, bottom-up methods are gaining more consideration from the scientific community due to the tunability of their properties. In fact, the possibility of synthesising CDs from several and different molecules via single- or multicomponent reactions gives rise to CDs with versatile and reproducible features in terms of morphology, structure and photoluminescence. In specific cases, the CDs also retain some of the precursors features, enduring the achievement of defined goals. Operationally, these methods can be performed in absence or presence of solvents, *i.e.* pyrolysis or solvothermal synthesis, under the action of microwaves or electrochemically.[8] The bottom-up methods rely on green chemistry guidelines and allow the employment of cheap, eco-friendly, biodegradable and biocompatible sources ranging from low molecular weight molecules, such as sugars, amino acid, small amines, or even waste products[9] to polymers, as starch,[10] polyethyleneimine[11] or other natural polysaccharides.[6d]

From a mechanistic point of view, the precursors, especially when dealing with small molecular weight molecules, are firstly subjected to condensation, aldol reactions or polymerization to form small oligomers; then, these intermediates aggregate by covalent and non-covalent interactions. Lastly, the micro/nano-aggregates undergo carbonization, oxidation and/or dehydration to form fluorescent nanoparticles, that preserve some functional groups on the surface (*i.e.* carboxylic acid, aldehydes, phenols, amines, and hydroxyls).[12] Such groups confer most of the more appealing properties to CDs, as their water dispersibility, chemical reactivity and versatility, the possibility of post-functionalization and bioactivity.

When dealing with CDs, one aspect that is particularly crucial is the step of purification. Water soluble CDs can be purified from crude reactions by filtration, centrifugation and dialysis, while organic soluble CDs can be subjected to solvent extraction and/or precipitation. Although these strategies are able to remove most side products and unreacted synthetic precursors, in some cases CDs purification requires more sophisticated techniques, such as HPLC or ion exchange chromatography and gel electrophoresis. Indeed, some promising properties are erroneously attributed to CDs, while instead, they belong to side products or unreacted precursors that are not properly removed by incomplete/insufficient purification steps.[6d,13] This factor is particularly crucial when dealing with the description of CDs fine use, structure/property relationship or scalability for real-word application, since, eventuality, some biological properties may instead arise from molecular-like artefacts rather than CDs.[14] Hence, it is compulsory to pay particular attention to the CDs purification, establishing straightforward protocols that avoid the misleadingly attribution of astonishing features to the wrong material and rigorous characterizations of the nanomaterials. The exhaustive description of the techniques used to characterise the CDs is far from the purposes of this article. For these details, we refer the readers to selected papers.[8,15]

Fungi Characteristics and Antimycotic Mechanism of Carbon Dots

The eukaryotic cells of fungi possess a rigid membrane that has the scope of supporting and protecting them from environmental stresses. The membrane is a dynamic structure mainly composed of glycoproteins, modified with N- and O-linked carbohydrates, glucans, among which 1–3-glucan is the mayor

component, and chitin, a semicrystalline polysaccharide made of beta-1,4-linked N-acetylglucosamine residues (Figure 2).[16] All the components, featuring their crosslinking degree, composition and tensile properties, strongly influence the rigidity and integrity of the membrane.[17] Mycotic eradication is severely hampered by the production of biofilms. In fact, following the adhesion to a substrate, the planktonic cells can proliferate and transit to their yeast/hyphal form.[18] Biofilms are constituted of microbial aggregates stored at a solid-liquid interface; they are enclosed in a matrix made of a conglomeration of highly hydrated extracellular polymeric substances (EPSs), such as polysaccharides, proteins, lipids and nucleic acids that constitute more than 90% of the dry mass. EPSs play a crucial role for the creation of a complex and three-dimensional architecture which confers unique adhesive and cohesive properties on biofilms and allows the fulfilment of many other biological functions. Also, EPSs provide extraordinary mechanical stability and viscoelastic properties to biofilms, making them elastic or rigid depending on the environmental conditions. Although biofilms can differ in terms of morphology and porosity, all of them are responsible for transiently sticking biofilm cells and permit the existence of interacting and hard-wearing mixed-species micropopulations.[19] These features allow the fungal cells' transition from the planktonic to the sessile form, responsible for the development of more demanding infections. Indeed, biofilm-associated infections are considered as a serious public health issue, because they are associated with the decrease of the efficacy and susceptibility to the traditional antifungal drugs, especially when having to deal with polymicrobial diseases. Also, these organisms can dramatically reduce the host immune response.[20]

Hence, the need for the development of new antifungal agents able to overcome such infections has been arising since the beginning of the XXI century, but only recently the attention of the scientific community has been moved on CDs.

Although the mechanism of fungi eradication by CDs is not completely understood, several recurring pathways can be acknowledged comparing the results reported (Figure 3). In any case, the initial CDs action is related to their ability to interact/enter cell membranes or to avoid biofilm formation, hindering the production of hyphae or the yeast-transition. For instance, similarly to other positive charged particles,[21] positive carbon dots display high electrostatic affinity towards the negative micromycetes membrane and can easily penetrate inside the cells. Once inside, the internal equilibrium of the cells is altered by dysfunctions of enzymatic processes, variation of charge concentration or reactive oxygen species (ROS) production. The latter aspect is especially referred to CDs rich in electron-donating functionalities, as nitrogen rich groups. As a consequence, damage to protein or DNA can occur, the membrane integrity is lost and the cytoplasmic material is released. Another mechanism to target mycotic cells is based on the synthesis of nanomaterials that can selectively interact with

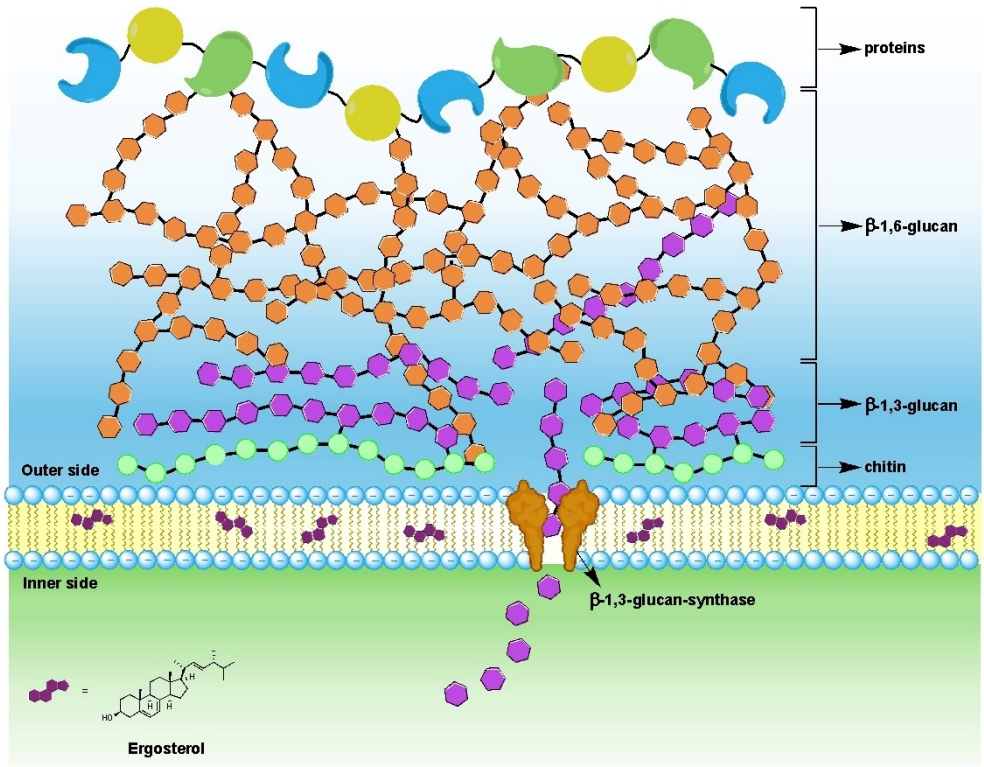


Figure 2. Representation of the basic structure of the fungal cell wall. From bottom to top, the β-1,3-glucan-synthase is immersed in the phospholipid bilayer, rich of ergosterol molecules, partially responsible for drugs internalisation. The enzyme synthesises β-1,3-glucan from free β-D-glucose molecules coming from the cytosol. β-1,3-glucan is one of the most important polymers of fungal cell wall, together with chitin and β-1,6-glucan: these components show high crosslinking degree and confer rigidity and integrity on the membrane, making it hardly crossable. At the top, there are glycoproteins mostly bonded via glycosylphosphatidylinositol remnants to β-(1,6) glucan and to the β-(1,3) glucan-chitin core.

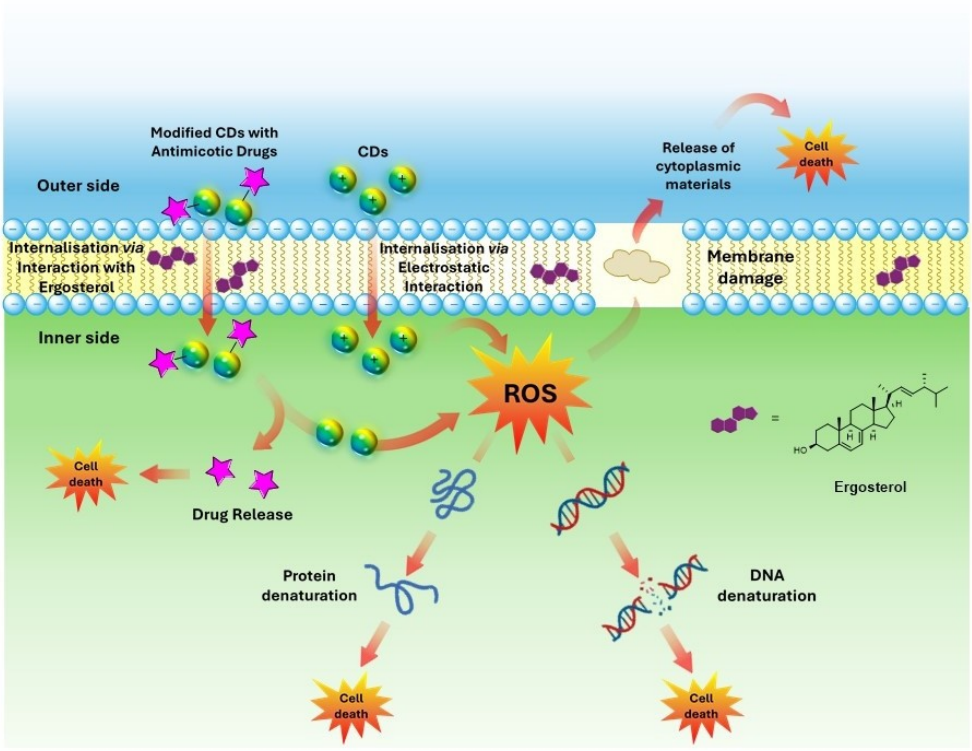


Figure 3. Antimycotic mechanism of CDs: their activity is related to their ability to permeate the fungal cell wall, achievable through interactions with its external negative charge or with ergosterol. Once inside the cell, CDs can promote the uncontrolled production of ROS, thus inducing the release of cytoplasmic materials and causing protein and DNA denaturation, with the result of the fungal cell death.

ergosterol, a sterol present in the microorganism's membrane. In this way, CDs can link to the membrane barrier and cross it.

According to specific reference methods, for the evaluation of the treatments efficiency for invasive infections caused by fungi, some standard definitions are adopted: foremost, the Minimum Inhibitory Concentration (MIC in μg/mL) or the Biofilm Minimum Inhibitory Concentration (BMIC in μg/mL) are defined as the lowest amount of antimicrobial agent that causes susceptibility or resistance in visible growth of an microbial strain in solution or in the formation of a biofilm.[22] To understand if the growth inhibition is caused by microbiostatic or microbicidal effect, the Minimum Fungicidal Concentration (MFC in μg/mL) is also tested. In this way, the concentration of a compound that is active in the eradication of 99% of the strain can be defined.

Heteroatom Doped Carbon Dots as Antimycotic Agent

One of the main reasons for CDs compatibility relies on the fact that they are mainly composed of carbon, hydrogen and oxygen. Then, the amount and type of doping with heteroatoms (non-metallic or metallic) can be tuned selecting among different precursors, synthetic pathways or post-synthesis procedures. All those parameters strongly influence the final chemico-physical, optical and biological performances of the CDs. For instance, non-metallic doping is frequently used to increase the fluorescence emission of the nanoparticles, a factor of big importance when dealing with bio-imaging studies.[23] On the other hand, the metal doping allows to impact the CDs with paramagnetic character, peculiar light absorption, or heating capability suitable for specific biomedical applications.[14] CDs doping is also crucial when dealing with microbial infections. It is well known that interactions between nanomaterials and pathogens are strongly influenced by the surface polarisation of nanoparticles, their chemical composition, superficial functional groups and amphiphilic character.[21]

For instance, microbicidal activity of boron (BGCD), sulphur (SGCD) and nitrogen (NGCD) doped carbon dots was studied and compared to glucose-based undoped CDs (GCD).[24] CDs were obtained by hydrothermal treatment of glucose in autoclave (200 °C, 6 h) in the presence of urea, sodium persulfate and boric acid for the N, S and B doping, respectively. Based on agar well diffusion method, the highest antifungal activity was expressed by NGCD, followed by SGCD and BGCD on *Aspergillus fumigaturs* (NGCD inhibition zone ~20.0 cm), *Fusarium solani* (SGCD inhibition zone ~12.5 cm), *Penicillium citrinum* (NGCD inhibition zone ~22.5 cm), *Candida albicans* (SGCD inhibition zone ~15.0 cm) and *Rhodotorula rubra* (NGCD inhibition zone ~21.0 cm). The NGCD exhibited also the highest MIC and MFC values against *A. fumigatus* (19 and 39 μg/mL), *P. citrinum* (19 and 39 μg/mL) and *C. albicans* (27 and 54 μg/mL) in the broth microdilution method. Interestingly, 72 h cytotoxicity on mouse fibroblast L929 cell line was not affected by CDs

chemical composition in a concentration ranging between 0.1 and 0.5 mg/mL.

CDs co-doping represents another successful strategy to obtain active nanomaterials. The development of halogen/nitrogen co-doped CDs endure the production of appealing antifungal nanomaterials. *C. albicans* has shown susceptibility versus nitrogen and chlorine co-doped dots, as reported by Gao and colleagues.[25] Starting from a deep-eutectic solvent comprised on choline chloride and urea, the authors obtained N,Cl-CDs with photocatalytic antifungal activity. Upon 80 minutes of visible light irradiation, the N,Cl-CDs inhibit the growth of planktonic *C. albicans* at an optimum experimental concentration of 7 mg/mL. The cell damage was visible on the outside envelopes that appeared distorted, deformed and wrinkled.

Xiao Li and coworkers synthesised nitrogen and iodine doped CDs (N,I–CDs) starting from 3-iodo-L-tyrosine, 3,5-diiodo-DL-tyrosine, and iopromide active against *C. albicans* strain.[26] The three sets of CDs exhibited good antifungal activity against *C. albicans*, in synergy with low amount of H_2O_2 under light irradiation. 90% of inhibition was achieved for N,I–CDs (I content of 2.72 mg/mL) in the presence of exogenous hydrogen peroxide (0.5 mM) under visible light irradiation for 120 min. The authors attributed the mechanism of antifungal action to a combination of peroxidase-like effect and photocatalytic activity, which seem to increase with the iodine content and surface charge value measured by Z-potential experiments.

The surface polarisation plays a pivotal role in the interaction with microorganisms since most of them possess negative out-barrier.[17] At this regard, our group investigated the fungicidal effect of positive (CDs-NH_2; +14.3 mV) and negative (CDs-CO_2H/NH_2; −22 mV and CDs-CO_2H; −36 mV) CDs on *C. albicans* as planktonic cells or in the form of biofilm.[27] The three series of CDs were prepared by microwave assisted synthesis of D-glucosamine and 1,3-diaminobenzene in H_2O/MeOH solution (CDs-NH_2), urea and citric acid (CDs-CO_2H/NH_2) and glucose and sodium polyacrylate in water (CDs-CO_2H). Our results highlighted the rapid internalisation of the sole positive CDs-NH_2 into *C. albicans* cells and their dose-response inhibition of the biofilm adhesion (79% reduction of adhesion at 500 μg/mL after 90 minutes of incubation) and formation (70% reduction of early and mature biofilm formation at 125 μg/mL). This aspect is of particular relevance if the virulence of *C. albicans* biofilms is considered.[28] Remarkably, the *in vivo* tests carried out on *G. mellonella larvae* treated with CDs-NH_2 showed that 90% the *larvae* survived to fungal infection in the presence of the CDs, evidencing the biosafety of the nanoparticles towards microorganisms with mammalian-like immune system.

Positive dots possess the characteristic of crossing physiological human barriers and simultaneously kill pathogenic fungi. In this regard, Chen et al. in 2024 synthesised small negative CDs (SN-CDs) by hydrothermal treatment of citric acid and L-glutathione and ultra-small positive carbon dots (SP-CDs), with the addition of polyvinyl polyamine to the same mixture.[29] Unlike SN-CDs, the SP-CDs were active against *F. solani*, which is responsible for eye fungal keratitis, at a concentration of 2.5 μg/mL. Similarly to what was observed by us, CDs fluorescence was visible inside the cell membrane indicating that the positive nanoparticles could easily enter biological membranes. CDs increase ROS production and no *hyphae* formation was detected at concentration of 20 μg/mL under optical microscope observation. Interestingly, the SP-CDs could also penetrate the corneal barrier, thus manifesting their therapeutic effect on different thickness.

Li et al. synthesised vitamin C based CDs (VC–CDs) through a one-step electrochemical method and tested their antifungal activity on two pathogenic fungi, *Rhizoctonia solani* and *Pyricularia grisea*, well known for causing rice diseases, rice sheath blight and rice blast.[30] After the addition of VC–CDs, both fungi were inhibited in a dose-dependent manner. In particular, VC–CDs showed a significant growth inhibition of *R. solani* and *P. grisea* at a concentration of 300 μg/mL, respectively after 48 h and 96 h (Figure 4). By using confocal microscopy, the authors demonstrated that VC–CDs were accumulated in the nuclei of hyphal cells, within which they could destroy nuclei themselves. Then, through DLS and PL experiments and agarose gel electrophoresis, the authors suggested that CDs with oxygen functional groups can adsorb on fungi cell walls, to damage it and to penetrate it through diffusion. Subsequently, they can interact with nucleic acids by non-covalent bonds, thus affecting genetic pathways of fungi and, also, they can inhibit the nonribosomal peptide synthetase (RsNPs) gene expression, involved in several biosynthetic processes.

Lastly, considering the eventuality that CDs may retain some peculiarity of the precursors properties, another outstanding strategy to obtain antifungal nano-agents relies on the use of natural active principles as starting materials for the synthesis of CDs. To this end, Wang and coworkers synthesised CQDs from *Forsythia* (a Chinese herbal medicine), urea and ethanolamine through a microwave hydrothermal treatment.[31] The so-called *Forsythia*@CQDs were tested against two pathogenic fungi of wood, *G. trabeum* and *C. versicolor*. Firstly, *in vitro*

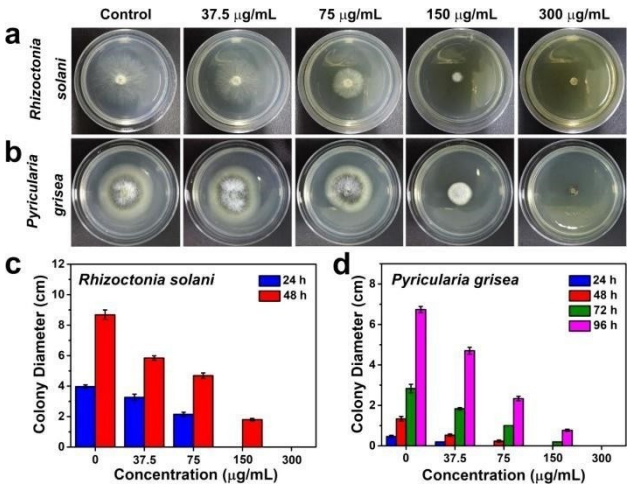


Figure 4. Photographs of (a) Rhizoctonia solani and (b) Pyricularia grisea treated with increasing concentrations of VC–CDs (0, 37.5, 75, 150, and 300 μg/mL). The histogram in (c) and (d) report the colony diameter of R. solani and P. grisea after different times (24–96 h) and increasing concentration treatments. Adapted with permission from[30] Copyright 2018 American Chemical Society.

tests were performed at different concentrations of the nanomaterial to evaluate both the antifungal activity and MIC specific values. The results showed that *Forsythia*@CQDs completely inhibited *G. trabeum* and *C. versicolor* at concentrations equal to 10% and 14% of *Forsythia*. Then, the corrosion resistance on *Pynus silvestris* and *Poplar* woods was tested after 12 weeks of treatment. Compared to 45% and 52% for two blank samples and to values ranging from c.a. 40% to 2% for *Forsythia*, the nanoparticles showed only a 1 (±0.5)% weight loss, suggesting fungistatic effects and a good corrosion resistance also in *in vivo* conditions. The authors attributed these astonishing antifungal properties to the small size and positive polarised *Forsythia*-based nanoparticles. Therefore, nanometric size of *Forsythia*@CQDs allow them to more easily penetrate into plant cells and to better exert the antifungal activity. The authors finally hypothesised that these effects may be due to the electrostatic interaction between negative fungal cell wall and positive surface of *Forsythia*@CQDs, because of amino groups presence and to the alteration of the fungal permeability.

Post-Synthesis Modified Carbon Dots as Antimycotic Agent

Besides the doped CDs with intrinsic antimycotic character, different CDs-based nanomaterials have been developed to face microbial infections. The abundance of surface functional groups and the presence of π-domains into the nanoparticles structure endure their derivatization as drug, pesticide, antimicrobial or gene delivery platforms. Post-synthetic modifications of the CDs are generally conducted following physical absorption, by hydrophobic/hydrophilic interactions or hydrogen bonding, or chemical functionalization with delivery molecules, forming amidic, ester, imide or etheric bonds. Furthermore, due to their ultra-small dimensions, the drug loading capacity of CDs is particularly high, thus encouraging the unravelling of engineered CDs-based systems.[6a]

In a recent article, flumorph, a commercial pesticides belonging to the morpholines family, was loaded onto CDs (Fluomorph-loaded CDs), obtained by hydrothermal treatment of seaweed, to fabricate nanostructure with enhanced antifungal capability.[32] Thanks to the large π-conjugated nanostructure and the richness of amino and hydroxyl groups of the CDs the authors gained a drug loading efficiency of *circa* 47%. The fluomorph-loaded CDs were tested against *Pseudoperonospora cubensis* in *in planta* experiments. Unmodified CDs exhibited low antifungal activity when compared to Flumorph-loaded nanoparticles. Furthermore, the latter exhibit an EC_{50} value lower than that of the sole flumorph (27.8 and 40.2 mg/mL respectively) meaning that the CDs permeability and water solubility possess a positive impact on microbicide performances.

In recent studies, glucose-based CDs (gCDs) have called attention to their capability to cross the blood brain barrier (BBB), which is known as one of the most hampered barriers to pass over in the body,[33] opening the way for their use, alone or drug-loaded, as therapeutic carriers for brain diseases.[34] The crossing of those class of CDs could involve the glucose transporter protein 1 (GLUT1),[34] especially if, after the synthesis, some glucose features remain in nanoparticles structure. Accordingly, in 2021 gCDs self-assembled with the antimycotic epigallocatechin-3-gallate (EGCG) were fabricated (gCDs–E) and studied as nano-agent to counteract *C. albicans* infections, involved in the development of Alzheimer's disease (AD).[35] The gCDs were prepared by solvothermal treatment of glucose in water at high temperature and the EGCG was loaded onto the nanoparticles exploiting hydrogen bonding,π-π stacking and electrostatic binding, as confirmed by FTIR, XPS and UV-vis and fluorescence spectroscopy. After 24 h of treatment, the survival rate of *C. albicans* decreased with the increase of gCDs–E (11.5% with concentration of 480 μg/mL). After the treatments with gCDs–E, serious morphology changes in cell barriers were evidenced by SEM images. Differently from the sole gCDs, 80 μg/mL of gCDs–E nanoparticles were able to suppress almost 67% of *C. albicans* viability, by standard plate count method. The gCDs/EGCG acted as multi-target therapeutic nanocomposite inhibiting the misfolding and aggregation of amyloids, whose deposit is responsible for some pathological traits of AD and preventing C. albicans spreading in vitro. Remarkably, the drug gCDs–E confirmed their cytocompatibility on NRK cells treated with up to 160 μg/mL, hemocompatibility and their ability to cross the BBB in vitro and in vivo.

In an effort to increase internalisation, gathering and antifungal activity of poor-water soluble drugs drugs, Li and coworkers covalently bonded Amphotericin B (AmB) to citric acid/ethylenediamine guanylated CDs (CD-Gu^+).[36] 1H-Pyrazole-1-carboximidamide hydrochloride was used to guanylate the CDs-NH_2 moieties whereas the -COOH groups were functionalized with AmB via EDC/NHS coupling. Afterwards, the nanoplatforms were evaluated against *C. albicans* cells and mature biofilm. After 24 h, the CD-Gu^+-AmB conjugates displayed a MIC value of 15.63 μg/mL against planktonic cells, thereby preserving the antifungal effect of AmB, while CD-Gu^+ were not active on either planktonic or sessile cells. Instead, the CD-Gu^+-AmB conjugates showed a comparable antibiofilm activity with free AmB, displaying an interesting BMIC value of 10 μg/mL. (Figure 5) Adhesion and internalisation of unmodified and AmB-functionalized CDs were evaluated by fluorescence imaging. Notably, only the AmB-modified CDs were visible inside the cell membranes while the majority of bare CDs adhered to the cell walls. The authors suggested that the action of the CD-Gu^+-AmB could rely on the binding of the AmB to the ergosterol through the mycosamine group in the fungal cell wall. Thereby, the membrane permeability is dramatically altered and the cell wall integrity is affected by aggregation phenomena of AmB that cause self-induced formation of pores and channels.[37] Hence, only the AmB modified dots could penetrate and accumulate in *C. albicans* leading to the cell death.

Nanocomposites grounded on CDs and Ag-nanoparticles (AgNPs) have been synthesised by Tenkayala and coworkers.[38] The CDs/AgNPs composites were prepared exploiting the reducing activity of CDs, derived from citrus sinensis peel, in the

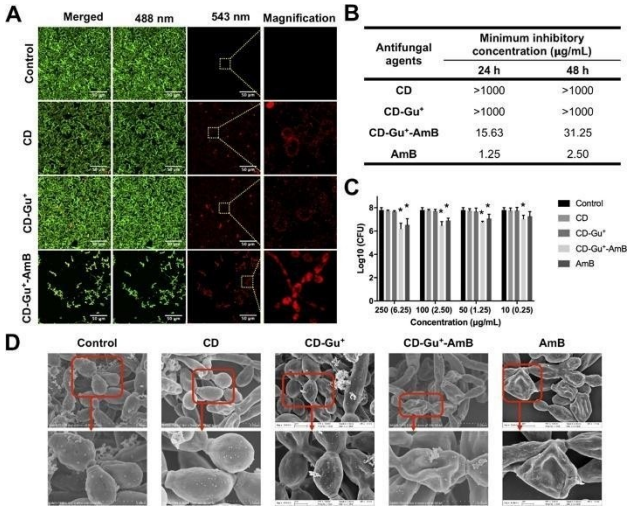


Figure 5. (A) Confocal images of two-day-old *C. albicans* (SC5314) biofilms after treatment with CD, CD-Gu$^+$ and CD-GU$^+$-AmB at concentration of 100 μg/mL. SYTO9 (λ_{ex} = 488 nm) was used for staining green-emitting fungal cells, while HeNe laser (λ_{ex} = 543 nm) was used for red-emitting CDs imaging. (B) MIC values of CD, CD-Gu$^+$, CD-GU$^+$-AmB and free AmB against planktonic form of *C. albicans* (SC5314) after 24 and 48 h.(C) Histogram representation of Log_{10} of the colony-forming units (CFUs) of two-day-old *C. albicans* (SC5314) biofilms as a function of decreasing concentrations of CD, CD-Gu$^+$ and CD-GU$^+$-AmB (250, 100, 50, and 10 μg/mL) and free AmB (6.25, 2.50, 1.25, and 0.25 μg/mL, equivalent to functionalized AmB of CD-Gu$^+$-AmB concentrations). The asterisk stands for relevant difference in values between the treatment and control groups ($p < 0.05$). (D) FEG-SEM images of *C. albicans* after being treated with CD, CD-Gu$^+$ and CD-GU$^+$-AmB (100 μg/mL) and free AmB (2.50 μg/mL). Adapted with permission from[36] Copyright 2019 American Chemical Society.

formation of AgNPs from $AgNO_3$ by solution heating. Through the interaction between the Ag + cations and -OH groups and by the contribution to the reduction of the oxygen-rich functionalities, the AgNPs could be simultaneously generated and stabilised by the CDs in situ, without the use of external reducing agents. Then, the CDs/AgNPs were examined, among the others, against A. niger using the agar diffusion method. The highest inhibition zone (40 mm) was achieved treating the strain with 100 μl of CDs/AgNPs solution (1 mg/mL) supporting the idea that CDs/AgNPS can be applied in the antifungal field.

The MIC, MFC and B-MIC values in μg/mL reported for as-synthesised CDs and modified CDs against *C. albicans* strain are collected in Table 1.

Carbon Dots as Fluorescent Nano-Trackers for Bioimaging of Fungi

The detection of pathogenic micromycetes is a factor of pivotal importance when dealing with infections, especially in the early stages of the spreading. To that, the fungi bioimaging is relevant for human safety, alongside with their eradication. Thanks to their peculiar optical properties, such as the high photostability, good brightness, high quantum yields, prominent biocompatibility and penetration capabilities, CDs demonstrated to be solid candidates as fluorescent and biosensing probes.[39] In fact, the interaction of the CDs with the microbial membrane can be exploited to sense and visualise cells by fluorescence spectroscopy.

At this regard, in our previous work,[27] we highlighted the possibility of using CDs-NH_2 as a fluorescent probe for the targeted detection of *C. albicans* planktonic cells (Figure 6): our results showed that, after only 5–10 minutes, the concentration of CDs-NH_2 into fungal cells was higher than in the external environment, presumably due to the positive surface polarisation of nanoparticles which promotes the cell membrane crossing. CDs derived from cornstalk and ethylenediamine, as carbon and nitrogen source respectively, were modified with water-soluble or alcohol-soluble AmB to detect *C. albicans*.[40] The results indicated that a change, *i.e.* increase, in the fluorescence signal was obtained only for the AmB-CDs derivatives in the presence of *C. albicans*, reasonably because of the specific interaction between AmB and the ergosterol in the

Table 1. Summary of the antimycotic effects of heteroatom-doped and post-synthesis modified CDs on *C. albicans* cell and biofilm.

Type of CDs1[a]	Precursors	Synthetic approach	Strain	MIC (μg/mL)	MFC (μg/mL)	B-MIC (μg/mL)	Ref.
S-doped CDs (ζ$^-$)	glucose, sodium persulfate	Hydrothermal	N. D.	78	152	/	[24]
B-doped CDs (ζ$^-$)	glucose, boric acid	Hydrothermal	N. D.	312	655	/	[24]
N-doped CDs (ζ$^-$)	glucose, urea	Hydrothermal	N. D.	27	54	/	[24]
N/Cl-doped CDs (N. D.)	choline chloride, urea, citric acid	Hydrothermal	N. D.	/	7000	/	[25]
N–I-doped CDs (ζ$^-$)	iopromide, ethylenediamine	Hydrothermal	N. D.	/	2720 [b]	/	[26]
N-doped CDs (ζ$^+$)	glucosamine hydrochloride, 1,3-diaminobenzene	Hydrothermal Microwave-Assisted	ATCC 1023	397	>500	125	[27]
EGCG-loaded CDs	glucose	Hydrothermal	ATCC 10231	80	480	/	[35b]
AmB-loaded CDs (ζ$^-$)	citric acid, ethylenediamine, formamide	Hydrothermal	SC5314	15.63	/	10	[36]

[a] In the following column the different kinds of heteroatom doped-CDs and drug-loaded CDs are reported, together with the sign of the surface zeta-potential. [b] The test has been performed in presence of a 0.5 mM solution of H_2O_2.

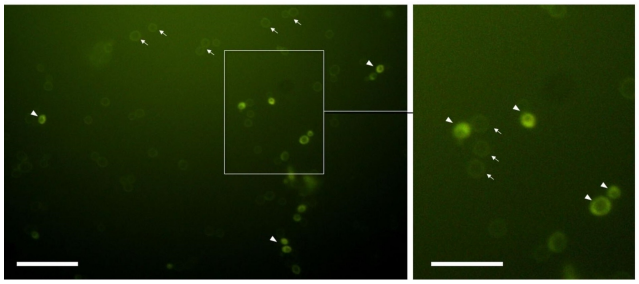


Figure 6. Epifluorescence micrograph of *C. albicans* (ATCC 1023) planktonic cells in presence of CDs-NH_2 as green-emitting probe. The squared area in the left image (scale bar 40 μm) is represented in detail in the right image (scale bar 5 μm). After 10 min exposure to CDs-NH_2, the cells exhibit fluorescence signal in the walls (arrows) and, some of them, in the cytoplasm (arrow head). Reproduced from ref.[27] Copyright 2024, The Authors.

membrane. The CDs modified with AmB showed a linear range of detection, between 2.60×10^5 and 1.99×10^8 cfu/mL, and good selectivity only towards *C. albicans*.

De Oliveira and coworkers synthesised blue emitting CDs by the microwave-assisted synthesis of chitosan, a natural polycationic polysaccharide, to mark inocula of *C. albicans*.[41] In this work, the authors varied different parameters, such as the synthesis time, the amount of doping agent and the molar ratio of reagents, to study their influence on CDs' quantum yield for bioimaging applications. The results highlighted that the amount of the doping agent and the molar ratio of the reactants was significant for increasing the quantum yield, ranging from 1.2 to 7.1%. After a brief exposition to the CDs, the cell culture presented a bright fluorescence indicating that the nanoparticle can easily permeate into the membrane and accumulate into the cytoplasm of the microorganism. As above, internalisation seems to be originated by the binding of ergosterol to the chemical functionalities, including amine, hydroxyl and carboxylic groups, present on the CDs surface.

Multi-color CDs were used as fluorescent imaging agents to track *Aspergillus aculeatus* and *Fomitopsis sp*, which are known to cause severe plant disease. Green and yellow emitting CDs were synthesised from well chopped Manilkara zapota fruit reacted with H_3PO_4 in water at different reaction times.[42] The reaction of Manilkara zapota fruits, which are rich of flavonoids, carbohydrates and vitamins, with a strong oxidant, as phosphoric acid, induced the formation of CDs enriched with numerous functional groups that plays a significant role in the tuning of fluorescence emission. Quantum yields of green and yellow emitting CDs were measured and estimated equal to 7.9 and 5.2%, respectively. Thanks to the instauration of hydrogen bonding between CDs and the fungi membranes, the nanoparticles were internalised in living cells via endocytosis mechanism and were visualised by confocal laser microscopy.

Non-toxic, blue-emitting CDs were synthesised from citric acid and polyethylene glycol (PEG): they displayed good quantum yield (5.7%) and thus were used as fluorescent nanotracker to image *C. albicans* yeast and hyphae.[43] The CDs exhibited no toxicity against human cell lines, such as NIH-3T3 and RAW-264.7, or the microbial strain up to high concentration (5 mg/mL). Furthermore, the nanoparticles did not show any intracellular ROS formation in *C. albicans* thus unaltering its growth in medium. The mycotic uptake was compared to that of rhodamine 6 G, used as positive control, and seemed to be based on passive membrane diffusion after 15 min of exposure. In addition, fluorescent images of *C. albicans* in the hyphal form were obtained thanks to CDs internalisation.

Polymeric Films Blended with Carbon Dots for Protective Food Packaging

In recent years, the need for novel and adequate packaging materials has become a relevant postharvest challenge in food product shelf life and quality. As a matter of fact, traditional plastic packaging is not able to prevent food from fungal and mycotoxin contamination. Filamentous fungi are considered as the major threat for food packaging because they can be entered, infected and broken down. Furthermore, several fungi are able to form moulds that can secret mycotoxins. The latter are well-known for causing mycotoxicosis thus causing carcinogenic, immunosuppressive, lethal, mutagenic or teratogenic effects on animals and human beings. Considering all these reasons, it appears clear that the development of new active packaging (AP) materials represents a primary purpose for the prompt inhibition of fungi and their secondary metabolites. Within this context, the most appealing solution is based on the development of AP-systems able to enhance food shelf life and improve its safety, while being safe for the users. AP systems can act in different ways, but, generally, they are made of polymers mixed with nanoparticles, in which the polymeric film is the structural component while the nanoparticle exerts antimicrobial activity and UV and atmosphere shielding. Considering the excellent luminescent properties of the CDs, several authors have investigated their use as polymeric filler in the fabrication of PA, thus increasing time of storage, food appearance and sterility.[44]

To this regard, Safitri et al. prepared composite films made of chitosan/pectin mixture and chitosan-based CDs for fruit active packaging applications.[45] The agar test diffusion method was used to evaluate the inhibition of the CDs-biofilm blends against *Colletotrichum sp.* The zone of inhibition increased with the amount of CDs loaded in the biofilm (1% and 2% loading gave 15.5 mm and 24.6 mm diameter, respectively). Then, the protective role of the biofilms was tested directly on mangos coated with the active package for 24 days. The CDs-biopolymer coatings were effective in the protection of the fruits, preventing them from weight loss and formation of large areas of fungi contamination during the experimental times (Figure 7).

Another successful example of protective packaging is represented by the work of Ezati et al.[46] The authors developed cellulose nanofibers-based thin films blended with two types of CDs as potential antioxidant and antimicrobial material for food packaging. In particular, the CDs were synthesised by glucose (GCD) or glucose and urea (NGCD) in a one-step hydrothermal

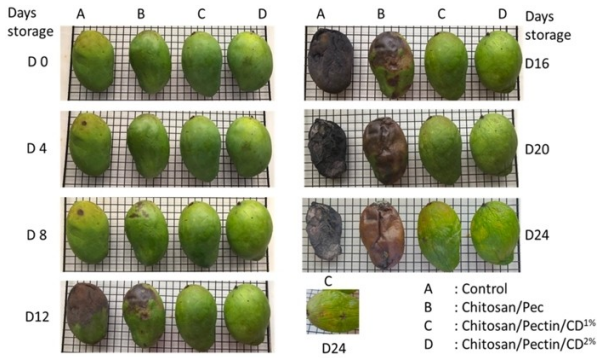


Figure 7. Change in Appearance during Storage at 25 °C of Mangoes Coated with Chitosan/pectin-based Coatings. Reproduced from ref.[45] Copyright 2024, The Authors.

process and mixed with cellulose matrices (1 wt% based on polymer). After that, the antimicrobial activity of the film with GCD or NGCD was evaluated on *A. flavus* mold. Compared to the control film, the NGCD-cellulose packaging entirely inhibited the growth of *A. flavus* after two days of incubation while the activity of the GCD-cellulose film was more moderate, highlighting once again the leading role of doping when dealing with antimycotic activity of carbon dots. SEM images showed that both CDs-implemented films caused severe damages to the mycotic membrane, altering the integrity of the barrier. Interestingly, the authors pointed out that the presence of nitrogen-containing groups enhances the electron-donating capability of the film, therefore inducing the generation of reactive oxygen species which are detrimental for microorganisms' eradication.

Summary and Outlook

In this work we attempted to give the readers a full overview on the recent advances in the production of CDs as antifungal agents, outlining some of the most relevant examples that have dealt with such a novel topic. At first, we highlighted some of the main and most appealing features of CDs in terms of classification, synthetic strategies and importance of purification procedures, to better address the possible structure/property relations. Then, to better understand the antimycotic effects of these nanoparticles on fungi, we described the structural properties of fungal wall cells, distinguishing between planktonic and sessile forms, especially focusing on the infective character of the latter. We outlined the most accredited mechanistic hypothesis regarding the antifungal properties of CDs, which are mainly based on internalisation *via* electrostatic or chemical interaction with ergosterol, followed by the uncontrolled production of ROS, able to denature biomolecules and to cause the leakage of cytoplasmic materials, with the consequent death of fungal cell. Thus, we reported to the readers' attention the current works regarding heteroatom doped and post-functionalized CDs employed as antifungal agents, by highlighting the biological effects on different fungi and the relative minimum inhibitory and fungicidal concentrations. The mentioned examples show that, although the topic is in an early stage of study, CDs could reasonably act both as fungistatic or fungicidal agents, depending mainly on the superficial net charge, on the heteroatom doping and on the superficial charge/composition. Biological screenings revealed that most of the antimycotic effects of CDs are associated with the production of ROS and with the irreversible alteration of the common biosynthetic processes of fungal cells. On the other hand, thanks to the small size, to the surface rich in functional groups and to the penetration capabilities, CDs have been employed as nanostructured drug delivery systems for molecules with intrinsic antimicrobial activity, whose free administration is often complicated or not recommended.

Also, because of high photostability and good brightness, CDs have been exploited as prominent fluorescent and biosensing probes for the detection of pathogenic fungi, in order to view and to analyse fungal cells by means of fluorescence and microscopy. The tests performed revealed that, in general, CDs could penetrate fungal cells after the chemical interaction with ergosterol or *via* endocytosis, displaying wide linear ranges of detection and good cell viability, thus unaltering the regular fungal growth.

Lastly, we evaluated the possibility of incorporating CDs in polymeric films for the design of nanocomposites able to improve food packaging's performances. At the time, the only two works published for antifungal purposes proved that these materials present enhanced antimicrobial and antioxidant activity, making them suitable candidates for food packaging's applications.

Therefore, this concept may have provided the necessary information to the readers for the comprehension of the great and promising potential of CDs as novel, tuneable, biocompatible and selective antifungal agents. However, for real world application some problems, such as large scale production, cost assessment, legislation protocols and environmental impact, still need to be addressed. We hope that this work could serve as an inspiring guide for an in-depth analysis of the CDs antimycotic character and of the mechanisms that regulate this feature, to better modulate and broaden CDs applications at both laboratory and industrial level.

Conflict of Interests

The authors declare no conflict of interest.

Keywords: Carbon dots · Antifungal · Antimycotic · Bioimaging · Protective food packaging

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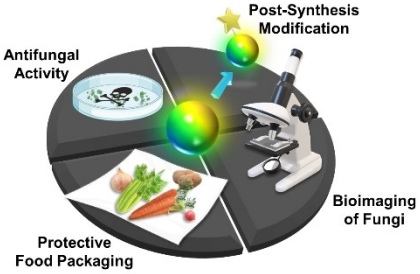
CONCEPT

We review the use of carbon dots (CDs) as novel, fluorescent and functionalizable antifungal nanomaterials. After highlighting the features of fungi and the antimycotic mechanisms of CDs, we demonstrated that these nanoparticles could exert antifungal activity by themselves, with promising applications for the development of food packaging materials, after loading with antimicrobial drugs, or as photoluminescent nano-trackers for fungal imaging.



E. Sturabotti, A. Camilli, F. Leonelli, F. Vetica**

1 – 12

Carbon Dots as Bioactive Antifungal Nanomaterials