New hybrid functional materials based on carbon: synthesis, characterization and study of properties
Zygouri, Panagiota

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Chapter 5

Hydrophilic oxidized carbon nanodiscs: A promising multifunctional material for bioapplications

Oxidation of industrially prepared carbon nanodiscs using a simple, versatile, and reproducible approach based on the Staudenmaier method yields a new hydrophilic form of nanocarbon. As a result of the strong acid treatment, graphene planes detach from the discs while the surface of the carbon nanodiscs becomes decorated with various oxygen-containing functional polar groups. Thus, the completely insoluble carbon nanodiscs convert to a hydrophilic derivative dispersible in many polar solvents including water. The new carbon structure is expected to have a wide range of applications in several fields including bioapplications. As test applications, these nanostructures were investigated whether these functionalized carbon nanodiscs can act as cytotoxic agent and as support for the development of nanobiocatalytic systems.

5.1 Introduction

There is no doubt that among the series of nanomaterials that have been synthesized or isolated the last decades carbon nanostructured materials occupy the most prominent position. This has to do with the intriguing capability of carbon to form many allotropes with bonds based on sp$^3$, sp$^2$ and sp hybridization. Carbon nanostructures include various low-dimensional forms

such as the 0D fullerenes, the 1D carbon nanotubes and 2D. The importance of their discovery is mirrored by the two Nobel prize awarded the last two decades (Kroto, Smalley, Curl in 1996 for the discovery of fullerenes and Novoselov and Geim in 2010 for the discovery of graphene). Due to their structure, size, low density, high specific surface, tunable pore structure, chemical stability and excellent electronic, thermal and mechanical properties carbon nanostructured materials have been considered promising candidates for a wide range of applications including organic electronics photovoltaics, biological and medical applications, catalyst supports, field emission devices, nanopores, sensors, semiconductor devices, composite materials (polymeric or ceramic), nanoelectronics, gas separations, supercapacitors, and energy storage materials.\[1\]

Carbon nanodiscs (CNDs) represent an interesting alternative to bulk graphite; they are produced through the so-called pyrolytic Kværner Carbon Black & H\(_2\) (CB&H) process,\[2\] which decomposes a continuous flow of hydrocarbon (typically heavy oil) into carbon and H\(_2\) with the help of an industrial-scale carbon-arc plasma torch generator operating at a temperature around 2000 °C. CB&H provides an unusual carbon product consisting of different turbostratic graphitic microstructures which may also exhibit disclination defects in their hexagonal network, resulting from the presence of pentagons in the specific seed from which they grew. Flat CNDs (no pentagons) are predominantly present in this mixture but also conical carbon structures (1-5 pentagons) and amorphous carbon (soot) occur, with volume fractions around 82%, 5% and 13%, respectively.\[3\] CNDs may be isolated by subjecting the crude material to liquid phase oxidation because soot and conical structures are preferentially oxidized.\[3b\]

CNDs are ultra-thin, quasi two-dimensional particles with diameter 1-4 μm, and the co-existing carbon cones are of similar size. A combined electron diffraction and electron microscopy study has indicated that they are both multilayer structures with a graphitic core and outer non-crystalline layers.\[4\]
More specifically it has been proposed that CNDs may start to form during the pyrolytic process through the growth of a thin planar graphite crystal with regular facets, which is then progressively encapsulated by additional non-crystalline carbon layers. Although a different number of carbon layers may be deposited depending on the local growth conditions, each CND is considered to have homogeneous thickness, typically in the range 10–30 nm.\textsuperscript{[4]} The degree of graphitization can be greatly promoted by post heat-treatment at 2700 °C under argon.\textsuperscript{[5]} Indeed AFM and XRD analysis of thermal treated nanodiscs and cones has shown that the annealing under such conditions leads to high structural organization and few surface defects, without affecting the geometry (and the thickness) of the particles. Annealed CNDs and cones have appeared to be almost single crystalline in the c-direction and comprise a limited number of stacked graphene layers (usually < 100). CNDs may thus be considered the next thicker structure following few layer graphene.

The particular size of these disc-like carbon nanoflakes is crucial for maintaining important properties such as electrical conductivity and on the other hand, small enough and even ideal in some cases, for application in bionanotechnology, nanomedicine and drug delivery, micromanipulation, nanochemistry etc. Like all other carbon structures, CNDs are insoluble in polar solvents and makes them difficult to process. An additional drawback in various applications is that they are immiscible with most media because of their great tendency to establish strong van der Waals and π-π interactions. A solution to this problem is the chemical modification of their surface which improves their dispersibility in organic solvents and water and makes them more compatible with other materials, facilitating the preparation of composites. Towards this aim, here we report a simple, versatile, and reproducible approach for the chemical oxidation of CNDs, based on the well-known Staudenmaier method\textsuperscript{[6]} that has been applied with great success for the chemical oxidation of graphite.\textsuperscript{[7]} As a result of the strong acid treatment, which also enables the separation of CNDs from the mixed starting material,
graphene planes detach from the discs, while the surface of the CNDs becomes decorated with various oxygen-containing functional polar groups such as hydroxyl, carboxyl and epoxy groups. Consequently the completely insoluble CNDs convert to a hydrophilic derivative dispersible in many polar solvents including water and are expected to have a wide range of diverse applications in several fields such as chemical industry, pharmaceutics, electronics, etc. Here we focus on representative case studies addressing cutting edge processes of great importance in biomedicine and biocatalysis such as the use of these nanostructures as efficient drug delivery systems and supports for the development of nanobiocatalytic systems. A variety of analytic techniques was used to fully characterize the produced nanocarbons, namely Fourier transform infrared (FTIR), Micro-Raman and X-ray photoelectron (XPS) spectroscopy, X-ray diffraction (XRD), thermal analysis (differential thermal (DTA) and thermogravimetric analysis (TGA)), as well as atomic force (AFM), transmission electron (TEM) and scanning electron (SEM) microscopy.

5.2 Results and Discussion

The strong oxidation of carbon nanodiscs is expected to generate a derivative similar to graphene oxide with oxygen-containing groups covalently attached to its layers. In detail, oxidized carbon nanodiscs are expected to exhibit a lamellar structure with randomly distributed unoxidized aromatic regions (sp²-carbon atoms), six-membered aliphatic regions (sp³-carbon atoms) as a result of oxidation, and a high concentration of oxygen-containing functional groups, like hydroxyl, epoxy, and carboxyl, grafted to layers and edges. A first indication for the successful oxidation arises from the exceptional solubility behaviour in water of the functionalised nanodiscs (Figure 1a, b). Further proof for the successful solubilization of the oxidized carbon nanodiscs is the Tyndall scattering effect for a clear oxCND aqueous colloid (2 mg/mL) using the beam of a laser pointer as shown in Figure 1c. The oxygen-containing groups attached to the surface of the discs and to its edges impart an enhanced
dispersibility in water and other polar solvents. Such a high hydrophilicity is expected for polar oxygen-containing groups, distributed more or less homogeneously around the carbon material.

Figure 1: Left panel: photographs of pristine carbon nanodiscs (a) Insoluble in water and of water-dispersible oxidized carbon nanodiscs (b); right panel: photograph of an aqueous colloidal dispersion (2 mg/mL) of oxCND (c) showing the Tyndall scattering effect.

FTIR and XPS spectroscopies were employed to confirm the presence of oxygen-containing functional groups covalently attached to the oxCNDs. The FTIR spectra of pristine and oxidized carbon nanodiscs are shown in Figure 2 (left panel). Oxidized carbon nanodiscs present intensive vibrational bands in the region of 1000-1750 cm\(^{-1}\), differently from the initial CNDs sample, which is an IR inactive material in all the frequency range. More specifically, the band at 1060 cm\(^{-1}\) is assigned to stretching vibrations of C–O groups, while the peak at 1412 cm\(^{-1}\) is attributed to bending vibrations (deformation) of hydroxyl groups C-OH groups.\[^{[8]}\]\ The bands at 1630 cm\(^{-1}\) and 1733 cm\(^{-1}\) are due to C=O stretching vibrations of the –COOH groups.\[^{[8-9]}\]\ The weak band at 1224 cm\(^{-1}\) is assigned to asymmetric stretching of C-O-C bridges in epoxy groups and/or to deformation vibrations of O-H in the carboxylic acid groups. Finally, the appearance of the peak at 3424 cm\(^{-1}\) is ascribed to the hydroxyl stretching vibrations of C-OH groups.\[^{[8]}\]\ The presence of all these characteristic vibrations confirms the successful oxidation of the pristine carbon nanodiscs. The formation of an oxygen-rich derivative is further supported by XPS results.
Figure 2 (right panel) shows the C 1s core level region of the XPS spectrum of oxCNDs. The spectral analysis entailed mathematically reconstructing the spectrum with a minimum number of peaks consistent with the raw data and the molecular structure. Four major contributions to the carbon 1s core-level region can be identified. The first peak at a binding energy of 285 eV is assigned to the C-C bonds of the graphitic framework\textsuperscript{[10]} and accounts for 28 \% of overall carbon intensity. A second peak at 286.1 eV is due to C-O bonds and corresponds to 16 \% of the overall intensity.\textsuperscript{[11]} Additional contributions at 287.2 eV and 288.4 eV stem from C=O bonds\textsuperscript{[12]} (43 \% of the total C 1s intensity) and O-C=O bonds\textsuperscript{[13]} (13 \% of the overall intensity) respectively. It is important to highlight that similar contributions from hydroxyl, epoxy, carbonyl and carboxylated groups are widely reported in the corresponding XPS spectra of graphene oxide sheets produced via acidic treatment of pristine graphite similar to the one applied here to the CNDs.\textsuperscript{[14]}

The successful oxidation of pristine CNDs was revealed also by Raman spectroscopy. The Raman spectra of pure and oxidized carbon nanodiscs are shown in Figure 3. Pristine and oxidized CNDs show the characteristic first-order G and D bands at around 1600 and 1350 cm\textsuperscript{-1}, respectively. The G-band
originates from the doubly degenerate zone center $E_{2g}$ mode, associated with $sp^2$-hybridized carbon atoms. On the other hand, the D band is correlated with $sp^3$ hybridized carbon as it requires a defect for its activation by double resonance, and thus indicates the presence of lattice defects and distortions.\cite{11}

The ratio between the D and G band intensities ($I_D/I_G$) is indicative of the quality of the graphitic lattice and was found to be equal to 0.57 for pristine CNDs. Upon oxidation, the CND sheets show a noticeable increase in the D/G ratio from 0.57 to 0.93\textsuperscript{t}, which is attributed to the change in hybridization of the carbon atoms from $sp^2$ to $sp^3$ when oxygen-containing groups (hydroxyl, carboxyl or epoxide) are created.

![Raman spectra](image)

**Figure 3:** Raman spectra of (a) pristine (CNDs) and (b) oxidized carbon nanodiscs (oxCNDs).

Thermogravimetric analysis (TGA) and thermal analysis (DTA) measurements were performed on pristine and oxidized carbon nanodiscs and the results are shown in Figure 4. For pristine CNDs, a sharp exothermal peak at 635 °C, followed by the complete decomposition of the material, indicates the thermal destruction of the graphitic network. In the case of oxidized carbon

\textsuperscript{t} The D/G ratio must be at least at the minimum 1.1 since it can be estimated by the relative increase of the D' band (\~1620 cm$^{-1}$) which exceeds the intensity of the G band.
nanodiscs a rather continuous weight loss upon heating is observed in the temperature range between 140 and 320 °C and attributed to the removal of the oxygen containing groups (hydroxyl, carboxyl, epoxy) covalently attached to the graphitic layers. The drop in the mass is estimated to be 30 wt%, indicating that a high degree of functionalization has occurred upon oxidation of the pristine CNDs. Moreover, the decomposition of graphitic lattice, identified by the sharp exothermic peak at 440 °C takes place at lower temperatures than for pristine CNDs since the presence of oxygenated species facilitates the combustion of the graphitic network. Finally, the amount of adsorbed water deduced from the TGA data points to a largely increased hydrophilic nature of these novel oxidized nanodiscs as compared to pristine CNDs. From the weight loss up to 120 °C, the percentage of adsorbed water was estimated to be ~20 wt%, a very high value for a carbonaceous material.

**Figure 4**: DTA/TG curves of (a) pristine (CNDs) and (b) oxidized carbon nanodiscs (oxCNDs).

The X-ray diffraction patterns of pristine and oxidized carbon nanodiscs are presented in Figure 5. Pristine CNDs display a well-defined peak, which is attributed to the 002 reflection of the graphite lattice (HOPG) at 26.6° and corresponds to a d_{002} spacing of 3.4 Å. After oxidation this diffraction peak disappears and a new sharp one emerges at lower angles (~11.7°). The latter is due to the principal 001 reflection and corresponds to a basal spacing of d_{001} = 7.5 Å, consistent with the successful oxidation of graphitic layers and the
creation of oxygen-containing groups that are randomly distributed on the basal planes and at edges of the nanodiscs.

Figure 5: X-ray diffraction patterns of (a) initial (CNDs) and (b) oxidized carbon nanodiscs (oxCNDs).

The morphology of the oxidized nanodiscs was examined thoroughly with a combination of SEM, TEM and AFM microscopies (Figure 6). Isolated disc-like nanoflakes with a mean diameter in the range of 1 and 2 μm are clearly visible in both TEM and SEM micrographs. The majority of these nanodiscs have a uniform round shape. Moreover, as a result of the strong acid treatment, single graphene layers detach from the discs as evidenced by AFM images as the one shown in the Figure 6g together with the topographical height profile. Isolated single layer graphene nanodiscs like this one with thickness 0.8 nm can be seen all over the scanned area. However, nanodiscs with thicknesses between 2 and 5 nm are also present indicating that exfoliation of few layer graphene discs also occurs during the oxidation process.
Figure 6: SEM (a, b, c), TEM (d, e, f) and AFM (g) micrographs of oxCNDs.
In view of possible applications for these new hydrophilic nanocarbons we tested whether oxCNDs can be used as cytotoxic drug delivery agent. Doxorubicin hydrochloride (DOX), which is considered as one of the most effective anticancer drugs,[16] was used as a model drug to be loaded onto the oxCNDs surface. In fact it is expected that oxCNDs can effectively load these drug molecules due to π–π stacking interaction between quinine part of DOX and the graphenic layers[17] strong hydrogen-bonding with the hydroxyl and carboxylic groups on the surface of oxCNDs as well as ionic interactions with hydroxyl and amino groups present in DOX.

The loading of DOX onto oxCNDs was determined from the absorbance of the unloaded drug in the supernatant, measured employing UV–Vis spectroscopy. The DOX-loading capacity was consistently found to be ca. 45 % w/w, which is much higher than that of some common drug carrier materials, such as liposomes,[18] where the loading capacity is always below 10 %. DOX loading also affected the ζ-potential of oxCNDs. Indeed, while the ζ-potential value of unloaded oxCNDs was found to be -24±1 mV, for the DOX-loaded oxCNDs we registered a value of 20±1 mV because binding to doxorubicin hydrochloride induces a positive charge on oxCNDs [isoelectric point of DOX=8.25].[19]

Following physicochemical characterization, we proceeded with administration of oxCNDs-DOX to A549 cells. Our first aim was to prove that the nanocarriers effectively deliver their DOX cargo to the cells. To achieve this, we employed optical fluorescence microscopy and exploited the inherent fluorescence of DOX. The representative images of fluorescence microscopy on A549 cells incubated with DOX and oxCNDs-DOX are shown in Figure 7. Cells incubated with free DOX (Fig. 7A) as well as with oxCNDs-DOX (Fig. 7B) clearly show DOX-fluorescence in their nuclei i.e. at the site of DOX action.[20] Therefore, oxCNDs did not prevent DOX from entering the cells but DOX was efficiently delivered to the cell nuclei, where it is expected to retain its action.
We also investigated the anti-tumor efficacy of oxCNDs-DOX on A549 cells. For this purpose, A549 cells were incubated with oxCNDs-DOX and free DOX in various concentrations and the viability of the cells was assessed 24, 48 and 72 h post incubation by standard MTT assays. The cell viability was found to be dose dependent for both oxCNDs-DOX and free DOX as illustrated in Figure 8. The MTT cell viability assay demonstrated that both oxCNDs-DOX and DOX efficiently kill A549 cancer cells in vitro. The toxicity of DOX increased progressively with concentration and assessment time, with a maximum cytocidal activity of ca. $\sim$80 % ($\sim$20% survival) determined 72 h post incubation for a 10 μM concentration, while oxCNDs-DOX at the same concentration (10 μM) and after the same post incubation time (72 h) exhibited a significantly higher cytocidal activity, i.e. 96 % (4 % survival). It is worth to note that unloaded oxCNDs was found to be almost non-cytotoxic for A549 cells. Therefore, oxCNDs is a very promising new material capable for use as effective drug-delivery system.
Figure 8: Comparative cytotoxicity of oxCNDs, free DOX and oxCNDs-DOX. The cytotoxicity was assessed at 24, 48 and 72 h post-incubation by standard MTT assay. The survival rates are relative to media only controls. MTT data are shown as means of at least twelve independent values with error bars representing one standard deviation.

Another possible future application of oxCNDs is their use as supports for the development of nanobiocatalytic systems. Cytochrome c (cyt c) was used as a model protein in order to investigate the effect of oxCNDs on the catalytic behavior (peroxidase activity and stability) of the protein. Cyt c is a small heme protein found in mitochondria and involved in the respiratory chain; in the presence of an electron acceptor such as hydrogen peroxide it is also able to catalyze peroxidase-like reactions in vitro. Due to its excellent catalytic attributes, cyt c is among the best characterized redox proteins and appears to be an excellent model protein to quantitatively assess the effect of carbon nanomaterials on the structure and the catalytic behavior of proteins and enzymes.\textsuperscript{22}
To study the effect of oxCNDs on the stability and peroxidase activity of cyt c, this protein was incubated at 40 °C in a buffer solution containing guaiacol (25 mM) and the remaining peroxidase activity was estimated at predetermined time intervals by addition of H$_2$O$_2$ and monitoring the oxidation of guaiacol. As seen in Figure 9(a), the presence of oxCNDs significantly increases the thermal stability of cyt c. A denaturation half time of 40 h was calculated for cyt c in the presence of oxCNDs. This is almost 4-fold higher than the denaturation half time observed in the absence of these nanomaterials. The stabilizing effect of oxCNDs was even more pronounced when cyt c was incubated in the presence of H$_2$O$_2$. It is well known that cyt c is rapidly inactivated by an excess of H$_2$O$_2$ in the absence of reducing substrate.[23] As seen in Figure 9(b), in the presence of oxCNDs, cyt c almost totally retained its peroxidase activity after 30 min of incubation with H$_2$O$_2$, while in the absence of oxCNDs, the remaining activity of cyt c was less than 20 %. Similar stabilizing effects by various carbon nanomaterials for different enzymes were observed recently and attributed to specific electrostatic and hydrophobic interactions between protein molecules and nanomaterials.[22, 24] It is interesting to note that the stabilizing effect observed for the small oxCNDs is higher than that observed for significantly larger GO nanomaterials[22] indicating that the size of nanomaterials is crucial for this stabilizing effect. Compared to GO, the small-sized oxCNDs are expected to interact stronger (because of the higher amount of active sites) with cyt c molecules and these interactions could lead to a more rigid structure of the protein molecule and thus to an increased stability.[25]
Figure 9: Effect of oxCNDs on the (a) thermal stability of cyt c after incubation up to 24 hours at 40 °C and (b) stability of cyt c against H$_2$O$_2$ after incubation for 30 min at 40 °C. 100 % indicates the activity at t = 0 min.

5.3 Conclusions

Insoluble carbon nanodiscs produced through the so-called pyrolytic Kværner Carbon Black & H$_2$ process were successfully oxidized using a simple, versatile, and reproducible approach based on the well-known Staudenmaier method. As a result of the strong acid treatment single round graphene layers detach from the discs and the surface of the CNDs becomes decorated with various oxygen-containing functional polar groups such as hydroxyl, carboxyl and epoxy groups. Thus the completely insoluble CNDs transform into a hydrophilic derivative, which is dispersible in many polar
solvents, including water. These hydrophilic nanostructures obtained were tested as cytotoxic drug delivery agents of one of the most effective anticancer drugs, to A549 cells. oxCNDs-DOX exhibited a significantly higher cytocidal activity than free DOX, while pristine oxCNDs showed almost non-cytotoxic activity on A549 cells, indicating that oxidized carbon nanodiscs is a very promising material for use as an effective drug-delivery system. Finally, oxCNDs were tested as supports for the development of nanobiocatalytic systems. This new nanomaterial was found to increase the thermal stability of cytochrome c as well as its stability of the peroxidase activity against H₂O₂. The stabilizing effect observed by the small-sized oxCNDs is even higher than that observed for significantly larger oxidized graphite nanomaterials reported in the literature indicating that the size of nanomaterials is crucial for this stabilizing effect.
References


