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Carbon Dot Therapeutic Platforms: Administration, Distribution, Metabolism, Excretion, Toxicity, and Therapeutic Potential

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Ultrasmall nanoparticles are often grouped under the broad umbrella term of "nanoparticles" when reported in the literature. However, for biomedical applications, their small sizes give them intimate interactions with biological species and endow them with unique functional physiochemical properties. Carbon quantum dots (CQDs) are an emerging class of ultrasmall nanoparticles which have demonstrated considerable biocompatibility and have been employed as potent theragnostic platforms. These particles find application for increasing drug solubility and targeting, along with facilitating the passage of drugs across impermeable membranes (i.e., blood brain barrier). Further functionality can be triggered by various environmental conditions or external stimuli (i.e., pH, temperature, near Infrared (NIR) light, ultrasound), and their intrinsic fluorescence is valuable for diagnostic applications. The focus of this review is to shed light on the therapeutic potential of CQDs and identify how they travel through the body, reach their site of action, administer therapeutic effect, and are excreted. Investigation into their toxicity and compatibility with larger nanoparticle carriers is also examined. The future of CQDs for theragnostic applications is promising due to their multifunctional attributes and documented biocompatibility. As nanomaterial platforms become more commonplace in clinical treatments, the commercialization of CQD therapeutics is anticipated.

1. Introduction

Nanoparticle-based therapeutic systems often have added layers of complexity when compared to chemical systems because of their varying structural, chemical, mechanical, and biological makeup. As a result, determining the pharmacokinetics (fate of the nano-therapeutic within the body) and safety of these constructs is difficult to predict.[1] Numerous studies have highlighted the biodistribution and excretion of nanoparticles in the body;[1–5] however, each nanoparticle formulation is unique and possesses distinct physiochemical properties, which require individual investigation into their theragnostic potential.

Carbon dots (CDs) are an emerging class of fluorescent nanoparticles which have, in recent times, gained attention for their biocompatibility and versatility for cancer therapeutic and diagnostic (theragnostic) applications. Their potential is

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likely to have far reaching impact for combatting numerous health ailments; however, their application for cancer therapeutics currently dominates the literature. There are many forms of CDs, each possessing different attributes suited to various applications. Some of these dots include; graphitic carbon dots, graphitic carbon nitride dots, carbon black dots, carbon quantum dots (CQDs) (amorphous), polymeric dots, polymer/carbon hybrid dots, and co-doped (hetero atom) CDs.[6] For simplicity, henceforth, all dots within this review will be referred to as "carbon dot" (CD) unless specified otherwise.

Carbon dots are amongst the smallest biocompatible nanoparticles documented (<10 nm diameter), and when below 6 nm, can be rapidly excreted through urine following treatment, a characteristic that most other engineered nanomaterials lack. Their small size allows them to either act alone or be integrated with other nanotherapeutics to impart additional functional capacities which are beneficial for therapeutic applications including: 1) Excellent heat absorbing capacities valuable for photothermal therapies.[7,8] 2) Can be doped with metals to give them additional functional properties (i.e., catalytic, conductive, increased affinities for specific analytes, density etc.).[9] 3) Their carbon core may be modified to contain several varying functional groups without the need for several additive functionalization steps which increase their hydrodynamic diameter (HD).[10,11] 4) They can facilitate the transportation of drugs through the body to regions where the drug alone could not travel by altering the drug/dot constructs polarity, overall charge, and solubility (i.e., transportation of drugs across the blood brain barrier).[11–13] 5) They can be doped with contrasting agents (for magnetic resonance imaging or computerized tomography (CT) scanning) or can serve as a contrasting agent alone for photoacoustic imaging applications.[14] 6) Used for fluorescent trackable gene delivery platforms.[15] 7) Used as a fluorescent probe for sensing of proteins, biomolecules, anions, and cations through specific interactions (electrostatic interactions, π–π interactions, electron transfer, covalent bonding) with low photobleaching tendencies.[16] 8) Their binding affinity for hydrophobic small molecule drugs with ring structures via weak π–π conjugation interactions may facilitate the transport of drug molecules to target tissues.[16] 9) They possess high and stable monodispersity.[17,18] 10) They can be tuned to act as an antioxidant or pro-oxidant depending on final intended use.[19,20] 11) Can be synthesized to be highly hydrophilic or hydrophobic.[21,22] 12) Show potential for use as a multi-theragnostic platform (i.e., targeted drug/gene delivery + drug release through photothermal treatment + trackable in vivo with photoacoustic imaging).[23] 13) They can be integrated into other nano/micro composites for added functionality.[24]

Advances in nanotechnology has revealed the great potential offered by these particles for therapeutic applications. However, a cautious approach for adopting nanoparticles for human therapy is currently in place with less than 60 nanomedicines approved for intravenous administration by the Food and Drug Administration (FDA).[25] This can be attributed to i) unpredictable and complicated pharmacokinetics when compared to chemical counterparts, ii) extended bodily retention, iii) non-specific biodistribution, iv) toxicity or production of bodily toxicity responses, and/or v) unfavorable interactions with biomolecules within the body. The majority of particles approved for healthcare applications are carbon based; however, for the most part are made from soft biodegradable polymers or lipids.[26] Limitations of other carbonaceous nanoparticle-based therapeutic platforms (i.e., increased hydrophobicity and bio-persistent size ranges) are circumnavigated with many CD platforms and therefore offer attractive potential for future marketable applications.

Despite the publication of encouraging results related to the suitability of CDs for biomedical applications, a complete investigation into their fate within bodily systems remains underexplored. The following review sheds light on the journey of CDs throughout the body and in doing so, highlights their potential as multifunctional cancer theragnostic platforms (**Figure 1**).

2. Valuable and Tuneable Carbon Dot Properties

2.1. Core and Surface Composition

Carbon dots can either be synthesized via nucleated growth into a carbonaceous core or by physical/chemical breakdown of larger carbonaceous precursors. Numerous reviews give detailed descriptions on the various synthesis approaches.[27–31] Irrespective of the synthesis approach, the carbon skeleton of CDs possesses sp^2/sp^3 hybridized atomic domains with a π-conjugated structure. Aromatic ring structures dominate the composition of most CDs cores allowing for π–π stacking interactions which have been used to transport small molecule drugs to tumor environments.[32] The core make-up of CDs can vary significantly between the different variations. For example, graphene quantum dots (GQDs) possess a crystalline and ordered carbon core whilst carbon polymeric dots exhibit a carbon/polymer hybrid structure which is mostly amorphous in nature. Heteroatom CDs have non-carbonaceous elements integrated into their core structure whilst CDs synthesized using various small molecules can have increased lattice defects with protruding functional groups/molecular structures derived from the parent precursor molecule(s). With such diversity in core properties, a plethora of different modification/synthesis strategies can be implemented to generate material with characteristics suited to specific applications.

The generation of CDs with cores possessing specific functional groups allows for simple subsequent functionalization strategies. Particle functionalization is central for fine-tuning CDs with properties suited to different nano-theragnostic applications, that is, CD/receptor recognition and specific binding, hydrophobicity/hydrophilicity tunability, electrostatic interactions, and evading immune responses. Carbon has tremendous capacity to bind to itself and other elements in many ways which allows CDs to present a great diversity of functional surface ligands. In some cases, post functionalization may not be necessary as CD cores can be generated directly with a molecular structure of interest; for example, CDs generated with folic acid as a sole carbon source for targeting of the folate receptor overexpressed on numerous cancer cell lines.[33]

2.2. Carbon Dots Classifications and their Properties

According to the nature of carbon core materials, there are four broad categories of CDs which all aforementioned subsets may fall under. These include GQDs, CQDs, carbon nanodots (CNDs),

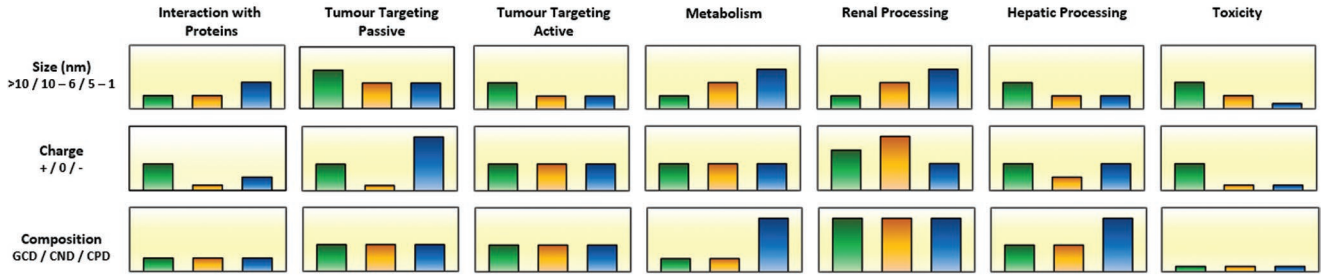

Figure 1. Size: (1) As the size of CDs decreases, the surface area available to interact with proteins increases; (2) Carbon nanoparticles >10 nm are increasingly endocytosed into cells over smaller counterparts; (3) larger particles have more functionalization strategies available (i.e., attachment of antibodies and aptamers) which can actively target cancer receptors; (4) decreased particle size means results in faster degradation of carbon core through enzymatic attack; (5) particles below 6 nm are generally renally excreted whereas particles >8 nm seldom pass through this route; (6) larger particles more often find their way to the liver for hepatic processing. Larger particles are more readily recognized by this system; (7) particles which do not participate in renal processing may bioaccumulate and therefore interact with the body for longer periods of time. Charge: (8) Charged molecules have increased propensity to interact with charged proteins. Positively charged nanoparticles have been shown to possess increased protein interactions over negatively charged particles; (9–10) positively charged particles may have increased cellular uptake over negatively charged particles but also interact with negatively charged biological entities (i.e., nucleic acids, proteins, etc.) which limits their targeting capacity; (11) no links between the differences in CD charge and their rate of metabolism have been reported. (12) Positively charged CDs often bind negatively charged biological species resulting in their HD being increased past the point of filtration size threshold. Strongly negatively charged particles may be repelled by the negatively charged membrane in the GFB. Neutral particles readily pass-through renal processing; (13) positive and negative charged CDs have not been shown to be processed differently by hepatic routes. Neutral particles have fewer interactions with biological species and may not be recognized by hepatic removal processes; (14) positively charged CDs have been shown to interact with negatively charged biological species (i.e., nucleic acids) and have also been implicated in increased toxicity. Core composition: (15–17) core composition of carbon dots has not been implicated in preferential binding to proteins or is beneficial for active/passive targeting; (18) polymeric dots have been shown to be readily biodegraded and not bio-accumulative. Solid core species may take longer for enzymatic attack; (19) All CD species can be readily excreted in the urine if they possess the correct size and charge properties; (20) polymeric dots are more readily susceptible to degradation in hepatic processing; (21) All CD species are biocompatible and show little toxicity if they possess the correct size and surface properties.

and polymeric dots (CPD).[25] Their compositions and mechanisms of photoluminescence generation are compared in **Table 1**.

In addition to above general types of CDs, heteroatom doping in CDs has been developed into an adaptable approach to effectively modify the chemical composition and structure of CDs, providing them with additional novel properties.[36]

2.3. Size

Although CDs have a relatively narrow size range (1–10 nm), within this window, variations in size can have significant implications for cellular uptake, biomolecule interactions, and excretion properties. The transition from bulk solids to nanoparticles results in significant changes in their electronic structure as described by the size quantization effect relating to molecular orbital theory. As nanoparticles fall below the quantum size range (<10 nm) their solid-state behavior becomes less pronounced and the line between solid and molecular state energy properties becomes blurred (i.e., the particles possess distinct molecular orbitals).[37,38]

In addition, charging energy that is experienced in quantum dots and CDs is comparable to the ionization energy found in atoms. It is therefore anticipated that interactions between CDs and (bio)molecules may be more interpersonal compared to nanoparticles above 10 nm in size and can infer novel properties.[37–40]

Table 1. Carbon dot types and their composition.

Carbon dot[34,35]	Composition	Origin of photoluminescence (PL)
Graphene quantum dots	1 to 10 layers of graphene sheets with certain crystallinity and functional groups on the edge of each sheet and/or within the interlayer defect.	Mainly from carbon core state due to conjugated π-domain with a quantum confinement effect and PL is affected by the surface or edge structure. Molecular states are also present.
Carbon quantum dots	Spherical/cylindrical with clear crystal structures and chemical groups on the surface.	Quantum confinement effect of the CQDs' size along with molecular state fluorescence.
Carbon nanodots	High degree of carbonization with some chemical groups on the surface, but usually no obvious crystal lattices structure.	PL mainly originates from the defect/surface state and subdomain state within the graphitic carbon core. Molecular states are also present. No quantum confinement effect of the particle size.
Carbon-based polymer dots (CPDs)	Polymeric entangled crosslinked structure comprising of abundant functional groups/polymer chains on the surface and throughout the amorphous condensed polymeric core. These dots may or may not possess carbonized regions within the polymeric network.	Surface state, subdomain state, molecular state, and crosslink enhanced emission (CEE) effect. Crosslink enhanced emission is not found in the other kinds of CDs.

Size is not only relevant to the novel interactions these particles can have with biological entities but is also important for tissue accumulation and excretion. The small size of CDs can be favorable for crossing different types of endothelial cells monolayers to escape the circulatory system and enter cells microenvironments. Conversely, particles which are too small may be required in ample concentrations to facilitate energy dependent cellular entry. Receptor driven cellular uptake often requires specific size, charge, ligand densities, and enthalpies for internalization, which have been reported to be optimal in the 30–60 nm size range.[41]

A property which can be seen as an attribute and a limitation for CDs is their rapid renal excretion if they are below 6 nm in size. If interactions with target tissues are not strong enough to retain the CDs, they are likely to be rapidly excreted and will not re-enter the circulation for a second pass to seek the target tissue. This clearly minimizes nanoparticle toxicity; however, it also reduces their therapeutic efficiency. Subsequent dosing or functionalization strategies which confer strong target interactions may need to be implemented for effective theragnostic applications. These aforementioned concepts will be discussed in more detail later in this review.

2.4. Shape

Carbon dots are often thought of as spherical, and indeed, there are very few reports on CDs possessing controllable shape profiles. One exception to this was shown with the synthesis of triangular CDs possessing tuneable and narrow fluorescence emission profiles.[42] Although in most cases CDs may look spherical under TEM images, atomic force microscopy often reveals that their shape is more representative of cylindrical discs due to their heights being different from their width.[43–45] This may result in prolonged bodily retention when compared to their spherical counterparts.[26]

2.5. Fluorescence

CQDs are renowned for their intrinsic fluorescence properties which are particularly useful for in vitro cell culture applications. However, most CDs generated to date show blue–green fluorescence outputs limiting their use for in vivo biomedical applications due to insufficient tissue penetration depths.[46] To combat this drawback, recent research has been focused on generating CDs possessing red or near infrared (NIR) emissions profiles increasing tissue penetration capabilities.[47–49] Carbon dots are often biocompatible and quite resistant to photobleaching which provides them with added benefits over traditional fluorescent dye-based probes. The ability to functionalize their surface with fluorescent probes further allows for ratiometric sensing applications.[50]

Research conducted by Jiang et al. (2020) demonstrated that solvothermally synthesized heteroatom CDs doped with nitrogen and fluorine (N-CDs-F) were capable of strong absorption in the full spectrum of UV–vis–NIR wavelengths.[48] In vitro, these N-CDs-F showed minimal influence on cell viability and cytotoxicity to HepG2 and HeLa cells following a 24 h incubation period. In vivo application of these dots for imaging applications were investigated and showed deep tissue penetration capacity with a fluorescence excitation wavelength of 735 nm and emission at 785 nm.

Unlike metallic semiconductor quantum dots whose fluorescence output is strongly correlated with size, CD fluorescence may originate from various mechanisms.[48] The fluorescence output of CDs is influenced by the relationship between their carbon cores composition and adjoining chemical groups.[51] Different accepted mechanisms for explaining CDs fluorescence include:

2.5.1. Molecular States

Bottom-up preparation methods for CDs commonly involves microwave irradiation or hydrothermal/solvothermal treatments of carbon, nitrogen, and other functional precursors in solution. As the carbon containing precursor solutions are heated to temperatures often between 150 and 250 °C, the organic species transform into organic ringed structures possessing inherent fluorescence. These fluorescent small molecules can be integrated into the CDs core structure or act as surface functionalities.[52] In carbon dots synthesized at low temperatures, molecular state fluorescence is the main contributing element for particle fluorescence. As the synthesis temperature increases, the carbon core becomes increasingly crystalline and the dominant mechanisms of fluorescence shift. The resulting mechanisms of fluorescence such as "bandgap transitions of conjugated π-domains" or "surface defect states" have an increasingly active role.[53]

2.5.2. Bandgap Transitions of Conjugated π-Domains

Like semiconductor quantum dots, some carbon dots generate fluorescence through a quantum confinement effect of conjugated π electrons. This mechanism of CD fluorescence is generally limited to species with crystalline structures and fluorescence output is governed by size dependent coulomb interactions. Briefly, electrons from the CDs conduction band move to empty states of the valence band resulting in direct recombination of electrons and holes. The energy of recombination results in the generation of a photon of light.[51,53]

2.5.3. Surface Defect States

The region on a CDs surface where its solid core ends and surface molecular structures dominate relates to its surface state. Surface defect states result when the symmetry of a surface state is disordered mainly arising through surface oxidation.[54] During the often harsh and minimally controlled synthesis conditions of carbon dots, great diversity in chemical groups derived from sp^2 and sp^3 hybrid carbon domains, dangling functional groups or other surface functionalities are present. Sites with imperfect sp^2 carbon domains result in the formation of surface energy traps which can capture excitons of specific wavelengths bringing the particles to a higher energy state. Radiative relaxation from excited state to ground state results in the emission of photons.[6]

For cellular and sub-cellular tracking applications, fluorescence quenching or turn-on fluorescence interactions between

dots and biological microenvironments/biomolecules may not be desirable as they can overestimate or underestimate cellular uptake and distribution capacities. However, when CDs are designed for use as biosensors, selective and sensitive interactions with target analytes resulting in varying fluorescence outputs is of high interest. Numerous mechanisms of CD quenching/turn-on fluorescence have been reported including:

2.5.4. Dynamic Quenching

Dynamic quenching of CD fluorescence occurs when collisions between CDs and a quencher result in charge or energy transfer which brings the particle from an excited state to a ground state. Meng et al. (2021) synthesized carbon dots using neutral red and ethylenediamine as precursors and used them as photoluminescent probes for sensing berberine in SMMC7721 cells. Fluorescence quenching of these red emitting (λex 474 nm/ λem 620 nm) was shown to be from a dynamic quenching mechanism and confirmed through an increase in CD fluorescence lifetime in the presence of berberine.[55]

2.5.5. Static Quenching

Static quenching occurs when a CD binds to a quencher and forms a non-fluorescent ground-state complex when it absorbs light. Huang et al. (2016) investigated the interaction between CDs synthesized with citric acid and PEG-200 as chemical precursors. These particles were shown to quench in the presence of human serum albumin (HSA) and this was confirmed by a i) decrease in Stern–Volmer quenching constant K^{SV} values with increasing temperature, ii) complex formation verification through shifts in CD/HAS conjugate absorption spectrum, and iii) lack of significant fluorescence lifetime differences between CDs in free and complexed states.[56]

2.5.6. Fluorescence Resonance Energy Transfer

Fluorescence resonance energy transfer (FRET) is a non-radiative energy transfer process which occurs through intermolecular long-range dipole–dipole coupling between two fluorophores.[57] In this system, energy is transferred from a donor with high energy to an acceptor with low energy. Carbon dots which employ FRET as a sensing mechanism are usually designed to possess their own intrinsic fluorescence whilst also carrying the fluorescence of another conjugated fluorescent molecule/dye with differing emissions characteristics.[58] In a turn-on FRET system, the fluorescence of CDs is initially removed due to its complexation with a fluorescent quencher, however, in the presence of a target anolyte, the quencher is displaced, and the CDs fluorescence is restored. Conversely, in a turn-off system, the fluorescence of the donor decreases with increasing anolyte concentration.[59] Fluorescence measurements from both the CD and the displaced fluorescent quencher allow for ratiometric measurements which are more reliable when compared to single fluorophore quenching on turn-on systems.[60]

2.5.7. Photoinduced Electron Transfer

In CDs, photoinduced electron transfer (PET) is an electron transfer process in which electrons are transferred between the fluorescent CD and their bound anolytes. PET can result in either an increase in fluorescence or fluorescence quenching.[61] During fluorescence turn-on, electrons are transferred from the anolyte to the CD fluorophore because the donors highest occupied molecular orbital energy is lower than that of the acceptors due to an increased redox potential.[62] Conversely, when excited CDs transfer electrons to an anolyte, there are less electrons returning from excited state to ground state, resulting in fluorescence quenching.[63]

2.5.8. Inner Filter Effect Observation

An inner filter effect (IFE) occurs when the excitation or emission spectrum of a CD overlaps with the absorption spectrum of a "quencher." When this happens, the photons from excited CDs are absorbed and lose flux intensity through a medium resulting in a decrease in measurable fluorescence. An IFE is not a mechanism of fluorescence quenching but rather a selective filtering of light.[50,64]

Detailed fluorescence quenching/turn-on mechanisms for sensing applications are beyond the scope of this review but are addressed in numerous recent publications.[51,65–67]

One notable biomedical application of a CD containing a FRET based probe was developed by Ding et al. (2015). During this study, a FRET based sensor was developed for the detection of Muchin 1 cancer marker using a graphene oxide template functionalized with a carbon dot/Muchin 1 aptamer complex. In the presence of Muchin 1, the carbon dot/Muchin 1 aptamer complex dissociates from the graphene oxide template resulting in a fluorescence turn-on phenomenon. The aptasensor was selective for the Muchin 1 peptide (known to be overexpressed on breast, ovarian, lung, bile duct, and pancreatic cancer cells) and possessed a linear fluorescence relationship with Muchin 1 between 20 and 804 nM.[68]

2.6. Density

Nanoparticle density is often overlooked when it comes to identifying the best type of particle for specific therapeutic delivery capacity. Siddique et al. (2018) reported CDs to possess a density of 1.5 g cc^{-1} which was in good agreement for the density of graphite-like amorphous carbon.[69] This density may be modified by incorporating metallic elements into their structure through heterodoping approaches. Other biologically relevant nanoparticles of similar size often possess much higher densities, that is, gold spherical nanoparticles, which possess a density of 19.3 g cc^{-1}. Gold nanoparticles (4–22 nm) have been shown to be ionized by cells and then recrystallize into bio persistent structures which raises concerns with regards to their toxicity and efficient excretion.[70] Therefore, the use of carbonaceous particles for the same therapeutic end may be preferred.

The association between nanoparticle density and the degree of tumor accumulation requires further investigation with

consistent conclusions yet to be reached. The Stokes-Einstein law for diffusion states that particles of the same size (irrespective of particle composition) should have the same diffusion coefficient. However, gravitational acceleration, inertia, and momentum of particles moving throughout the body is strongly dependent on particle density.[71]

Black et al. (2014) compared the efficiency of gold nanoparticles with different shapes (spheres, rods, discs, and cubical cages) on tumor accumulation. Although spherical particles had the greatest tumor uptake, gold crucible cages showed the greatest tumor penetration depths due to their reduced density having fewer interactions with pressurized fluid flow within extracellular spaces.[72]

Conversely, Tang et al. (2016) compared the tumor accumulation of gold and silver nanoparticles with the same size (≈3 nm) and surface functionalizations but differing densities. Silver particles with lower densities were rapidly excreted from the body and passive tumor targeting decreased linearly as particle density decreased. In addition, decreases in particle density were also implicated in increased bodily distribution but lower retention times in target tumor tissues.[73]

The density of particles may also be an important design factor for other theragnostic applications such as, heat retention or use as a contrasting agent for photoacoustic imaging.

2.7. Thermal Stability

The capacity for materials to retain heat can be useful when combined with thermal responsive materials (i.e., drug release in polymer matrices) and has application in cancer therapy for hyperthermia therapy. For example, temperatures above ≈40 °C can damage and destroy cancer cells with minimal damage to normal tissues.[74] Bao et al. (2018) generated sulfur containing CDs via a solvothermal synthesis approach which not only targeted tumor tissues but also possessed a photothermal conversion efficiency of over 59% when irradiated with a laser at a wavelength of 655 nm at 1 W cm^{-2}. These biocompatible dots were able to reach 52.7 °C after 600 s of irradiation and completely destroyed tumor tissue within mice following 5 min of low powered laser irradiation.[75]

Photothermal conversion capacities of CDs are generally found between 30% and 45%; however, CDs which can absorb NIR I or NIR II radiation have recently shown increased capacity to act as efficient photothermal therapeutic agents achieving between 58% and 80% efficiency.[8,75,76] Geng et al. (2020) demonstrated that CD photothermal efficiency increased with an increase in graphitic nitrogen in their lattice structure. These CDs were able to achieve photothermal conversion efficiencies over 80% when irradiated with 1064 nm laser at low laser power densities.[76]

2.8. Particle Charge, Hydrophobicity, and Hydrophilicity

Carbon dot therapies which rely on crossing cellular membranes (i.e., cellular imaging, gene delivery, drug delivery, targeted theragnostic) rely heavily on particles charge and hydrophobicity/hydrophilicity. Due to the vast potential for tuneable carbon core and surface functionalities, CDs can be modified to possess varying charges and solubility profiles. Tuneable surface charge is particularly important for creating electrostatic attractions or can be used to prevent binding of specific biomolecules through electrostatic repulsion forces. Positively charged CDs have shown an increased capacity to internalize within cells due to interactions between the cationic dots and negatively charged components on the cell membrane (i.e., proteoglycans)[15] which facilitates adsorptive endocytosis; however, strongly localized positive charges on CDs are also linked with increased cytotoxicity and opsonisation.

Most documented CDs possess a hydrophilic nature; however, hydrophobic variants have been developed and may hold promise for increased cellular internalization or adipose tissue targeting. The hydrophilicity of CD has been shown to be transferable to hydrophobic molecules through solid dispersive nanoconjugation. When the hydrophilic CD binds to a hydrophobic drug (i.e., Doxorubicin), the complex possesses an increased hydrophilicity when compared to that of the drug alone. An example of this was shown in a recent study where CDs increased the solubility of vitamin B9 by more than 6200 times.[77] This may lead to the reassessment of the potential of hydrophobic medications which were deemed to have low bioavailability. Roughly 40% of commercial drugs and nearly 90% of molecules in the drug development pipeline have poor water solubility. Poor water-solubility is the main reason why drugs fail to become commercial products and is also a considerable factor behind undesirable drug bioavailability and increased toxicity.[78,79]

2.9. Heteroatom Doped Carbon Dots

Carbon atoms are versatile as they can form a variety of allotropic structures through their sp, sp^2, and sp^3 hybrid chemical bonding arrangements. This enables carbon to bind to itself with great structural diversity, that is, chained, branched, and cyclic arrangements. In addition, carbon has the capacity to bind up to four different elements simultaneously, further increasing its structural diversity and providing material with additional functional properties.[80,81] The integration of non-carbonaceous elements into carbon dots core is a strategy termed heteroatom doping, and both metallic and non-metallic elements have shown application using this approach.

Nitrogen doping is by far the most common form of heteroatom doping strategy and is present in most CD formulations. The incorporation of nitrogen adds electrons to the carbonaceous core, and by doing so, modifies the internal electronic environment resulting in greatly enhanced photoluminescent output.[9] Numerous examples of non-metallic heteroatom doping have been reported.[82–84] One such example was demonstrated by Su et al. (2018) who generated biocompatible iodine and nitrogen doped-CDs (I-CDs) fabricated through the hydrothermal processing of citric acid and Iohexol. The I-CDs possessed high fluorescence with a quantum yield of 18%, and their conjugation with an antibody was used to target EGFR-overexpressing cancer cells for fluorescence imaging. The iodine ions found within the cetuximab molecular structure gave the probe additional contrasting properties and made CT imaging possible.[14]

Metals are generally better electron donors than non-metallic heteroatoms, they possess larger atomic radii and have more

unoccupied orbitals.[85,86] When metallic ions are introduced into CDs, significant changes in charge density and charge transition are expected, often resulting in improved physicochemical attributes. Additionally, the photoluminescence properties of CDs can also be enhanced due to surface plasmonic resonance effects offered by the doped metals.[87]

In 2020, Yue et al. synthesized ruthenium-doped CDs (Ru-CDs) via a hydrothermal method using citric acid and a ruthenium (II) complex (Ru-Aphen). These Ru-CDs exhibited intense red PL emissions, were capable of generating high concentration of reactive oxygen species (ROS), could cleave DNA when subjected to light and were biocompatible when not irradiated by light.[88] Heteroatom doping of carbon dots may greatly increase their functionality, however, care should be taken to ensure that this strategy does not result in added toxicity.

3. Administration and Cellular Uptake Mechanisms

3.1. Administration

CDs are typically administered into the body via parenteral routes (i.e., subcutaneous, intravenous, or intertumoral).[89,90] For laboratory-based studies, the intravenous injection is favorable over non-injectable approaches because particles are rapidly distributed throughout the entire body and smaller doses are required for therapeutic action and bioavailability. As this route bypasses first-pass metabolism, there are less interactions which have the potential to modify the surface and efficacy of the dots. When comparing varying injectable routes for CD administration, particular routes can be suited to different applications.

Huang et al. (2013) synthesized CDs (HD of 4.1 nm) via the laser ablation of a carbon soot followed by post functionalization with diamine terminated PEG-1500N and near-infrared dye ZW800.[91] These dots were administered to athymic nude mice via intravenous, subcutaneous, or intramuscular injection. The group administrated subcutaneously showed increased tumor retention of dots over 24 h when compared to intravenous or intramuscular routes. These dots were all efficiently cleared from the body via a dominant renal route within 24 h without any significant organ accumulation (**Figure 2**).

Once in the bloodstream, CDs will travel through the circulatory system and may have numerous interactions which can influence cellular uptake or excretion (i.e., plasma components, platelets, red blood, white blood cells, endothelial cells). Before dots can interact with target tissues, they need to leave the circulatory system by crossing the vascular endothelium. Their small size is likely to enhance their passive capacity to

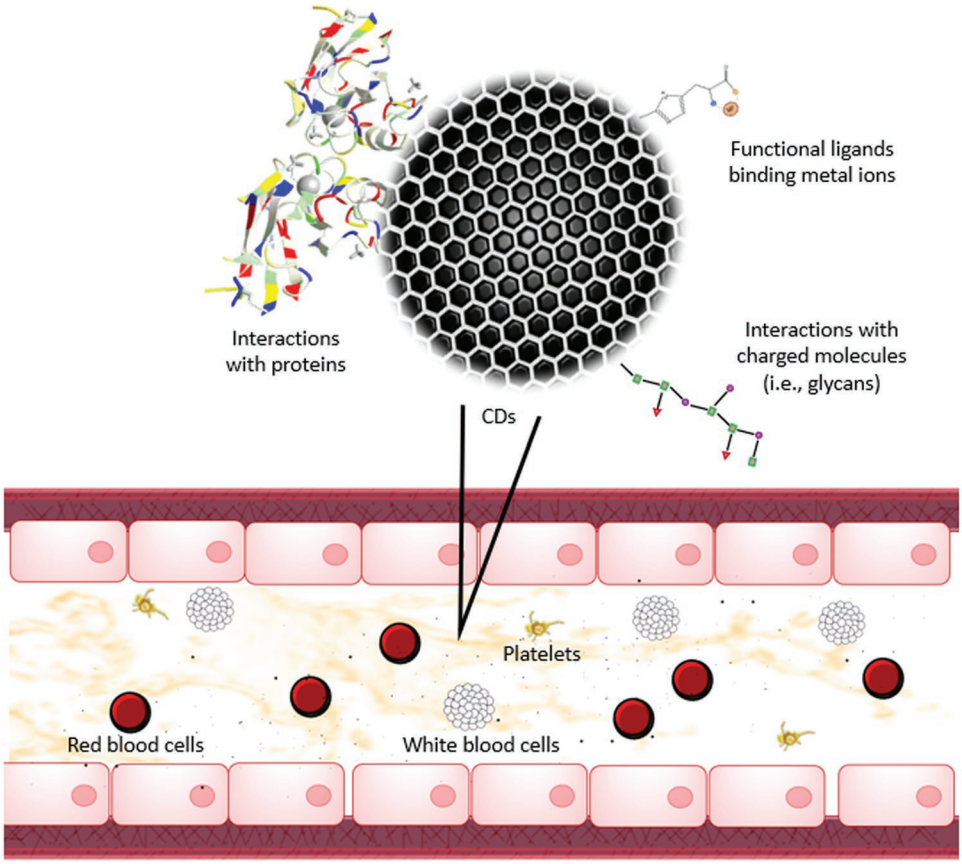

Figure 2. Carbon dots which are administered through injectable routes enter the bloodstream where they encounter numerous biological entities. Apart from the larger blood constituents (i.e., red blood cells, white blood cells, and platelets), within the plasma, proteins, hormones, glycans, vitamins, and waste products can also have intimate interactions with CDs.

pass the different physical barriers within this system. The vascular endothelium consists of a monolayer of endothelial cells (EC) which line all arteries, veins, and capillaries. This barrier between blood and interstitial spaces of tissue has numerous functions including the control of vascular permeability, regulation of blood fluidity, facilitation of signaling, development of angiogenesis, and regulation of the immune system. The properties of the vascular endothelium vary in different regions of the body with alterations in cell surface receptors, intracellular junctions, glycocalyx composition, or transport vesicle type and density.[92] The endothelium may be continuous, fenestrated, or discontinuous. The continuous endothelium facilitates the movement of nutrients, oxygen, and small molecules and has a passive cut-off size of <1 nm when tight junctions are present (blood-brain barrier, spinal cord, retina) or <5 nm when adherent junctions are present (skin, muscle, heart, lung, adipose tissue). Fenestrated endothelia have passages that may allow particles up to 15 nm through (skin, kidney, endocrine glands) whilst the discontinuous endothelium may allow particulate matter over 100 nm to pass through (liver, spleen).[93,94] In certain ailments (i.e., cancer and artheroscelerosis) the endothelial layer in these regions can become leaky and the resulting in vasculatures with passages over 700 nm in diameter. Numerous nanoparticle-based delivery approaches have used these conditions for targeted tumor therapies,[95] although recently, leaky vasculature has been considered only a minor uptake route (≈3%) in human tumors with transcytosis dominating particle uptake.[96] Aside from passive transport between junctions of the endothelium, various transcellular pathways which pass materials through the cell are implicated in cross membrane trafficking. Caveola vesicular transport is the dominant form of intra-cell transport on endothelial cells and can comprise of up to 70% of the endothelial cell membrane.[96] Cellular transport of CDs will be addressed in more detail elsewhere within this review.

Once across the endothelial cell membrane, CDs find themselves within the interstitial compartment (cellular microenvironment). This space is composed of collagen, proteins, and glycosaminoglycans which form the extracellular matrix. Interstitial fluid within this space facilitates the movement of ions, proteins, nutrients, and other entities to the cells surface where further interactions are required to screen for appropriate cellular uptake.[95] This microenvironment comprises numerous chemical and physical barriers which may alter the properties of dots and cellular accessibility.[97] In fact, targeting of cancer tumor environments with nanoparticles is a common strategy exploited by researchers to take advantage of specific different conditions (i.e., anoxic and acidic) for the triggered release of therapeutics.[97] For biological applications, CDs need to be engineered to not only seek out target tissues or microenvironments of interest, but also to have minimal interactions with non-target constituents.

Although infrequently documented, CDs have been administered to the body without the intrusiveness of injections. Intranasal instillation, oral administration, and topical ocular delivery have all been successfully applied.[98] One such example was shown by Pierrat et al. (2015), who synthesized CD based nanocarriers and used them to successfully deliver CD/DNA to the lungs of mice by intranasal instillation.[99] This nucleic acid delivery approach had similar efficiency to the current gold standard for pulmonary administration of DNA (cationic lipid formulation -GL67A) and showed lower toxicity.

Intranasal instillation is often less effective at delivering therapeutics to the lung when compared to aerosol approaches.[100] However, investigations into aerosol delivery of CDs are seldom reported in the literature. Carbon nanoparticles such as graphene and carbon nanotubes have reported no-observed-adverse-effect levels of 3.02 and 0.98 mg m^{-3}, respectively, in rats.[101] However, the large sizes and poor water solubility of these particles compared to that of CDs are likely to result in decreased in particle clearance from the lung. The potential for hydrophilic CDs to be administered through aerosol needs further investigation but may prove to be promising, particularly for respiratory conditions. Care must be taken to design particles that do not persist in off target lung tissues for extended periods of time to prevent chronic lung disease (i.e., use of rapidly biodegradable polymeric CDs).

3.2. Cellular Uptake Mechanisms

Mammalian cell membranes are composed of numerous barriers which restrict what comes in and out of the cell (i.e., hydrophobic lipid bi-layer, membrane channels, transporters, protein interactions, etc.). To pass the cell membrane and enter a cell, nanoparticles can utilize several different cellular pathways. Larger particles (300–2000 nm) are typically taken up via phagocytizing cells (i.e., neutrophils and Kupffer cells) whereas smaller nanoparticles <100 nm employ numerous different dominating mechanisms (endocytosis, pinocytosis, diffusion, defective barrier entry, etc.).

Vesicular transport pathways which are more relevant to non-phagocytic cell types and can facilitate CD cellular uptake include but are not limited to; clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME), clathrin/caveolae-independent endocytosis, and macro/micro-pinocytosis.[102] The pathway for cellular uptake of cargo is dependent on numerous factors including particles size, shape, surface charge, surface functionalization, band gap energy, porosity, crystallinity, solubility, and associations with other dots and macromolecules. Each of these factors will have varying associations with different cell types.[15]

Entry into the cell is not often exclusively attributed to one type of mechanism and may proceed via multiple simultaneous pathways. This can result in difficulty predicting target destination transport dynamics. For example, Hua et al. (2018) hydrothermally synthesized CDs using m-phenylenediamine, l-cysteine and NaOH as the precursors. The cellular pathways for CD cell delivery were investigated by testing their capacity to enter HeLa cells when endocytosis inhibitors (genistein, chlorpromazine, methyl-β-cyclodextrin, 5-(*N*,*N*-dimethyl)-amiloride hydrochloride) and an energy inhibitor (NaN_3) were added to cells. Not only did the researchers determine that cellular uptake of the dots was mainly energy and temperature dependent, but elucidated that CME, macropinocytosis, and CvME were all dominant mechanisms of cellular uptake (**Figure 3**).[103]

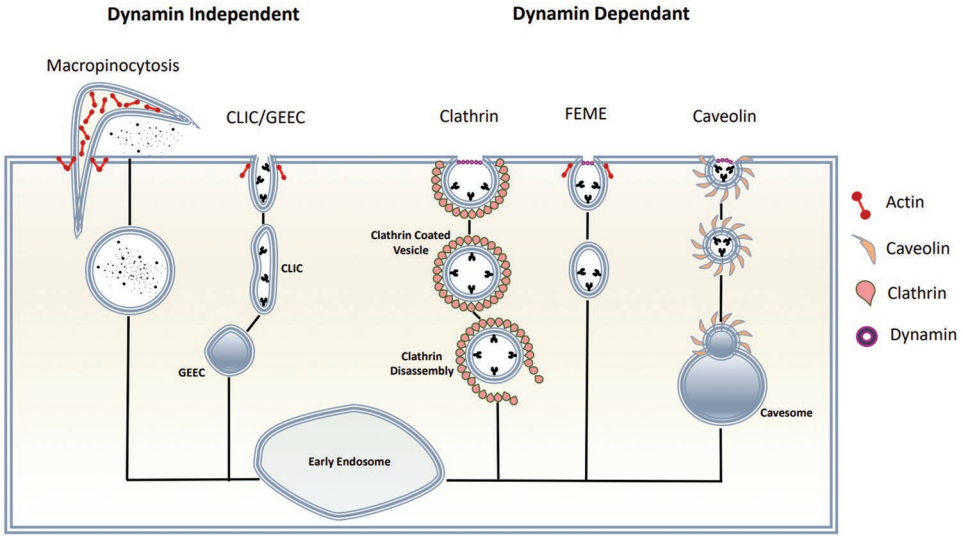

Figure 3. CD cellular uptake is predominantly mediated through numerous active cellular uptake mechanisms, namely macropinocytosis, clathrin mediated endocytosis and caveolin mediated endocytosis. The FEME and CLIC/GEEC endocytosis pathways may hold particular promise for CD bulk cellular uptake and tumor specific delivery.

3.2.1. Clathrin-Mediated Endocytosis

CME is the primary mechanism most cells utilize to uptake macromolecules, nutrients, growth factors along with selected plasma membrane components (cholesterol, iron, etc.).[104] Uptake occurs through numerous steps including i) initiation, ii) cargo selection, iii) coat assembly, iv) scission, and v) uncoating. CME usually begins when chemical ligands interact with the cell surface resulting in the initiation of a nucleation process resulting in the initial formation of a membrane pit. An adaptor protein (AP2) binds to numerous different adaptor molecules which have receptors for specific cargo (i.e., transferrin, low-density lipoproteins, bulk CDs functionalized with specific ligands) which are then selectively permitted to enter the cell. Clathrin triskelia subsequently move to the pit and polymerise to form a clathrin barrier around the pit. Once formed, the clathrin coated pit is pinched away from the cell membrane by the activity of dynamin and enters the cytoplasm where the clathrin coat is disassembled to produce an endocytic vesicle containing the cargo.

Although this process has traditionally been thought to be purely selective for allowing molecules with specific surface functionalization access to the cell (receptor-dependent CME), non-specific absorption via this mechanism is also commonly experienced. Rather than ligand specific interactions between nanomaterials and the cell membrane, nonspecific charged surfaces, and/or hydrophobic nature can trigger pit formation via receptor independent CME up-taking extracellular fluid and its constituents.[97]

3.2.2. Caveolae-Mediated Endocytosis

The initiation of CvME does not start at the plasma membrane but rather in the Golgi complex where caveolin-2 interacts with caveolin-1 to form a complex. This complex further interacts with cholesterol to dissociate it from the Golgi complex and facilitates its movement to the plasma membrane. Once there it fuses with previously assembled caveolar vesicles on the cells surface and is anchored by cytoskeletal components.[105] These formed caveolae are typically 50–100 nm in size and composed mainly of cholesterol and sphingolipids which create a highly hydrophobic environment. Numerous signaling molecules are known to accumulate within the caveolae and these can be activated by receptor ligands.[106] Not all caveolae undergo endocytosis; however, specific binding of ligands to caveolin/caveolae, cross-linking of caveolar components and internal accumulation of receptors within the pits result in downstream signaling events which facilitate the separation of the caveolae vesicle from the membrane and into the cytoplasm.[107] CvME often escapes lysosomal processing, reducing the potential for the modification of CD surface functionalities.

3.2.3. Clathrin/Caveolae Independent Endocytosis

Clathrin- and caveolae-independent endocytosis involves the formation of early endosomes without the employment of clathrin or caveolae-dependent processes. Three dominant initiation mechanisms of clathrin and caveolae independent endocytosis have been identified, including i) acute signaling-induced membrane remodeling (macro/micro pinocytosis), ii) cargo capture by binding to cytosolic proteins (FEME pathway), and iii) extracellular lipid or cargo clustering (CLIC/GEEC pathway).[108]

The activation of clathrin- and caveolae-independent endocytosis require different stimuli which may include interactions with chemical ligands, charge, crosslinking of receptors, distinctive hydrophobic moieties (i.e., bacterial toxins or viral proteins) along with appropriate membrane fluidity, curvature, and tension. As most clathrin and caveolae independent

endocytosis pathways are still poorly understood, further research in this area is required to exploit these pathways for CD uptake.[108,109]

3.2.4. Macropinocytosis

Macropinocytosis is arguably the most characterized clathrin- and caveolae-independent endocytosis pathway and has been implicated in CD cellular uptake. During macropinocytosis, actin polymerizes at the cell membrane resulting in ruffling of the membrane due to cytoskeleton remodeling. This creates appendages which protrude out of the cell and engulf extracellular constituents into vacuoles called macropinosomes which are then internalized. The process is initiated by prolonged signals from receptor ligands such as growth factors, toll-like receptors or chemokines but is not considered to be cargo specific. Macropinocytosis has been identified as a viable mechanism for CD cellular uptake; however, tuning the physiochemical properties of CDs to be specific for this uptake mechanism needs further research focus.

3.2.5. FEME

Fast endophilin-mediated endocytosis is a dynamin-dependent but clathrin-independent mechanism of endocytosis which is initiated by interactions between endophilin protein and G-protein-coupled receptors (or other specific intermediate proteins).[110] Pit formation via this endocytic mechanism is fast (<10 s) and therefore rapidly transports cargo across the cell membrane. As this process is strongly mediated by specific receptor interactions, CDs alone are unlikely to utilize this cellular uptake approach unless they are bound to cytosolic proteins, or their composition mimics the physiochemical properties of appropriate receptors. Future research may use CDs to decorate G-protein-coupled receptors for rapid cellular entry via FEME.

3.2.6. Clathrin Independent Carrier/GEEC

Cellular uptake via the clathrin independent carrier (CLIC)/GEEC pathway is a continuous dynamin- and clathrin-independent endocytosis process.[3] This pathway is responsible for the uptake of GPI-anchored proteins, CD44, specific lectins, and integrins, along with other glycosylated cargoes. It also mediates cellular fluid and bulk membrane uptake for plasma membrane repair and homeostasis in certain cell types. The primary CLICs develop as a result of cargo clustering at the plasma membrane at regions where Cdc42 (a plasma membrane associated small GTPase), and specific membrane bound enzymes ($PI(4,5)P_2$ and $PI(3,4,5)P_3$) are present.[110] This GTPase is then activated by cholesterol which results in the recruitment of actin polymerization machinery and subsequent CLIC formation. This process is rapid (<15 s) and the continuous budding converts into GPI-anchored protein-enriched early endosomal compartment (GEECs) with the help of Rab5 and EEA-1proteins.[111] In addition, membrane tension has a major influence on CLIC/GEEC initiation with increased membrane tension preventing its initiation.

This pathway is responsible for bulk fluid uptake in fibroblasts cells (more than three times that of the clathrin dependent pathway) and may therefore find specific application in the delivery of nanoparticles to tumors. During bulk fluid uptake, initiators and extracellular constituents without receptors can be all internalized in large concentrations.[112] Receptors known to activate the CLIC/GEEC pathway include the folic acid receptor and hyaluronic acid receptor. This may open scope for enhanced CD delivery approaches as folic acid and hyaluronic acid are used as tumor targeting ligands. If CDs functionalized with folic acid or hyaluronic acid are complexed to these receptors prior to administration, their uptake capacities may be enhanced. Tumorous tissues are stiffer than ordinary tissues[113] and therefore, investigations into their ability to facilitate CLIC/GEEC initiation may be reduced because of increased membrane tension. Developing strategies to overcome this may be fruitful for enhanced CD tumor uptake.

Cellular uptake via FEME and CLIC/GEEC pathways is often mistaken with that of the CvME pathway (i.e., cellular uptake of the folic acid by CLIC/GEEC has traditionally been thought to proceed by the CvME pathway). Specific endocytosis inhibitors are yet to be developed for rapid and routine cellular uptake via these mechanisms.[3]

3.2.7. Transcytosis

Transcytosis is a cellular transport mechanism whereby macromolecules are initially internalized into cells via endocytosis and are then enclosed into vesicles which are exocytosed at the other side of the cell.[114] Transcytosis is initiated by either receptor mediated uptake or through the interaction between positively charged molecules and the negative cell membrane. Numerous cell types have been shown to use transcytosis for transporting materials across the cell including neurons, osteoclasts, intestinal cells, and endothelial cells.[115] Nanoparticles employing this mechanism of transport for tumor therapeutics have shown increased tumor penetration depth, which is crucial for effective tumor therapy.[116] The small size of CDs may limit their initial endocytic uptake into cells and therefore having them encapsulated into larger nanoparticle constructs may be beneficial for receptor mediated transcytosis. If CDs reach the cell in large enough concentrations, interactions between the cells and CDs may facilitate transcytosis; however, strongly positively charged CDs have shown elevated toxicity profiles. Additional research is required to exploit this approach for tumor targeting.

3.2.8. Antiporter-Mediated Uptake

Antiporters are membrane-bound proteins which are responsible for maintaining cellular homeostasis through the movement of one or more molecules or ions into the cell whilst moving other entities out of the cell via secondary active transport. Antiporters such as the large amino acid transporter 1 (LAT1) have been used as cellular uptake mechanisms for CDs

to target cancers due to their overexpression on tumorous tissues. Li et al. (2020)[11] screened the capacity of CDs to enter numerous cancerous cell types over healthy cell types. Different cancerous cells (n = 39) and non-cancerous cell types (n = 19) were equilibrated with the CDs and flow cytometry was used to determine dot concentration based on fluorescence output. All cancerous cells had an uptake efficiency between 95% and 100% whereas non-cancerous cells could only achieve between 1.7% and 29.5% efficiency. The elevated uptake was attributed to an increase in large amino acid transporter 1 (LAAT-1) receptors overexpressed on cancerous tissues which were receptive to CDs which possessed paired α-carboxyl and amino groups. These results were replicated in murine models with tumor specific uptake found in brain tumors and human tumor xenografts.

3.2.9. Passive Transport

Passive diffusion, as the name suggests, does not rely on active processes for cellular uptake. Carbon dots with polar physiochemical properties often require active mechanisms for cellular uptake as they cannot freely pass the hydrophobic phospholipid cell membrane. Ultrasmall nanoparticles with zwitterionic or hydrophobic surface properties have been able to utilize passive transport for cellular uptake.[117] One notable example was demonstrated by Chen et al. (2017), who synthesized amphiphilic CDs by the thermal pyrolysis of paprika which showed negligible reliance on energy-dependent pathways for HeLa cell cellular uptake.[118] Cells were incubated at 37 and 4 °C in the presence of Cyto D (macropinocytosis inhibitor), nystatin (caveolae endocytic inhibitor), nocodazole (inhibitor of microtubule formation), and NaN_3 (inhibitor of ATP-dependent endocytosis). Cells under the influence of all treatments and temperatures showed similar fluorescence outputs in the cytoplasmic region of the cell indicating that cell penetration is independent of energy driven mechanisms. However, when these amphiphilic dots were refluxed in NaOH, their capsanthin functionality was lost, resulting in the particles possessing a hydrophilic nature which could not enter cells.

Another example was shown by Sri et al. (2018), who synthesized zwitterionic CDs through microwave irradiation of citric acid and L-cysteine aqueous solution. These dots were capable of transmembrane movement by both active and passive mechanisms. Incubation of the CDs with oral squamous carcinoma cell lines (Cal27 and FaDu) at 4 °C and without the presence of ATP reinforced their cellular uptake capacity in an energy independent manner.[119]

4. Sub Cellular Distribution of Dots

Mammalian cells are not composed of a soup of unorganized biological constituents but are rather highly compartmentalized, with each constituent responsible for performing specific functions. Although each compartment has its own role, the working of the entire cell is reliant on the proper functioning of each component.[120] Specific diseases may be particularly reliant on the functionality of one or several specific cellular organelles or other cellular compartments. For this reason, functionalizing CDs to selectively seek out compartments of interest holds great promise for targeted theragnostic. Due to the small size of CDs, developing particles which possess multiple functional ligands in sufficient densities for different applications may be limited. For example, generating CDs which can target a specific cell type may require one type of functional ligand; however, for this particle to selectively target the nucleus, another ligand may be required. For cancer therapy, numerous examples of preferential cancer cell accumulation blended with specific organelle accumulation have been documented.[121–124]

The inherently photostable fluorescence (which may range from UV to NIR-II range) of CDs is particularly useful for identifying their cellular localization and the destination of any therapeutics they may be shuttling. To verify organelle specific localization, commercial fluorescent chemical probes with specific organelle affinity are introduced to cells along with CDs with different fluorescence profiles to the chemical probes. Fluorescence images are then taken of the cells and if there is overlap between the dot's fluorescence and the probes fluorescence, organelle specific uptake can be verified. Common probes use for selective fluorescence staining include MitoTracker for mitochondria, LysoTracker for lysosomes, ER-Tracker for endoplasmic reticulum, BODIPY TR ceramide for Golgi complex, and Hoechst 33 342 for nuclear DNA.

For clarity, we refer to a study completed by Gao et al. (2017) who synthesized a silicon/CD composite by heating 3-aminopropyltrimethoxysilane and glycerol to 260 °C for 4 h within an autoclave. These dots were used as a fluorescent probe to distinguish between cancerous and non-cancerous cells. The dots generated were shown to be biocompatible and capable of selectively targeting the mitochondria in cancer cells. Pearson's correlation coefficient was used to compare fluorescence localization between the dots and MitoTracker identifying a 0.91 (91%) correlation. Localization of dots in the lysosomes, Golgi complex, ER only showed correlation coefficients of 0.17, 0.14, and 0.51, respectively, when compared against their corresponding organelle selective probes.[125]

5. Targeted Tumor Delivery

Targeted delivery of therapeutics is highly valuable for minimizing the influence of therapeutics to non-target cells. Early chemotherapeutic drugs have minimal tumor targeting capacity and as a result, healthy bodily systems are damaged resulting in adverse and often life-threatening side effects. The toxicity of these medications to cancerous cells and non-cancerous tissues alike prevents concentrated doses of chemotherapeutic to be administered which are often required to completely prevent tumor proliferation and progression. Targeting of cancerous tissues with CDs is accomplished through passive and/or active targeting. Active cancer targeting relies on the binding of nanoparticle surface ligands to receptors which are overly expressed on cancer cells surfaces,[126] whereas passive targeting solely relies on exploiting the conditions within the tumor microenvironment for preferential uptake and therapeutic benefit. Leaky vasculature structure has traditionally

been considered a dominant route for nanoparticles to pass endothelial cell junctions and reach the tumorous mass. However, recent research has attributed leaky vasculature to only 3% of nanoparticle transport across the endothelium in humans. Instead of this passive route, an active transcytosis mechanism has been shown to be responsible for >95% of nanoparticle uptake in multiple significant studies.[127,128] This form of endocytosis may be activated through charge, receptor recognition or through other uncharacterized active mechanisms.

Carbon dots can be either functionalized themselves to seek out cancerous tissues or can be encapsulated within larger nanocarriers which have cancer targeting functionalities. Larger nanocarriers have the added benefit of accommodating surface functionalities which are too large to act as a surface ligand for CDs (i.e., antibodies and other proteins) and may be favored for cellular uptake via endocytosis. Irrespective of the active or passive cancer targeting strategy one is exploiting, favorable interactions with the tumor microenvironment are central to effective tumor theragnostic activity.

5.1. Tumor Microenvironment

As immortal tumor cells proliferate, they acquire an increased demand for nutrients and growth factors. To supply the tumor with what it craves, pro-angiogenic factors are generated in disproportionate amounts resulting in angiogenesis within the tumor microenvironment. However, these newly formed blood vessels are often deformed when compared to those formed in healthy tissues. Angiogenesis within the acidic, oxygen depleted and pressurized tumor environment results in decreased vessel stability.[129] This loss in stability results from an altered function of several systems and often involves: i) Pericytes detaching from capillaries, ii) enhanced vasculature permeability due to the junctions between endothelial cells becoming weak, iii) the basement membrane loses its evenness, becoming thicker in sections and completely lost in others, iv) decrease perfusion, v) increased interstitial pressure, vi) a leaky vasculature (<3%), vii) and altered transcytosis (now considered the dominant nanoparticle uptake mechanism into solid tumors), to name but a few.[128,130] In addition to the altered functionality to the vasculature system, the tumor microenvironment is rich in ROS which create an inhospitable environment which also prevents immune cells from destroying cancerous cells.

Even in the face of harsh conditions, the tumor microenvironment offers numerous different environmental niches which can draw in CDs and prolong their retention so they can impart their therapeutic effects. Conditions facilitating CD active and passive targeting can include: 1) Complimentary transcytosis receptors (i.e., charge or ligand mediated activation). 2) Leaky vasculature. 3) Lowered pH (pH 6.5–7.0). 4) Unique redox conditions. 5) High ROS and inflamed conditions. 6) The presence of cancer specific markers (i.e., folic acid receptor, transferrin receptor, CD44). 7) Cancer specific mitochondrial targeting. 8) Cancer specific immune checkpoint (ICP)-targeting. 9) Nanoparticle retention through low lymphatic drainage.

However, despite the plentiful targets, the high interstitial pressure of tumors, coupled with the propensity of fluid to flow outward from the tumor and its microenvironment creates a substantial barrier for all therapeutics to penetrate deeply into tumorous tissues. The pressures experienced in the tumor environment is equivalent to that found within its neighboring vasculature, resulting in the absence of a pressure gradient. This results in an environment where only diffusion can be permitted. Due to the small size of CDs (when compared to other nanotherapeutics) and their commonly hydrophilic nature, the capacity for diffusion into the tumor microenvironment is enhanced. However, due to the movement of fluid seeping from the tumor in an outward direction, the efficiency of diffusion for all therapeutics (including CDs) entering deep into the tumor is hindered.[131]

The core of the tumorous mass is the true intended target for targeted cancer therapy as this is where the most aggressive sub-clonal growth stems from resulting in increased proliferation, somatic copy number, and Fuhrman grade.[132] To reach the core, additional strategies may be employed to facilitate movement into this region including the functionalization of CDs with "enhanced permeability and retention" agents or triggering CDs with external radiation stimuli (i.e., ultrasound, radiotherapy, photodynamic, or hyperthermia) to physically enhance access (**Figure 4**).[24]

5.2. Combination Therapy

Cancer has a remarkable way of adapting to traditional chemical-based therapeutics resulting in drug resistant cancers which are particularly persistent and difficult to treat.[133] Cancer therapy has traditionally used nonselective cytotoxic agents best exemplified by chemotherapies which have off-target interactions with healthy body systems resulting in debilitating side-effects and reduced therapeutic efficiency. Over the past decade, research focus has shifted to a targeted combinational therapy approach. By simultaneously attacking cancers using multiple approaches, single cancer coping strategies are inadequate to alleviate the damage, resulting in more effective therapeutic endpoints, that is, enhanced cancer cell death with minimal side effects.[134] Carbon dots possess many attributes which make them ideal for combinational therapy, including: 1) Their hydrophilicity can be transferred to sparingly soluble drug molecules through a process called solid dispersion, greatly increasing drug bioavailability. 2) Surface functionalization of CDs with cancer targeting ligands can deliver therapeutic cargo to its site of intended action, preventing off target interactions. 3) Their carbonaceous core can absorb NIR light and convert it to heat energy for photothermal therapy. 4) They can be functionalized with photosensitizers, heat responsive therapeutics, heteroatoms or other entities which can be triggered by external radiation or altering environmental conditions (i.e., light, ultrasound, pH, temperature, etc.). 5) Have their own intrinsic fluorescence which can be used for diagnostic applications. 6) Can act alone or be used to functionalize larger nanoparticle carriers to add functional properties.

The potential of these multi-theragnostic nanoparticles is only recently being realized. With the numerous possible variations of core composition and surface functionalization blended with the capacity for external stimuli enhancements ensure that this research area has plenty of room for novel theragnostic applications. Not only can these particles be used to

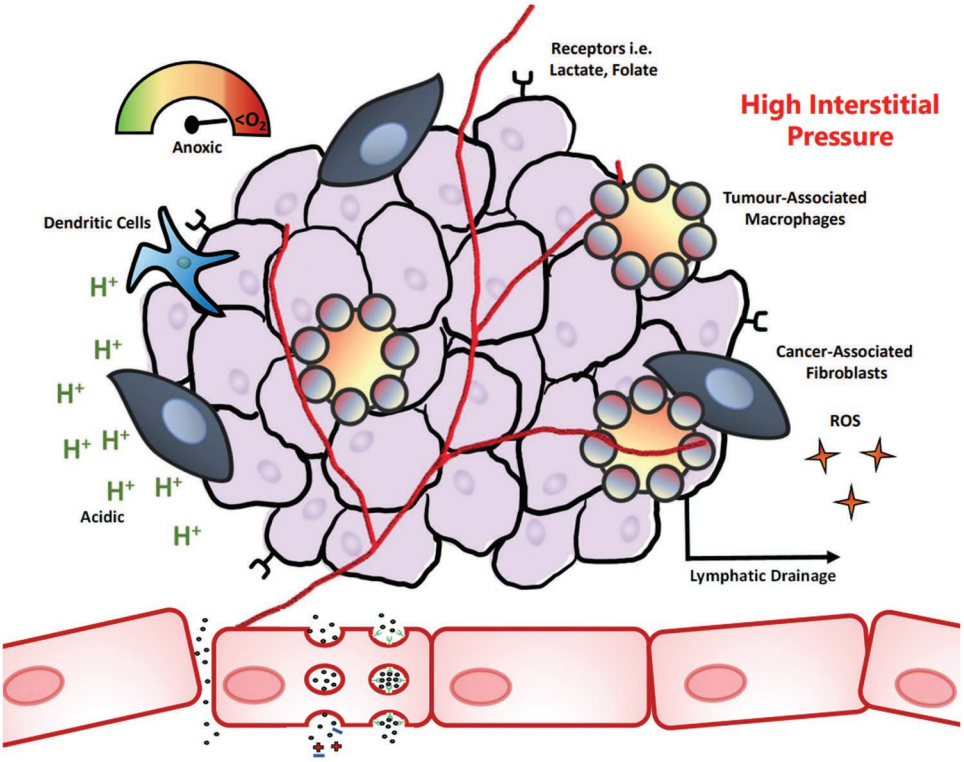

Figure 4. The tumor microenvironment presents numerous niches which can be utilized for tumor targeting (i.e., acidic conditions, cancer specific receptors, and fenestrated endothelium); however, barriers such as, high interstitial fluid pressure and lymphatic drainage prevent diffusion of nanomaterials deep into the tumorous mass.

destroy cancers through photothermal,[11] chemodynamic,[135] and immune[136] based combination therapies, but may also hold promise for the development of healthy bodily systems via regenerative medicinal therapy.

Luo et al. (2021) generated a liposomal carbon dots nanohybrid containing iron doped CDs and used them as tetra-modal contrast agents, gene transfection carriers, and photothermal/chemodynamic therapeutic platforms. These constructs possessed photothermal capacities over 63%, twofold gene transfection efficiencies in animal models, significantly enhanced photoacoustic signal. The synergistic photothermal and chemodynamic therapy resulted in significantly reduced breast tumor growth and 80% survival within female Balb/c mice for more than 50 days.[135]

6. Organ Distribution and Retention of Dots

Once administered, CDs enter the bloodstream and their distribution largely depends on their surface functionalities and their interaction with differing cell types, macromolecules (i.e., proteins, DNA, carbohydrates) or internal organelles. For therapeutic applications, non-specific distribution of treatment results in dilution, cellular uptake discrepancies and may also have severe consequences for non-target cells. Of importance is the preventative accumulation of CDs in non-target organs.

The adsorption of plasma proteins to the surface of CDs (opsonisation) is one of the most influential forces driving their non-target organ retention. CD size, shape, charge, surface chemistry, and hydrophilicity all influence the type of proteins which may have an affinity for specific CDs.[137] Nanoparticles which have adsorbed proteins from the circulatory system are often removed from circulation and processed by the mononuclear phagocyte system (MPS). The proteins on these nanoparticles interact with the surface receptors on phagocytes which results in them being taken up by the cells, transported to the phagosome, and fused with lysosomes for enzymatic degradation.[138] Developing strategies to prevent unwanted opsonisation whilst retaining functionality are therefore of great importance. An alternative approach involves tailoring CDs to bind specific proteins with low affinity. This reduces other protein interactions and once the dots reach their intended destination, the weakly bound proteins can be replaced by stronger interactions of target receptors.

To identify the organ accumulation and/or dominant excretion routes of CDs, the organs of mice are usually harvested at differing time points following intravenous administration and their associated fluorescence is measured. Unlike semiconductor dots where fluorescence output can be crosschecked with metal concentration via ICP-MS analysis, there are limited alternative verification tools for identifying carbon within the body. Although this approach can give semi-quantitative indications for CD movement and accumulation, varying factors which can influence fluorescence may give false positive or false negative readings. Some of these factors include: If CD concentrations are too high, fluorescence output can decrease;

measurement of fluorescence through different biological fluids can result in a masking effects; certain biological entities, that is, digestive enzymes may damage surface ligands and alter their fluorescence output; metallic ions may react with surface ligands and alter their fluorescence; particle aggregation can lead to either quenching or increased fluorescence, and differences in pH can alter fluorescence outputs of some CDs.

A rare study using mass spectroscopy (combined with fluorescence imaging) to track the organ distribution of carbon nanoparticles, including CDs, provided insightful information relating to their retention.[139] It is worth noting that the CDs used in this study were synthesized by the decomposition of carbon soot through refluxing with HNO_3, followed by pH adjustment and subsequent reduction with $NaBH_4$. The study showed that larger carbon nanomaterials, that is, carbon nanotubes and graphene oxide, showed high retention in the lung with 55–43% ID g^{-1} of their injected dose being retained there. Carbon dots however only showed 2% ID g^{-1} of their injected dose in the lung and 5% ID g^{-1} in the spleen. The organ showing the highest retention of CDs was the liver with just under 12% ID g^{-1} retention. The CDs used in this study showed prolonged retention with low quantities remaining in the heart, liver, spleen, lung, and kidney after 30 days. This retention time is seldom reported for fluorescence-based measurements; however, varying surface functionalities will also promote excretion over organ retention. Despite the extended retention time, the CDs in this study showed high biocompatibility with no adverse effects, whereas the carbon nanotubes and graphene oxide brought upon minor abnormalities in renal and liver function.

The fluorescence of harvested organs is by far the most utilized approach for identifying CD organ retention and secretion. A study conducted by Su et al. (2020) demonstrated the capacity for CDs to be tumor targeting whilst showing negligible organ retention.[11] Using CDs hydrothermally synthesized from an anthraquinone dye and citric acid as drug delivery vesicles, targeted tumor delivery and retention was found in hepatic (A549), ovarian (HeLa), and brain (U87 glioma) tumors. Following 8 h of exposure, tumor retention of dots was high whilst concentrations found in the heart, liver, lungs, spleen, and kidneys were considered negligible. This indicated that the CDs had insignificant organ accumulation and efficient body excretion. Interestingly, the organ retention of CDs generated through the thermal pyrolysis of citric acid was also assessed after 8 h in this study and showed significant organ retention in the lung, kidneys, and liver.

The small size of CDs means that there is less chance for them to become lodged in the capillaries and therefore are preferentially removed from the blood circulation via renal routes or processed by the liver (and small amount in the spleen) through the MPS. Therefore, their extended retention of CDs found in the lungs, heart, and brain tissues is infrequently reported unless surface functionalities have strong affinity for these regions, or their size is above 8 nm.

7. Metabolism

CD cores are quite recalcitrant to modification and degradation within bodily systems and are largely thought to be excreted from the body without being metabolized. If CDs are not rapidly excreted or undergo biodegradation within the body, they may accumulate within vital organs and cause chronic health problems. Persistence of CDs within the body is linked not only with their size but also the stability and nature of their functional groups. Therefore, it may not be necessary to completely metabolize the entire CD, but removal or modification of unfavorable surface functionalities (i.e., those which bind to proteins or other macromolecules) may increase clearance through the body. Recently, it has been shown that in the presence of H_2O_2, nitrogen doped CDs generated free radicals which altered their physiochemical properties. The toxicity of these dots was examined and after a month, no indications of toxicity were apparent in mouse models. CDs below 6 nm in size have an increased capacity to be excreted through the body via renal clearance.[140] Therefore, only slight degradation of dots with larger hydrodynamic radii are required to make them less than 6 nm and facilitate bodily excretion via renal avenues.

Recently, it has been shown that enzymes found within the body have the capacity to "eat-away" at the carbon core of CDs and facilitate their biodegradation.[141,142] Martin et al. (2019) hydrothermally synthesized graphene dots with 1,3,6-trinitropyrene and NaOH as the precursors.[141] The resulting dots were subjected to human myeloperoxidase and eosinophil peroxidase enzymes (in the presence of H_2O_2) and enzymatic degradation potential was investigated. Transmission electron microscopy images of dots before and after treatment showed visual "eating-away" of the dots surface and Raman spectroscopy verified a progressive loss of "defect" and "graphitic" regions of the dots indicating dot degradation. In addition, fluorescence intensities of dots subjected to enzymatic treatment dropped to below 30% of that in the positive control following 5 h of incubation. Both H_2O_2 alone and enzyme alone did not result in degradative processes for the dots.

To further support the potential for enzymatic degradation of carbonaceous species within mammalian systems, numerous studies have shown that carbon nanotubes can biodegrade through enzymatic attack. Carbon nanotubes are made from building blocks which have a similar make up to CDs and therefore, enzymatic reactivity between them and CDs is likely to proceed via similar mechanisms. Kagan et al. (2010) showed that carbon nanotubes can be degraded by neutrophil myeloperoxidase in mice.[143] In acidic conditions, hypochlorite, reactive free radical intermediates, and free radicals associated with myeloperoxidase are employed to break down the CNT structure.

Carbon nanotubes with functional groups often present on CDs (carboxyl groups) and π–π stacking capabilities were capable of binding to HSA proteins and facilitate the production of reactive hypochlorite ions via the human neutrophil enzyme, myeloperoxidase.[144] The generated conditions were favorable for the degradation of carbon nanotubes; however, to date similar macromolecular interactions and subsequent enzymatic reactions have not been investigated in CDs (**Figure 5**).

8. Excretion of Dots

Excretion is a process which decreases toxicity and damage by removing compounds from the body's circulation, helping to

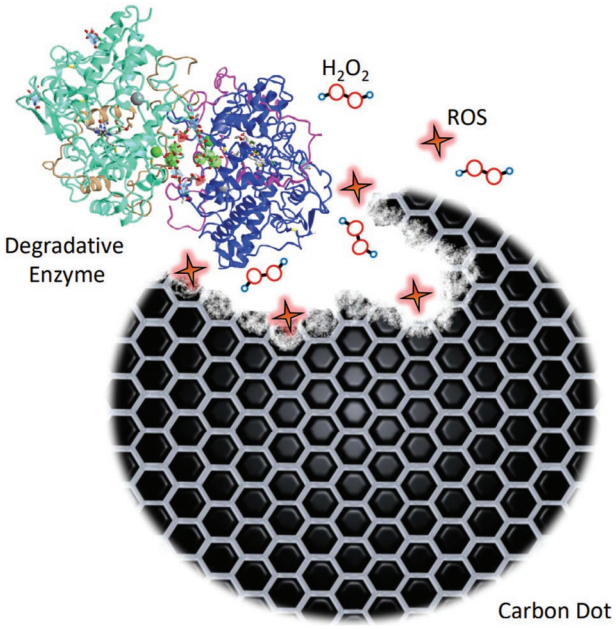

Figure 5. Carbon dots which are not rapidly excreted may have their structure degraded by degradative enzymes (i.e., human myeloperoxidase and eosinophil peroxidase) in the presence of hydrogen peroxide. Once their core has been degraded, they may be reduced in size and be excreted through renal routes.

regulate bodily tissue and fluid composition. Renal (urine) and hepatic (bile to faeces) pathways are the two dominant excretion routes used for eliminating nanoparticles including CDs. Minor pathways include excretion through the breast milk, tears, saliva, sweat, and lungs. Renal excretion is generally a rapid process with particles being removed from the body following intravenous administration within hours to days. In contrast, particles which are processed via the hepatic pathway can remain in the body for extended periods of time (hours to months). If particles do not degrade and are unable to be processed by renal or hepatic routes, they may be processed by the MPS generating immune responses and be present within the body for months to years.[145] Extended bodily retention may result in inflammatory stress responses and chronic toxicity. The effectiveness of a nanotherapeutic is irrelevant if it cannot be removed from the body following application and therefore, necessary excretion is a prerequisite for commercially viable nanotherapeutics.

8.1. Renal Excretion

The kidneys can be thought of as pressurized molecular sieves which maintain homeostasis. These organs filter the blood and selectively maintain optimal levels of beneficial macromolecules (glucose, electrolytes, low molecular weight proteins, etc.) whilst excreting metabolic waste products. Excretion of CDs through the kidney is strongly dependent on their size and charge.[146] Within this system, renal arterial blood gets transported through the afferent arteriole and into glomerulus capillaries. Blood constituents are then filtered through the glomerular filtration barrier where molecular size and charge have a significant influence on their capacity to pass and enter the bowman's space for further processing within the nephron's lumen.[147]

The glomerular filtration assembly has three main cellular barriers which facilitate the filtration process including the fenestrated endothelium (FE), glomerular basement membrane (GBM) and epithelial podocytes (EP).[148] The FE is composed of endothelial cells with slits between them of ≈70–90 nm which prevents the movement of large particles.[146] In addition, this barrier is also negatively charged due to the presence of the glycocalyx (made up of sulphated proteoglycan, glycoproteins, and glycosaminoglycans) which can limit the passage of particles with strong negative charge through electrostatic repulsive forces. Once through the FE, CDs must pass through the GBM composed of the lamina rara interna, lamina densa, and the lamina rara externa filtration matrices which facilitate the movement of particles based on charge and size (2–8 nm).[149] The external layer of the glomerular filtration assembly is made up of specialized epithelial cells called "podocytes." These podocytes have cytoplasmic projections which branch out and interlock forming a matrix with 4–14 nm pore size. Through the numerous different barriers that must be passed, dots possessing hydrodynamic diameters below 6 nm can readily filter through this assembly whilst particles above 8 nm often continue to circulate within the blood and often require processing through the liver[26] (**Figure 6**).

Rapid renal excretion of CDs below 6 nm has been reported by numerous researchers including Bao et al. (2018) who generated CDs to be used for photothermal therapy and as photoacoustic imaging agents.[7] Citric acid and urea were dissolved in DMSO, subjected to solvothermal conditions and the resulting dots were sized between 2 and 5 nm in diameter and possessed a zeta potential of −20.1 mV. Following intravenous administration, dots accumulated in tumor tissue along with the kidneys and liver. The liver was cleared of a fluorescence signature within 3 h post intravenous administration whereas the kidneys possessed fluorescence output up to 24 h. Urine was also collected at numerous time points and was assessed for CD related fluorescence. Time points between 30 min–1 h possessed the greatest fluorescence outputs, indicating the highest excreted concentration. After 1 h, fluorescence intensity decreased and disappeared within 24 h. It was concluded that the dots were rapidly excreted within the body and showed excellent biocompatibility with negligible biotoxicity.

Size may be a major determining factor for CD excretion route; however, particles charge also mediates elimination efficiency. During renal processing, the bodies negatively charged sulphated proteoglycan, glycoproteins, and glycosaminoglycans slow the excretion of negatively charged particles through the glomerular filtration membrane due to electrostatic repulsion. Numerous studies have assessed cellular uptake capacity of differently charged CDs;[151–153] however, limited studies have reported on their charge-related excretion capacity. In stating this, both molecular and semiconductor quantum dot nanoparticle indicators support the well adopted concept that strongly negatively charged entities are more slowly excreted through renal routes when compared to neutral ones, which are more slowly excreted when compared to positively charged ones.[151]

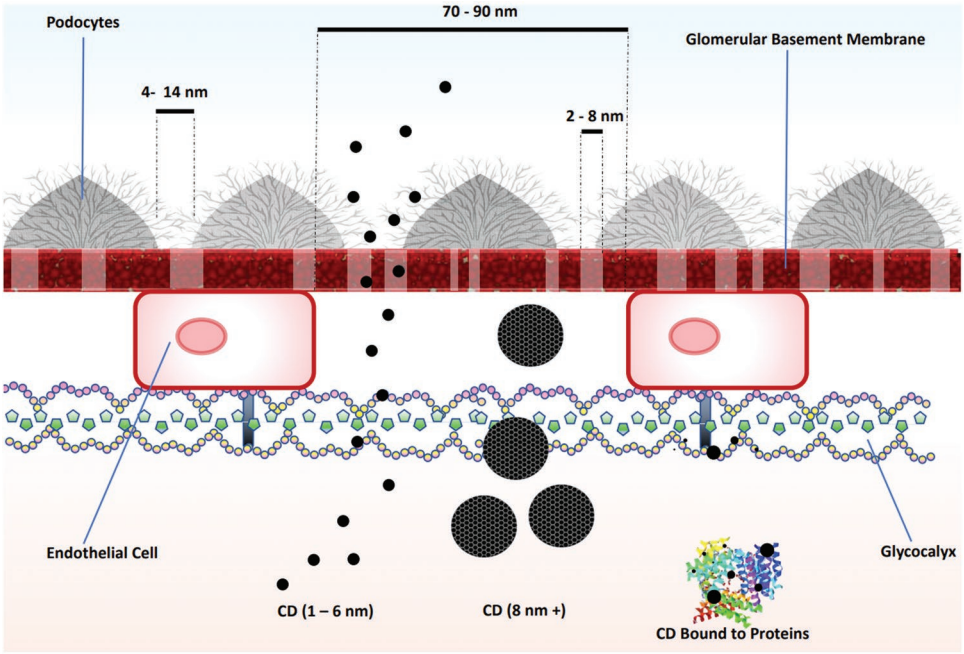

Figure 6. Renal excretion: Only dots with sizes <6 nm have a favorable pass through the numerous size and charge exclusion barriers in the glomerular filtration assembly (Redrawn from ref. [149,150]). However, size alone does not ensure unhindered passage to the Bowmans space as the strongly negatively charged glycocalyx may repel strongly negatively charged CDs. CDs bound to proteins or possessing strong hydrophobicity will also not pass through this molecular sieve.

Liang et al. (2016) synthesized mercaptosuccinic acid-capped quantum dots (3.7 nm) possessing a negative charge (−52 mV) and polyethylenimine-conjugated CDs (5.7 nm) with a positive charge of (23.4 mV). The study reported that the particles were not significantly bound to proteins (BSA and FBS in vitro) and therefore, particle charge could be related to excretion capacity without biomolecule complexing interferences. The study showed that positively charged particles were rapidly excreted into the urine of BALB/c mice following intravenous administration; however, negatively charged CDs were not detected in urine and accumulated in the liver.[154]

Many CDs-based therapies are frequently reported to possess high biocompatibility and rapid renal excretion; however, their short residence time in target tissues may be insufficient for effective therapeutic benefit. Therefore, modifications where surface functionalities can have stronger interaction with their targets, or encasing dots in larger biodegradable nanoparticles may be areas for future focus and optimization.

8.2. Hepatic Excretion/Clearance

CDs which are not rapidly excreted by the renal filtration enter the liver through the hepatic artery or the portal vein and are carried along with the blood flow to infiltrate the hepatic sinusoids.[44] These unique microvascular structures are lined up by liver sinusoidal endothelial cells (LSEC) forming a porous barrier between blood and hepatocytes. The average 100–120 nm diameter of human LSEC fenestrae allows the passage of small molecules including nanoparticles while capturing majority of infectious microorganisms.[155] The CDs interacting with hepatocytes can be transported back to the bloodstream and follow excretion into bile and eventually faeces.[156,157] Limited investigation into CDs processing mechanisms in the liver are present in the literature; however CDs retention has been demonstrated.[158,159] It is therefore hypothesized that CDs will be processed by the liver in a similar manner to “hard” nanoparticles of similar size which have been capped with carbonaceous coatings.[160]

Size is a dominant factor influencing the accumulation of CDs in the liver, as demonstrated by Nurunnabi et al. (2013)[161] where GQDs of 5, 7, 12, and 21 nm diameter showed gradually increased retention with increasing size. Nanomaterials with a 10 nm diameter have shown a twofold increase in retention in the hepatic sinusoid when compared to nanoparticles over 90 nm in size.[160] Furthermore, ultra-small nanoparticles show preferential processing in the hepatic sinusoid over other extra-hepatic regions.

Ultra-small nanomaterials entering the livers bloodstream interact with immune cells and their uptake is dependent on charge, size, surface functionality, and shape along with the cell's endocytic uptake disposition.[162] Those which are not removed by immune cells continue to pass through the hepatic sinusoid where blood velocity dramatically slows, allowing them to have longer interactions with numerous cell types (Kupffer cells, LESC, hepatocytes, etc.).

Kupffer cells are resident hepatic macrophages that demonstrate high phagocytic activity eliminating noxious substances from the hepatic circulatory system including nanoparticle below 10 nm in size.[160,163,164] These cells present dual mechanism of action with endocytosis and pinocytosis of dots on one

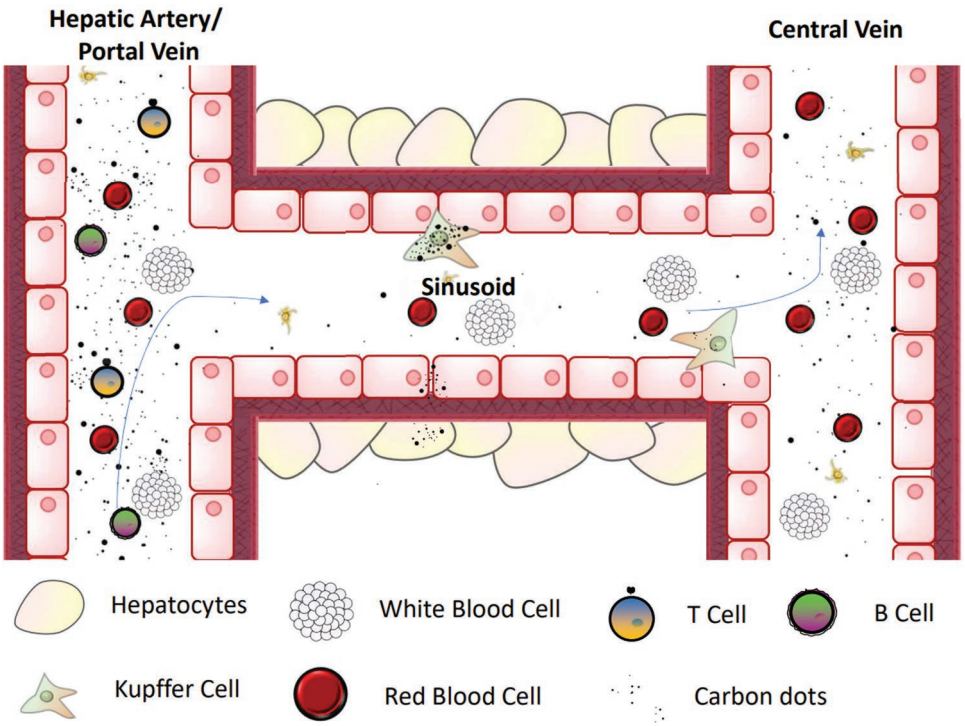

Figure 7. Within the liver, injected CDs travel through the bloodstream and interact with B and T lymphocytes. Those which are not removed enter the hepatic sinusoid where blood velocity decreases allowing increased interactions between CDs and numerous cell types (Kupffer cells, LESC, sinusoidal endothelial cells, hepatocytes, etc.) responsible for particle processing and excretion. Particles which escape this processing exit the liver through the hepatic veins and re-enter the circulatory system via inferior vena cava (Redrawn from ref. [160]).

side and cytokines secretion to illicit an immune response on the other hand.[162] Those CDs which have not been captured and processed by Kupfer cells, LESC or hepatocytes, exit the liver through the hepatic veins and re-enter the circulatory system via inferior vena cava (**Figure 7**).

Whilst nanoparticles with strong negative charges have increased propensity to be processed by the liver over the kidneys, particle-protein interactions are greatly facilitated by surface charge and electrostatic attraction with highly charged particles (either positive or negative).[165] Carbon dots which bind proteins typically form complexes above the filtration barrier threshold size (≈6 nm) and are processed by the liver and spleen.[166]

Hydrophilic entities, if not toxic, do not often need modification to be excreted by the body; however, hydrophobic entities require alterations to possess a more hydrophilic nature for excretion.[167] If dots are not hydrophilic, they may undergo direct oxidation, reduction, and hydrolysis by hepatic microsomal enzymes to modify their surface chemistry. This processing primes hydrophobic drugs (and likely hydrophobic ligands attached to dots) and facilitates covalent bonding between polar biomolecules (amino acids, glucuronic acid, glutathione, acetate, and sulphate) to non-polar objects and increases their solubility so they can be processed for renal or hepatic excretion.

Excretion by the liver may be an extremely slow process resulting in unwanted accumulation and long-term toxicity. However, the aforementioned evidence for CD enzymatic degradation can result in a reduction in core size which may favor renal excretion.[156] It is recommended that caution should be practiced when using CDs in animals with liver dysfunction or in parallel with antifungal or chronic corticosteroids treatments.

9. Toxicity of Carbon Dots

The core structures of most CDs are considered to be highly biocompatible across the range of graphitic, amorphous, and polymeric varieties. Under physiological conditions, the core of CDs do not ionize into toxic species, have low to negligible undesirable reactivity, can biodegrade/be subject to enzymatic breakdown (especially evident with polymeric CDs), show low levels of bioaccumulation (particularly when below 6 nm in diameter) and rarely generate prolonged or significant inflammatory responses. The functionalization of any nanoparticle construct with chemical structures possessing known toxicity should always be carefully considered. However, attachment of toxic ligands (including therapeutics with toxic side effects) to CD supports has, in some cases, shown increased bioavailability and reduced toxicity of these compounds.[168] Despite the overwhelming evidence for CDs possessing high biocompatibility,[11,169,170] deviations to this observation have been documented. Dominant factors which have been shown to bring upon undesirable bodily responses include i) multiple-dosing regimens, ii) activation by extended light irradiation, iii) HD sizes above 6 nm, iv) high positive charge, and v) concentrated localized charge densities.

9.1. Carbon Dot Dosage

As with any drug or nanotherapeutic, toxicity is usually dose dependent. Owing to the numerous possible variations in CD physiochemical properties, the potential for uncommon adverse interactions is also possible. Toxicity is therefore CD specific. Carbon dots generally show little toxicity below 200 μg mL^{-1} when considering a cut-off of 80% cell viability over 24 h.[151,171–173] Most reported studies have determined toxicity based on data from single administration dosages. However, in clinical settings, multiple subsequent doses may be required to achieve a desired therapeutic endpoint. Hong et al. (2018),[174] intraperitoneally administered CDs to mice every 2 days for 30 days and used ^{1}H NMR-based metabolomics to identify toxicity. After 30 days, 15 dosages of 24 μg mL^{-1} had been given to the mice and results highlighted that the immune system had been triggered, cell membrane function had been impacted and liver clearance function had been altered. These observations were not witnessed with biochemical analysis and histopathology alone indicating that ^{1}H NMR-based metabolomics provided deeper insight to CD toxicity over time.

Another relevant study by Zheng et al. (2015)[175] synthesized carbon dots with citric acid and two differing amine precursors, ethylenediamine (CD) and *N*-ethylethane-1,2-diamine (Et-IPCA). Repeated dose toxicity testing of these dots into ICR mice were performed with daily doses of 100 mg kg^{-1} for CD and 25 mg kg^{-1} for Et-IPCA respectively over a 1-week period. Percentage differences in organ coefficients, elevated white blood cell counts and decreased red blood cell counts suggest that acute inflammatory responses were produced following treatment. However, after a 90-days recovery period, blood chemistry, haematological parameters, body weight, organ coefficients, and organ histopathology exhibited negligible prolonged toxicity.

9.2. Light Irradiation

Carbon dots have great potential for use as NIR fluorescent diagnostic tools and also for tools in photothermal/photodynamic therapy. Both of these diagnostic and therapeutic applications require the particles to be irradiated with light to exert their function. Recently it has been discovered that CDs can photodegrade or produce ROS in the presence of laser light irradiation.[176,177] The generation of ROS can be utilized for effective cancer therapeutic applications; however, the photodegradation of carbon dots core results in the formation of toxic daughter molecular structures. Liu et al. (2021) demonstrated this conclusion using CDs made through microwave pyrolysis of polyethylene glycol (PEG) and glucose (and also through commercially sourced carbon dots, Sigma-Aldrich). These carbon dots showed significant photodegradation (28.6–59.8%) following 8 days of laser light irradiation under optimized conditions and were broken down into 1431 different species, 499 of which were identified as potentially cytotoxic. Photodegradation of the carbon core structure resulted in the generation of electrons and holes in the dots structure leading to the generation of hydroxyl and alkyl radicals. These radicals could then attack the dots structure and break the core into polyaromatic hydrocarbon fragments of varying molecular weight and structural composition. Although the generation of toxic species were confirmed, the conditions that led to this are not overly relevant for biomedical applications. Within this study, light irradiation of <420 nm showed the highest incidence of photodegradation; however, these wavelengths have low skin penetrability and are therefore not suitable for theragnostic applications. Most photothermal therapeutic studies utilizing CDs in rodents show laser treatment times of between 5 and 20 min.[169,177] With irradiation times of this duration, light exposure periods within this study exceeding 12 h are not likely to be relative for therapeutic applications.

9.3. Size

The size of CDs has a strong correlation with how they are processed and secreted by the body. As previously described, nanoparticles >6 nm struggle to pass the kidneys and be renally excreted. This results in extended bodily circulation and increases the interactions with different bodily systems and biomolecules. Carbon dots which possess a HD <6 nm and can be excreted through renal filtration often show negligible toxicity. Particles above 8 nm have an increased propensity to be processed by the MPS and although this is not necessarily adverse, extended retention may result in the triggering of the immune system, result in enzymatic attack, or lead to unfavorable interactions with other biological systems or biomolecules.

9.4. Charge and Surface Chemistry

Particle charge and charge density plays a dominant role in the affinity a CD has for differing biological processes.[178] Carbon dots with a neutral charge have been shown to be less toxic than those with a negative charge which in turn are less toxic than those with a positive charge.[151,173] Strong positive charges tend to bind to negatively charged biological entities (i.e., nucleic acids, negatively charged proteins,[179] and have a greater chance to become opsonized, which decreases their renal filtration capacity and increases bodily retention.

Havrdova et al. (2016)[173] compared what effects positively charged polyethyleneimine CDs, negatively charged CDs made from candle soot and neutrally charged PEG CDs induced on mouse fibroblast cell morphology, intracellular trafficking, and cell cycle function. Neutrally charged CDs showed negligible toxicity whereas negatively charged CDs induced morphological changes at high doses >200 μg mL^{-1} and cell cycle abnormalities were obvious at 350 μg mL^{-1} dosage. Positively charged CDs were considered to be the most toxic with migration into the nucleus apparent; morphological changes were realized at 50 μg mL^{-1}, and cell cycle abnormalities were obvious at 50 and 100 μg mL^{-1} dosage concentrations. Instilling positive charges to CDs can be useful for certain biomedical applications and should not be disregarded as a therapeutic tool despite a relationship between positively charged CDs and increased toxicity. Carbon dots possessing positive charges have been shown to demonstrate promising results for nucleic acid delivery and gene therapy applications.[180]

Overall particle charge is often used assess a particles interaction with certain biomolecules. Although this is a valuable tool for predicting such interactions, it does not consider charged functional groups in a particle showing an overall opposite charge. Weiss et al. (2021)[178] investigated the toxicity of cationic ligand density on carbon nanoparticles as opposed to overall particle charge. Results showed that highly localized regions of cationic charge (not overall zeta potential) brought upon oxidative stress, mitochondrial dysfunction, IL-8 release, and reduced integrity of lysosomes. Although these carbon particles were outside of the quantum size range (>10 nm), the study highlights that charge density is a greater determining factor for biological interactions and toxicity when compared to overall zeta potential.

To reinforce that overall charge is not sufficient to predict a particles interaction capacity, a study conducted by Xu et al. (2018)[181] showed that aminated CDs with net negative charge could still interact with and cross-link negatively charged DNA. These particles were synthesized to possess sizes <10 nm; however, in culture medium the particles agglomerated into clusters with HD >100 nm which was likely responsible for extended bodily retention.

10. Carbon Dots in Larger Nanoparticle Carriers

Although rapid uptake and localization of CDs in tumor cells has been reported, due to their small size, their rapid circulation through the body coupled with efficient renal excretion capacity often results in reduced interactions with cancers. Specific functionalization strategies have led to increased retention times in specific tissues resulting in sufficient tumor destruction. However, alternative strategies have been adopted to blend optimized cellular uptake of dots, increase their retention time, and also allow for renal excretion. Of these approaches, the "ultra-small in nano" is an area of research focus which holds great potential for targeted theragnostic applications.[26] Ultra-small in nano refers to the encapsulation of ultra-small nanoparticles (i.e., CDs) within larger nanoparticles. Liposomes, lipid microspheres, iron nanoparticles, polymeric micelle nanoparticle, and silica nanoparticles are all examples of FDA approved nanoparticles and have the potential to house ultra-small CDs. Housing CDs into larger nanoparticle carriers may facilitate enhanced endocytic cellular uptake or additional functionalization possibilities.[182]

Nanoparticles with a diameter below 30 nm do not possess sufficient enthalpy to drive membrane wrapping and cellular internalization efficiently, whereas particles exceeding 60 nm do not interact with enough cellular receptors to encourage optimal membrane wrapping and cellular internalization.[41] Therefore, encapsulating CDs into nanoparticles between 30 and 60 nm may increase endocytic uptake whilst retaining CD functionality. In addition, due to CDs ultra-small size, the functionalization of these particles with larger entities (i.e., antibodies, camelids, nanozymes, aptamers, peptides, DNA, RNA) is not possible. In fact, it is rather that these molecules are being functionalized with the CDs due to their smaller size. Encapsulating CDs into larger nanoparticles may increase their theragnostic potential as functional biomolecules with known affinity for a target can be utilized. Carbon dots have many properties which could bring upon added functionality to existing nano-platforms including fluorescence, photothermal capacity, sonodynamic capacity, transferable hydrophilicity, drug carrying, and shuttling potential, etc.

One such example highlighting the benefit of an "ultra-small in nano" approach was demonstrated by Ren et al. (2019). Within this study, hydrophilic NIR-fluorescent CDs were used as a drug carrier for the hydrophobic cancer drug—cinobufagin and were encapsulated into liposomes. This resulted in a construct with trackable tumor targeting drug delivery capacity. Furthermore, the construct possessed enhanced cellular uptake and improved anti-cancer activity when compared to cinobufagin alone (**Figure 8**).[183]

11. Conclusions and Future Perspectives

When deciding on which carbon core a CD should possess, the application in mind must first be identified. For example, if photothermal applications are of interest, a solid core capable of retaining heat is beneficial. However, microenvironments with varying pH or temperature may facilitate drug release from polymeric CDs. In addition, amorphous carbon dots may have increased solubility but less π–π stacking capacity to attach drugs whilst crystalline dots show high photothermal conversion efficiency and high drug conjugation via π–π stacking but are less susceptible to biodegradation. In any case, if particles are able to be broken down into non-toxic precursors or directly excreted, their persistence and accumulation should remain low, resulting in more biocompatible constructs. Although excretion and biodegradability are desirable, particles still need to bind specifically to the target site for long enough to exert their therapeutic function. With particles below 6 nm, rapid renal excretion

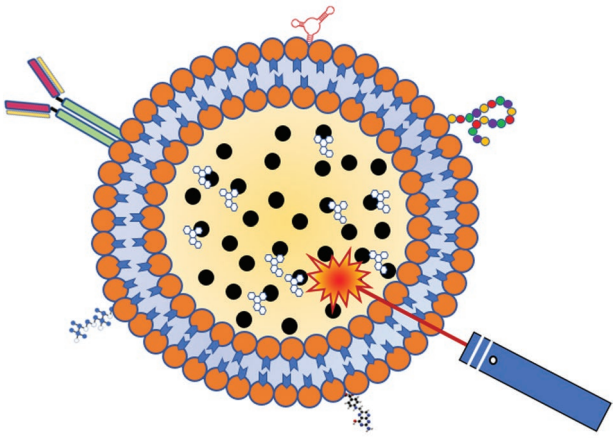

Figure 8. Ultra-small in nano: Ultra-small CDs may be housed within larger nanoparticle carriers (i.e., liposomes, polymeric nanoparticles, lipid nanoparticles, metal oxide nanoparticles, mesoporous silicon nanoparticles, or exosomes) which can accommodate an increased range in surface functionalities. These functionalities may include antibodies, peptides/proteins, aptamers, nanozymes, camelids, polymeric ligands, and chemical functional groups. External triggers may also be applied to the parent nanoparticle carrier to exert added therapeutic benefit.

may pose a problem and therefore, the design of ligands with strong target specificity and affinity is desirable.

Particle charge is a feature which should be factored in when designing CDs. If the desired application is to bind negatively charged biomolecules (i.e., nucleic acids, negatively charged proteins), then a positive charge may be necessary. However, strongly positively charged species have also shown increased toxicity due to the binding and disruption of mechanistic machinery of certain biomolecules. Zwitterionic, neutrally charged or negatively charged particles may all be suited to different applications and are generally considered to be highly biocompatible. With saying this, attaching negatively charged ligands with known toxicity to CDs, is likely to impart some toxicity to the structure, even though toxic drugs have shown increased biocompatibility when conjugated to CD particles.

Due to their very small size, the triggering of cellular surface receptors to initiate endocytosis in not energetically favorable for individual CD particles. Therefore, for active uptake, CDs need to be administered at concentrations which allow numerous particles to reach the cell at the same time and trigger numerous receptors simultaneously. Functionalization of particles with ligands known to trigger cellular uptake responses is likely beneficial, although charge and hydrophobicity can also facilitate such mechanisms.

The "perfect" carbon dot for biomedical applications should: 1) Be hydrophilic. 2) Fluoresce in the NIR region. 3) Facilitate drug transport across biological barriers. 4) Have a hydrodynamic diameter below 6 nm in size. 5) Have photothermal conversion efficiencies above 50%. 6) Be triggered by external radiation to impart additional functional properties. 7) Have minimal off-site interactions and high specificity to target site of interest. 8) Have high cellular uptake rates (if desired). 9) Be non-toxic and either biodegradable or readily excreted.

Various carbonaceous nanoparticle classes (i.e., carbon nanotubes, fullerenes, carbon nanoparticles above 10 nm in size, lipid, and polymeric nanoparticles) have shown significant potential for in vivo therapeutic applications. Despite this, only the biodegradable lipid and polymeric variants have been granted regulatory approval by the FDA/TGA. Carbon dots are relatively new to the biomedical landscape but possess multiple functional properties giving them numerous potential applications. When compared to carbon-based nanoparticles such as, graphene oxide and carbon nanotubes, their increased hydrophilicity and renal clearable sizes likely increase their bioavailability and in vivo suitability. Despite the numerous beneficial attributes of CDs, improvements can always be made to increase their suitability and potential to become marketable products.

Unlike chemical therapeutics which possess identical chemical structure compositions, the arrangements of individual CDs are similar but seldom identical. Bulk CD material made up of many slightly varying alterations of similar material, synthesized from the same precursors, under the same conditions. Although the ability to have each particle identical to the next is impossible, future research on particle homogeneity and standardized purification approaches is necessary. In addition, product yields are often low (below 30%) when synthesizing carbon dots using bottom–up approaches. Providing conditions which results in optimum particle nucleation, growth and homogeneity whilst maintaining ideal physiochemical properties needs further refinement and potentially novel methodologies.

The fluorescence of carbon dots usually resides between blue and green wavelengths of light. Although valuable for cellular uptake investigations, these wavelengths have limited tissue penetration depth and are therefore not useful for in vivo diagnostic applications. Recent research has gained traction with producing CDs which can be excited and emit in the NIR window for diagnostic and photoactivated applications. Increasing particle hydrophobicity, incorporating heteroatoms or NIR fluorescent molecules into the core structure may result in shifts in fluorescence to the NIR region.

Investigating the excretion and bodily accumulation is mainly identified via fluorescence but fluorescence intensity can be influenced by numerous factors (quenching upon interactions with biomolecules and various ionic species, tissue density, ligand degradation, etc.). The influence of these biological and chemical factors on CD fluorescence is often un-reported in the literature and can result in misleading interpretations. Designing particles to have specific, quantifiable, and expected fluorescence outputs without unwanted interference from external factors is important. Incorporating additional contrast agents (gadolinium, ytterbium, tantalum, tungsten, gold, iodine, and bismuth) into carbon dots structure may be used to further validate outputs from fluorescence measurements.

Carbon dots possessing optimum traits for biocompatibility often also suffer from short blood circulation times. This is one major limitation preventing their sole use as anti-cancer therapeutic agents. However, when combined with larger nanoparticle carriers or functionalized with ligands with strong affinity for cancers, effective site-specific retention can be realized. The role CD additives play with drug solubilization/dispersion, biocompatibility, site specific uptake, and bodily retention requires additional research focus. In addition, the targeting of non-cancerous sites within the body with CDs has little representation in the literature and may benefit from further investigation.

Optimizing cellular affinity and/or uptake strategies is important for any cell-based therapy. Individual carbon dots are too small to satisfy the enthalpy and entropy requirements for efficient receptor mediated cellular uptake. In addition, the strong hydrophilicity experienced in many CDs is not ideal for passive uptake mechanisms. Therefore, carbon dots often enter cells as "bulk material" as opposed to being passaged across the membrane alone. Strategies to facilitate particle uptake in these types of systems (i.e., co-administration with cell uptake facilitators, modifying CD physiochemical properties, encapsulating CDs into larger nanoparticle carriers) may prove beneficial for optimizing particle uptake and therapeutic effectiveness.

The administration of CDs in vivo is predominantly performed through injectable routes; however, studies are starting to emerge showing the potential for administration via less obtrusive approaches. Future development of CD containing formulations which can be taken through oral, suppository, ocular, intranasal instillation, or aerosol routes, will increase user friendliness and application potential.

Despite the aforementioned limitations and scope for future research, CDs offer promising attributes for numerous

biomedical applications. These biocompatible ultra-small entities are constructed from either aromatic, aliphatic or polymeric carbon (or a mixture of these) which facilitates numerous possible functionalization strategies. Their small size allows them to discretely interact with larger nanoparticle carriers or biological entities and can also facilitates drug transport across the blood brain barrier. Their multifunctional physiochemical characteristics (solid dispersion for increasing drug solubility, externally triggered photothermal and sonodynamic properties, tuneable fluorescence into the NIR range, pro-oxidant, or antioxidant properties, etc.) open exciting potential for numerous biomedical applications.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

ADME, cancer, carbon dots, metabolism, pharmacokinetics, toxicity

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