Carbon nanodots with opposite chirality possess the same major physicochemical properties such as optical features, hydrodynamic diameter, and colloidal stability. Here, a detailed analysis about the comparison of the concentration of both carbon nanodots is carried out, putting a threshold to when differences in biological behavior may be related to chirality and may exclude effects based merely on differences in exposure concentrations due to uncertainties in concentration determination. The present study approaches this comparative analysis evaluating two basic biological phenomena, the protein adsorption and cell internalization. We find how a meticulous concentration error estimation enables the evaluation of the differences in biological effects related to chirality.
While chemically similar, molecular isomers with different chirality can have significant different biological impact, such as pharmacological effect or cellular toxicity. This has been well investigated on the level of small organic chiral isomers, which are naturally existing or can be synthesized. In the last decades, the concept of chirality in biological interactions has gained ever more interest in the raising world of nanomaterials, such as nanoparticles (NPs) or assemblies of NPs. Apart from some atomically defined metal clusters, NPs in general do neither possess a defined molecular formula or structural formula. As thus NPs of one type are not identical but will have a (narrow) size-, shape-, and charge-distribution, the question is if chirality on the size level of whole NPs plays a role with the NP’s interaction with biology, such as protein adsorption and cellular uptake. In fact, reports exist in which adsorption of proteins, the so-called protein corona was found to depend on the chirality of the NPs. Previous studies on carbon nanodots (CNDs) have focused on studying the effect of opposite chiral carbon dots in their biocompatibility and toxicity to liver HepG2 cells. Previous studies on chiral diamines, such as (+)- and (-)-, stereo-extract, interaction with the proteins, tuning enzymatic activity, interaction with the Golgi apparatus, studying their effect on plant growth, as well as establishing methods for detecting the interaction between achiral carbon dots and proteins.

For a quantitative analysis, there are several complications. As the NPs of one type will not be identical, but there will be a distribution of their properties, a potential effect of NPs of the same type but with different chirality might be at a lower level than the inhomogeneity in effect due to the distribution of NP properties. Also, differences in chirality may involve additional distributions in the NPs properties, such as size, optical properties, colloidal stability, etc., as two types of NPs with different chirality will originate from two distinct batches of synthesis. The paramount requirement for analyzing the effect of chirality on the interaction of NPs with biology thus will be NPs with narrow distributions of their properties. In addition, in order to directly compare the biological impact of NPs with different chirality a metric needs to be defined on how properties of different NPs can be compared at the same concentration. Given the fact that surface coatings modify the molecular weight of NPs, it is not the same metric to measure at the same mass concentration or to measure at the same NP number concentration.

Here we report a detailed study on the error of quantification of the concentration of NPs with opposite chirality for the comparative analysis of their interaction with biology. We firstly analyze and evaluate different routes for the determination of NP concentration and consider the error of quantification for each route. Subsequently, we use such error as a threshold to evaluate if the biological variations could be related to merely difference of concentration, or to the chiral properties. The study reveals that only a proper quantification of NPs leads to attributable different biological responses to NPs with equal physicochemical properties except the chiral surface.

**Results**

**Synthesis and characterization.** Here we chose CNDs, i.e. quasi-spherical NPs with an amorphous carbon-rich core and diameter under 10 nm, as model system. Our synthetic protocol consists of a microwave-assisted hydrothermal bottom-up synthesis using arginine (Arg) and ethylenediamine (EDA) as precursors. The corresponding nitrogen-doped N-CNDs were shown to possess a nanoscale amorphous core that is covered by an amino-rich surface. Additionally, by substituting ethylenediamine with chiral diamines, such as (R,R) and (S,S)-1,2-cyclohexanediamine, chiral CNDs, termed here R-CNDs and S-CNDs, were prepared. Electronic circular dichroism of R-CNDs and S-CNDs verified the mirror-image relationship between the two NPs (Supplementary Fig. 1). The basic physicochemical properties of these chiral and achiral CNDs have been demonstrated to be highly similar, such as their diameter as determined by atomic force microscope (\(d_{AFM} = 2.47 \pm 0.84 \text{ nm}\) and 2.64 \(\pm 0.89 \text{ nm}\) for the N-CNDs and R/S-CNDs, respectively)\(^{32,34,35}\), while their structure and composition, as determined by Fourier-transformed infrared spectroscopy (FT-IR, Supplementary Fig. 2) and X-ray photoelectron spectroscopy (XPS, Supplementary Fig. 3) showed similar multiple oxygen and nitrogen functional groups between them\(^{32,34,35}\). However, the absorption and fluorescence emission properties of the three different CNDs (i.e. the achiral N-CNDs and the chiral R/S-CNDs) are different (Supplementary Fig. 4), due to the presence of different surface functionalities and/or emissive traps\(^{32,34,35}\). This introduces a general source of error.

**Metric for comparing the different types of CNDs.** As many biological responses to NPs are dependent on the NP dose (i.e. cellular uptake, toxicity, etc.), a metric is needed to apply the N-, S-, and R-CNDs at the same dose to allow for quantitative comparison. Due to the small size and the carbon composition of the CNDs, it is a big challenge to define a reliable metric and thus we will first discuss the different approaches in this regard. In this way, first, the error in not being able to apply the identical amount of N-, S-, and R-CNDs needs to be estimated. Only effects in biological response higher than the error in CND quantification may be considered a significant difference in the biological response to be related to the opposite chirality.

In order to determine number concentrations, i.e. the number of NPs per volume of solution or their molarity (with Avogadro’s number being the scaling factor between these two entities), the NPs in a fixed volume of solution need to be counted. For big enough NPs counting can be performed easily with optical microscopy. Due to their small size this however is not possible for the CNDs. In principle, small NPs can be counted by immobilizing them on a surface (optionally with evaporation of the solvent) and by imaging them with high-resolution microscopies, such as atomic force microscopy (AFM) or transmission electron microscopy (TEM). Note that for such single NP imaging, the resolution of the microscope given by the refraction limit does not necessarily need to be better than the size of the NPs. By working with strongly diluted solutions statistically each signal comes from an individual NP and agglomerates can be excluded, and thus counting of NPs can be performed without being able to resolve them. However, as in this case the number of NPs per image is low, there is a huge error in the counting statistics. In the case of the CNDs investigated in this study the relative error in counting, which determines the uncertainty in concentration determination is \(\Delta_{CND} C_{CND}^{-1} = 43\%\) (Supplementary Table 1). We performed also counting of the CNDs with TEM, which was complicated by their low contrast due to their carbon composition. As TEM with improved refraction limit allows for resolving of individual CND here higher CND concentrations could be used and thus the number of CND counted per image could be increased. However, here agglomeration of the CNDs on the TEM grid occurred, and the relative error in counting, which describes the uncertainty in concentration determination was determined to be \(\Delta_{CND} C_{CND}^{-1} = 68\%\) (Supplementary Table 2). Another common way for NP counting is nanoparticle tracking analysis (NTA). However, the here used CNDs are below the size limit recommended by the manufacturer of the frequently used Nanosight instrument (the manufacturer Malvern Panalytical recommends NPs > 10 nm diameter) and due
to their low fluorescence emission intensity individual CND does not provide sufficient signal to be detected. Only rare agglomerates of CND are detected, leading to artificial huge hydrodynamic diameter (Supplementary Fig. 5). Thus, for the here used CNDs standard counting methodologies cannot be applied due to the huge experimental error.

An often-used alternative method to NP counting for the determination of NP concentrations is mass determination. In case of metal NPs the elemental amount of metal from the NPs and thus their concentration can be conveniently determined for example with inductively coupled plasma mass spectrometry (ICP-MS)\(^3\). However, ICP-MS is not a convenient method for carbon-based NPs such as the here investigated CNDs. Also, simply adjusting the samples to the similar weight of the CNDs is not possible, as apart from experimental errors (limits in the precision of weighing the CND powder; association of water by the hygroscopic CND powder, etc.) the N-, S-, and R-CNDs as prepared in three different syntheses will not have precisely the same mean mass per NP and also the mass distribution of the different samples will not be identical.

For this reason, here concentration determination of the CNDs was performed based on their optical properties, i.e. molar extinction coefficient and quantum yield. Due to different absorption spectra and fluorescence emission intensities, it is however not possible to simply prepare CND samples of similar doses by adjusting the respective concentrations to yield solutions with the same absorption or fluorescence intensity. In order to estimate the error in concentration determination, we plotted the absorption at 280 nm (\(A_{280}\)) and the integrated fluorescence emission intensity \(I_{\text{max}}\) from 425 to 475 nm upon excitation at 405 nm of the CND samples dissolved in water at the mass concentration \(C_{\text{CNDs}}\) (Supplementary Table 4, Supplementary Figs. 8 and 9) for the different types of CNDs. This error is clearly better than the errors obtained by AFM and TEM counting and thus, in our hands, the best way to determine the CND concentrations. Consequently, only biological effects bigger than 22% of the different CND samples will be considered to be significantly above the error in concentration determination.

To probe the interaction of the CNDs with proteins and cells, the CND samples were adjusted to have the same absorption at 280 nm. As the R-, S-, and N-CNDs at the same concentration \(C_{\text{CNDs}}\) as determined by weight have slightly different absorption (Fig. 1b), we diluted the two samples with higher absorption than the third sample, until the R-, S-, and N-CNDs samples had the same absorption intensity at 280 nm. Upon dilution, the mass concentration of the CND samples has been slightly changed. We thus refer to the concentrations of the CNDs in the following as adjusted concentrations \(C'_{\text{CNDs}}\) which refers to their absorption value. For the undiluted sample \(C_{\text{CNDs}} = C'_{\text{CNDs}}\) for the samples diluted to match the absorption, the adjusted concentration \(C'_{\text{CNDs}}\) is defined as equal to \(C'_{\text{CNDs}}\) of the undiluted sample. For details, we refer to Supplementary Fig. 7.

For the uptake studies, the amount of internalized CNDs was quantified by their fluorescence. However, as the different types of CNDs have different quantum yields, from the absolute detected fluorescence intensity \(I\) as detected for the different CND samples, the CND concentration \(C'_{\text{CND}}\) (which is proportional to their absorption) could not be directly derived. Thus, a correction factor \(X\) had to be applied (Supplementary Table 4, Supplementary Figs. 8 and 9), that the different CND samples had the same fluorescence \(I' = X \times I\) at the same concentration \(C'_{\text{CND}}\). Note that the correction factor \(X\) had to be determined in the cell culture medium and for the used devices with which fluorescence was detected (i.e. fluorescence spectrometer, flow cytometry, confocal microscopy). Also, background correction was applied. Only by using this double correction \((C_{\text{CND}} \rightarrow C'_{\text{CND}}\text{ and } I \rightarrow I')\) the uptake of CND by cells could be compared for the different types of CNDs.

**Role of the protein corona.** As first impact of the interaction of the CNDs with biology, we chose the adsorption of different proteins. By measuring protein concentration-dependent size.

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**Fig. 1 Carbon nanodots concentration determination through UV-Vis and fluorescence emission spectrophotometry.** a Sketch of the N-, S-, and R-CNDs. b Absorption \(A_{280}\) at 280 nm of CNDs dissolved in water at the mass concentration \(C_{\text{CNDs}}\) (determined by weighing). The \(A_{280}(C_{\text{CNDs}})\) curve was fitted with linear regression to yield the slope \(\beta_{A_2}(\text{ml mg}^{-1}) = A_{280}(l) \Delta C_{\text{CNDs}}^{-1}\) (j = R-CND, S-CND, N-CND). \(R^2\) indicates the fitting reliability with linear regression (perfect fit: \(R^2 = 1\)). Data for the absorption at 405 nm \(A_{405}\) are shown in Supplementary Fig. 6. c Integrated fluorescence emission \(I_{\text{max}}\) ranging from 425 to 475 nm (excitation wavelength \(\lambda_{\text{ex}} = 405\) nm) of CNDs dissolved in water at the mass concentration \(C_{\text{CNDs}}\). The \(I_{\text{max}}(C_{\text{CNDs}})\) curve was fitted with linear regression to yield the slope \(\beta_{I_2}(\text{mAU}) = I_{\text{max}}(l) \Delta C_{\text{CNDs}}^{-1}\) (j = R-CND, S-CND, N-CND). From these slopes, the percentual differences \(\Delta \beta_{I_2}\) in the slopes between the R-CND and S-CND sample to the N-CND sample were derived for the absorption and intensity measurements as \(\Delta \beta_{I_2} = \beta_{I_2}(\text{R-CND} - \beta_{I_2}(\text{S-CND}) (l = A, l = 405 \text{ nm})\), and then the deviation \(\Delta \beta_{I_2}\) in these differences between the absorption and intensity measurements were obtained as \(\Delta \beta_{I_2} = |\Delta \beta_{A_2} - \Delta \beta_{I_2}(\text{j = R-CND, S-CND})|\). The percentual error in concentration determination was defined as the maximum of these values as \(\Delta C_{\text{CNDs}} = C_{\text{CNDs}}^{-1} = \max(\Delta \beta_{A_2}, \Delta \beta_{I_2})\). The values are enlisted in Table 1. The shown data were obtained with batch #1.
increase of the CND–protein conjugates by fluorescence correlation spectroscopy\textsuperscript{39–41}, absolute quantifiers describing the interaction could be obtained, namely the apparent dissociation constant $K_D$, the maximum number of proteins $N_{\text{max}}$ that can bind per CND based on the Hill model, and the maximum size increase in hydrodynamic radius $\Delta h,\text{max}$ upon protein adsorption. $K_D$ describes the protein concentrations at which half of the CND surface is covered with proteins. As model proteins, three representative serum proteins, i.e. human serum albumin (HSA), alpha microglobulin (α2M), and transferrin (Tf) were used. Data of batch #1 are shown in Fig. 2, and the data of the other two batches are depicted in Supplementary Fig. 10.

The data shown in Fig. 2 indicate that proteins in general only weakly bind to the CNDs. The $K_D$ value gives the protein concentration, which is needed to half-saturate the CND surface (i.e. to have half of the maximum possible number of bound proteins). In comparison to other NPs the $K_D$ values are higher, meaning that the CNDs are worse binders for the proteins than the other NPs. In the case of HSA $K_D$ values of around 5.1 μM have been obtained for polymer-coated FePt NPs\textsuperscript{39,42}, which is lower than the $\langle K_D\rangle$ of 32.3 μM (Table 2) of the CNDs. Transferrin binds only weakly to the CNDs and under the exploratory concentration range no saturation could be achieved, meaning that $\langle K_D\rangle>1000\text{ μM}$. This is much higher than $K_D$ values of around 26 μM\textsuperscript{40,42}, which have been obtained for polymer-coated FePt NPs. Alpha microglobulin does not adsorb to any of the tested CND surfaces within the tested concentration range. Thus, as with Tf, no quantitative values could be detected for the $K_D$ value, which will be $>1000\text{ μM}$. Only HSA resulted to adsorb sufficiently well to all CND surfaces and only for this protein a quantitative analysis based on $K_D$ was possible. The derived parameters for HSA for N-, S-, and R-CNDs of the three different batches are enlisted in Supplementary Table 5. A possible interference for quantitative analysis of the binding of proteins to NP surfaces is NP agglomeration\textsuperscript{43–45}. In case the size increase of the NPs upon adsorption of protein is much bigger than the size of the proteins (note that in many cases a monolayer adsorption of proteins covering the NP surface has been described), then this may be due to agglomeration effects. As shown in Table 2 (and Supplementary Table 5), the maximum change in hydrodynamic CND radius upon saturation of the CND surface with HSA ($\Delta h,\text{max}$) is around 2.5 nm (Supplementary Table 5), which corresponds to the size of one HSA molecule, in good agreement with previous studies\textsuperscript{39,46}. Of note, the size of one HSA molecule is much bigger than the size of one CND with a hydrodynamic radius of $r_{\text{h,0}}$ of around 0.7–1.0 nm (Supplementary Table 5). Due to the small size of the CNDs one CND can adsorb only 1–2 HSA molecules ($N_{\text{max}}$ Table 2 and Supplementary Table 5). We note that there is variation in $K_D$ values between the different CND batches (Supplementary Fig. 10 and Supplementary Table 5). In fact, for batch #2 and batch #3 under the exploratory HSA concentration range, no saturation of the CND surface with HSA could be reached, i.e. the $r_{\text{h}}(C_{\text{HSA}})$ curves did not reach saturation. Under these conditions, the determination of $K_D$ values is extremely prone to errors (Supplementary Table 5), and thus we only considered the values of batch #1 (Table 2) for quantitative analysis. However, the batch-to-batch variability has to be taken into account when discussing the biological significance of the results. The data reported in Table 2 demonstrate that S-CNDs are significantly worse binders to HSA than R-CNDs (batch #1: $K_D$ of 39.9 μM versus 22.7 μM). The difference between both is $\Delta K_D(K_D)^{-1}=0.36$, which is bigger than the error associated with determining the CND concentration $\Delta C_{\text{CND}}$ CNDs$^{-1}=22\%$ (Supplementary Table 3), though the CND concentration does at any rate only moderately influence the results for the $K_D$ determination\textsuperscript{41}. As mentioned, as no saturation of the CNDs with HSA is not though out of three CND batches only one could be quantitatively evaluated. Of note, for these batches (#2 and #3) also the hydrodynamic radii $r_{\text{h,0}}$ of the N-CNDs are higher than in the other cases (Supplementary Table 5), which might be a reason for the different $K_D$ values. Also in the UV-Vis absorption spectra of batch #4 of the N-CNDs some agglomeration (e.g. scattering at high wavelength) is visible (Supplementary Fig. 11). Thus, protein adsorption on N-CNDs might be influenced by slight agglomeration and differences to the R- and S-CNDs cannot be unequivocally related to changes in chirality.

We thus can summarize that CNDs in comparison to other NPs are very weak binders to proteins, and from the three investigated proteins here only HSA formed a clear protein corona. For HSA there is a significantly better binding of R-CNDs to the CNDs than of S-CNDs. The HSA binding of N-CNDs had large batch–batch variations and there was the same agglomeration, and thus these data cannot be interpreted quantitatively.

We want to mention that our data refer only to three selected serum proteins. As with our method, i.e. FCS, we only detect changes in the hydrodynamic diameter upon protein adsorption, upon exposure to blood we would not be able to tell which proteins had adsorbed and caused the increase in size of the NPs. In order to detail the composition of the protein corona typically mass spectrometry analysis is performed\textsuperscript{47}. However, for such measurements first unbound excess proteins have to be removed. For the small CNDs as investigated here to which only few proteins can weakly bind, such purification may significantly change the protein composition left on the NP surface\textsuperscript{41}. In contrast, diffusion measurements with FCS are performed in situ, without the need for purification. While such measurements do not allow for telling the composition of the adsorbed protein corona, and thus are best carried out in different solutions containing only one type of model protein, diffusion measurements can still verify protein corona formation in blood\textsuperscript{48}.

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|}
\hline
$\beta_{A,R}\text{-CND}$ & $\beta_{S,R}\text{-CND}$ & $\beta_{A,N}\text{-CND}$ & $\beta_{S,N}\text{-CND}$ & $\beta_{A,R}\text{-CND}$ & $\beta_{S,R}\text{-CND}$ & $\beta_{A,S}\text{-CND}$ & $\beta_{S,S}\text{-CND}$ & $\Delta A_{B=0}\text{-CND}$ & $\Delta C_{\text{CND}}$

\hline
0.00177 & 0.00225 & 0.00270 & 569422 & 842279 & 1187647 & 0.34 & 0.17 & 0.52 & 0.29 & 0.18 & 0.13 & 0.18

\hline
\end{tabular}
\caption{Error analysis in concentration determination by adsorption ($A_{B=0}(C_{\text{CND}})$) and fluorescence intensity ($I_{\text{max}}(C_{\text{CND}})$) measurements.}
\end{table}

**Cellular uptake.** Cellular uptake of the different types of CNDs (here batches #3, #4, and #5 were used) was quantified with previously established methods\textsuperscript{49–51} with two cell lines (see “Methods”). HeLa cells were used as a standard model system,
which is widely spread in general uptake studies. As second system THP-1 macrophages as derived by the differentiation of the human monocytic leukemia cell line THP-1 were used, modeling an exposure scenario which CNDs could encounter in vivo. Viability assays were performed, which demonstrated that under the used incubation conditions the CNDs are biocompatible (see "Methods").

Uptake of CNDs by cells as quantified by the mean CND fluorescence \( I \) per cell was detected with two independent methods, flow cytometry (see "Methods") and confocal microscopy (see "Methods"), as there may be discrepancies between the results of these two detection techniques. Also, time- and concentration-dependence of CND uptake was quantified, as well in serum-free as in serum-supplemented media. Probing such a range of different parameters serves as an internal control that CNDs were uptaken like other NPs. CNDs showed the expected general uptake behavior: uptake of CNDs increased with increasing incubation times, with increasing CND concentration, and was higher in serum-free than in serum-supplemented medium (see "Methods"). In this way, differences in the uptake of the different types of CNDs are due to differences in the CNDs and not due to other factors. The main findings of the uptake quantification study are summarized in Fig. 3. Here the time-dependence of the uptake is shown. The corresponding concentration-dependence is presented in the "Methods" section. The data show that consistently there is (at the same incubation concentration \( C_{\text{CND}} \)) lowest internalization of N-CNDs, and highest internalization for S-CNDs, as well for HeLa cells as for THP-1 derived macrophages (Fig. 3a versus Fig. 3b), quantified by both flow cytometry and confocal microscopy (Fig. 3b versus Fig. 3d). The question is now whether these differences in the uptake behavior of the different types of CNDs are significant. For this, the values at \( t = 48 \) h are analyzed in Table 3.

For N-CNDs there was always lower internalization than for S-CNDs and R-CNDs. As there was some agglomeration for the N-CNDs as discussed above this effect most likely is not related to the chirality of the CNDs, but to their colloidal stability, and thus will not be discussed further. For all investigated conditions there was more uptake for S-CNDs than for R-CNDs. Note, that the data shown in Table 3 are not "cherry-picked", and in fact, under different CND concentrations the uptake difference between R-CNDs and S-CNDs was even higher (Supplementary Fig. 13). This is an important internal control, that results are not related to only one particular incubation condition but are valid in general. While the data shown in Fig. 3 and Table 3 show that S-CNDs internalize best, for making a final statement the differences in uptake need to be related to the experimental errors. For example, while for THP-1 derived macrophages the tendency as obtained from flow cytometry is the same as from confocal microscopy data, the difference between both types of CNDs is more pronounced for the confocal microscopy data. In Fig. 1 we pointed out that there is an uncertainty of CND concentration determination of \( \Delta C_{\text{CND}} \), \( C_{\text{CND}} = 22\% \). From Table 3 the difference in uptake between S-CNDs and R-CNDs for HeLa cells is \( \Delta I'(T) \) = 16% and 12% for serum-supplemented and serum-free incubation conditions, respectively. According to our analysis the change in uptake behavior for HeLa cells might be caused by uncertainty in concentration determination \( (\Delta I'(T)^{-1} < \Delta C_{\text{CND}}, C_{\text{CND}}^{-1}) \) and thus no difference in the uptake behavior based on chirality should be claimed. In contrast, from Table 3 the difference in uptake between S-CNDs and R-CNDs for THP-1 derived macrophages is \( \Delta I'(T)^{-1} = 24\% \) and 38% for serum-supplemented and serum-free incubation conditions (flow cytometry data) and \( \Delta I'(T)^{-1} = 88\% \) and 110% for serum-supplemented and serum-free incubation conditions (confocal microscopy data), respectively. Thus, these differences are clearly above the error of concentration determination \( (\Delta I'(T)^{-1} > \Delta C_{\text{CND}}, C_{\text{CND}}^{-1}) \) and are thus related to the different chirality of the CNDs. As macrophages are designed to interact with “intruding” molecules/particles/materials, it is plausible that they can better distinguish “what to
take up", i.e. better differential of surface differences between S-CNDs and R-CNDs. We thus can summarize that for THP-1 derived macrophages there is significantly higher uptake from S-CNDs than for R-CNDs.

Apart from the uptake quantification we also performed colocalization assays and chemical blocker for known pathways of endocytosis (see "Methods"). All types of internalized CNDs colocalized to a high degree with lysosomes (Supplementary Fig. 15). Cellular uptake of the CNDs was blocked at 4 °C, suggesting that the internalization of the CNDs is an energy-dependent process (see "Methods"). The three types of CNDs were taken up by both different cell types largely via a
phagocytosis pathway, while N-CNDs could also be endocytosed via a clathrin-associated endocytosis pathway, similar to the results obtained for the THP-1 derived macrophages (see “Methods”). The different cellular uptake behavior of the N-CNDs may be related to their lower colloidal stability.

Discussion

Correlating the interaction of NPs with cells with the physicochemical properties of the NPs is by far not trivial. Several physicochemical properties may be entangled, and many time effects may not be due to a primary physicochemical parameter (such as e.g. chirality), but due to colloidal stability (as here in the case of the N-CNDs). In fact, we have shown here by FCS measurements that for the R- and S-CNDs chirality does not affect colloidal stability and only because for this case entanglement of chirality and colloidal stability was ruled out differences in biological effects can be related to chirality as physicochemical parameter. For the N-CNDs there was an effect on colloidal stability and thus differences in their biological effects cannot be related to their non-chiral nature.

For many types of NPs concentration determination is not unequivocal, and thus errors in the metric (here \( \Delta C_{\text{CNDs}} C_{\text{CNDs}}^{-1} \)) need to be taken as threshold whether detected differences in the interaction of the NPs with cells can be related to certain physicochemical parameters. In the present case, the uptake of S-CNDs is significantly higher than that of R-CNDs for THP-1 derived macrophages, and differences in uptake can be related to the different chirality between both types of CNDs. For the case of HeLa cells, the same tendency was observed, but differences in uptake might be also due to uncertainties in concentration determination.

Taking into account the protein corona data, HSA binds significantly better to R-CNDs than to S-CNDs (Table 2: \( K_0(R-\text{CNDs}) = 22.7 \mu M < K_0(S-\text{CNDs}) = 39.9 \mu M \)). It is not directly obvious why the different chirality between R-CNDs and S-CNDs leads to different adsorption behavior of HSA, as concerning the chemical composition, charge, etc. but surfaces are identical. As the surface of the CNDs will not be smooth and homogenous, most likely the different chirality has some local effect on the arrangement of the organic shell, like a different local density of the functional groups or different local conformation, which would explain why HSA binds differently to both surfaces.

R-CNDs are endocytosed to a significantly lesser extent by THP-1 derived macrophages than S-CNDs (Table 3: \( \langle I'(R-\text{CNDs}) \rangle < \langle I'(S-\text{CNDs}) \rangle \)). An easy way to interpret this scenario could be the following. There is more protein corona for R-CNDs. The presence of a protein corona for many NPs is often associated with lower uptake by cells (in fact there is lower uptake in serum-supplemented conditions where a protein corona can be formed than in serum-free conditions), which would explain the reduced uptake for the R-CNDs. However, this interpretation might be too simple. In comparison to other NPs the CNDs are very poor binders to proteins, which will be influenced by their small size. Only for HSA, but not for Tf and α2M saturation in binding could be achieved (Fig. 2). However, also for HSA there is on average only one single HSA molecule associated with each CNDs (Table 2; \( N_{\text{max}} \) parameter). Differences in uptake (\( \Delta I'(R-\text{CNDs}) \langle I'(S-\text{CNDs}) \rangle^{-1} \)) versus \( \Delta I'(R-\text{CNDs}) \langle I'(S-\text{CNDs}) \rangle^{-1} \) were larger in serum-free than in serum-supplemented medium. While also under serum-free culture conditions presence of some proteins in the culture medium cannot be excluded, it is unlikely that at low protein concentrations (\( c_p \) of proteins in serum-free medium \( \ll K_0 \)) a difference in the attachment of proteins from the medium between S-CNDs and R-CNDs is the reason for their different uptake by cells, in particular as the CNDs are poor binders for proteins. The surface of cells is not homogenous, and the lipid bilayer is patterned with proteins and sugars. Before a NP is internalized by a cell, it first needs to bind to the cell surface, where it may dwell for some time until it is endocytosed. We thus speculate that in addition to protein corona-related effects there might be better (non-specific) adsorption of R-CNDs to the cell surface than of S-CNDs, which then would relate to higher uptake. This demonstrates that while the general picture of particle uptake is quite well established, it is not that all details would be understood.

The data shown here demonstrate that also under more stringent considerations of errors in the concentrations determination of CNDs, it has been shown that chirality may affect the protein corona formation and in vitro cellular uptake of CNDs to an extent of >20%. It thus can be speculated that this difference would also influence the in vivo interaction of CNDs. Chirality itself does not influence the most important physicochemical properties of CNDs, such as fluorescence and colloidal properties. By using CNDs of different chirality thus different distributions of otherwise identical CNDs might be obtained.

### Methods

**Characterization and concentration determination of the CNDs**

*Basic optical characterization of the CNDs.* Different carbon nanodots (CNDs) were synthesized according to previously published protocols, i.e. N-CNDs, and R-CNDs and S-CNDs. Firstly, the optical properties of the CNDs were
characterized. Briefly, the obtained lyophilized solids of CNDs were first weighted and then fully dissolved in filtered Milli-Q water to form stock solutions with a concentration of $C_{\text{CND}} = 10 \text{ mg ml}^{-1}$. Then, the stock solutions were further diluted to $C_{\text{CND}} = 100 \text{ ng ml}^{-1}$ with filtered sterilized water for absorbance and fluorescence spectra measurements (Supplementary Fig. 4) using an UV-Vis absorption spectrophotometer (Agilent 8453, Agilent technologies, Australia) and a fluorescence spectrometer (FluoroLog-3, Horiba Jobin Yvon, USA). In the absorption spectra of CNDs (A) values assigned to 280 nm and 360 nm absorption peaks, while the N-CNDs only had one peak, at the same position as the R-CNDs and S-CNDs at 280 nm. Concerning the fluorescence properties, the three CNDs demonstrated a slight excitation-dependent emission shift upon excitation at different wavelengths $\lambda_{ex}$ ranging from 330 to 440 nm.

Concentration-dependent absorption/fluorescence intensity measurements. In order to account for different absorption and emission properties of the different CNDs, the concentration-dependent absorption and fluorescence spectra were measured for the different CND samples. For this objective, the CND solutions with concentrations ranging from $C_{\text{CND}} = 0.78 \text{ ng ml}^{-1}$ to 400 pg ml$^{-1}$ were prepared by diluting the CND stock solutions with filtered Milli-Q water. Then, UV-Vis absorption spectra $A(\lambda)$ and fluorescence spectra $I(\lambda)$ ($\lambda_{em} = 405 \text{ nm}$) were recorded (Supplementary Fig. 26). The three different types of CNDs all possessed a dose-dependent absorbance and fluorescence behavior as expected, i.e., the absorption and fluorescence linearly decreased with more diluted CNDs solutions. The absorption values $A_{\text{CND}}(\lambda = 280 \text{ nm})$ plotted against the CND concentrations are shown in Fig. 1 in the main manuscript. In order to probe the influence of the wavelength at which the absorption is measured for the error analysis, the same concentrations were diluted to 405 nm of the CNDs (Supplementary Fig. 6). As $A_{\text{CND}} < A_{\text{M}}$, all further evaluation was performed with $A_{\text{M}}$.

Concentration adjustment and fluorescence intensity correction. As the CNDs at different mass concentration $C_{\text{CND}}$ (as determined by weighting) showed different absorption (Supplementary Fig. 6), the concentrations were adjusted to lead to the same absorption at 280 nm. This was done to account for other concentrations via determination via absorption measurements. For this, the absorption values of the CND solutions were determined at 280 nm and the CND solutions with higher absorption values (which was always the R-CNDs) were diluted with filtered Milli-Q water until achieving all samples had the same absorption $A_{\text{M}}$. The R-CND solution remained undiluted at concentration $C_{\text{CND}}$. In the following the adjusted concentrations of the S-CND and N-CND solutions were assumed to be at the same concentration as the R-CND solution. Here “the same concentration” refers to equal absorption at 280 nm. These absorption-based concentrations are referred to as adjusted concentrations $C_{\text{CND}}’$, $C_{\text{CND}}’$ (S-CND) = $C_{\text{CND}}(\text{S-CND})$, $C_{\text{CND}}(\text{N-CND})$ = $C_{\text{CND}}(\text{R-CND})$. The CND concentration determination is shown in Supplementary Fig. 7. In order to determine the error in concentration determination absorption $A(\lambda)$ and emission spectra $I(\lambda)$ were recorded in dependence of the adjusted concentrations $C_{\text{CND}}’$. The spectra are displayed for the five different batches of CNDs used in this study in Supplementary Figs. 18 and 19. The concentration-dependence of the absorption at 280 nm and of the fluorescence emission as derived from the spectra was plotted for all five batches in Supplementary Fig. 27. Based on Supplementary Fig. 23 error analysis was performed as described in Fig. 1 of the main manuscript, and the results are displayed in Supplementary Table 3. As can be seen in Supplementary Figs. 7 and 27 at the same adjusted concentration $C_{\text{CND}}’$ (R-CND) and N-CND samples have different fluorescence intensity emission intensities. This needs to be considered when quantifying the uptake of CNDs by their fluorescence with flow cytometry and confocal microscopy. For flow cytometry was collected with a 450 nm/50 nm bandpass filter, and for confocal microscopy with an LP 420 nm long pass filter (Supplementary Fig. 7). In this way correction factors X taking into account the different fluorescence intensities at the same adjusted concentrations were defined. $S_{\text{ex}} = (I(\text{S-CND})) / (I(\text{R-CND}))$ is the ratio of the integrated S-CND fluorescence and the integrated R-CND fluorescence, at the same adjusted concentration of S-CNDs and R-CNDs. The integration range was used emulating the flow cytometry and confocal microscopy filters. $S_{\text{ex}} = (\Delta(\text{S-CND})) / (\Delta(\text{R-CND}))$ is the ratio of the integrated S-CND fluorescence and the integrated R-CND fluorescence at the same adjusted concentration of S-CNDs and N-CNDs. The resulting values are enlisted for all five used batches in Supplementary Table 4.

Concentration determination by CND counting with transmission electron microscopy (TEM). Transmission electron microscopy (TEM) measurements to count CNDs were performed using a Jeol JEM-1011 instrument operating at 100 kV. Two µL of the corresponding CND solution ($C_{\text{CND}}$ (R-CND) = 2.0 $\text{ mg ml}^{-1}$, $C_{\text{CND}}$ (S-CNDs) = 2.8 mg ml$^{-1}$) were drop-casted onto a copper grid (400 mesh, diameter 3.05 mm) coated with amorphous carbon. As can be observed from the TEM-micrographs shown in Supplementary Figs. 30 and 31, homogeneous coating was not achieved. For the R-CNDs different aggregates were observed, whereas for the S-CNDs some areas with dispersed CNDs were also found. It is not known, how much of the aggregates form during drying on the TEM grid. On both samples it was possible to differentiate single CNDs; however, due to the limited contrast, an exact determination of CND size was not possible. Based on micrograph analysis we could determine a mean diameter of $d_{\text{ex}}$ = 1.4 ± 0.4 nm for R-CNDs (216 CNDs investigated) and $d_{\text{ex}}$ = 2.4 ± 0.9 nm for S-CNDs ($N = 255$ CNDs investigated). These values are compatible with those obtained by AFM ($d_{\text{AFM}}$) and discussed in the main text. We emphasize, however, that due to the limited number of observed CNDs and limited contrast, the diameters as determined with TEM should be considered a rough estimate. We used the determined CND diameters to calculate theoretical concentrations, assuming spherical CNDs. With the density of amorphous carbon $\rho_{\text{C}} = 3.50 \text{ g cm}^{-3}$ (diamond has a similar density of $\rho_{\text{C}} = 3.51 \text{ g cm}^{-3}$) and the weight concentrations ($C_{\text{CND}}$ (R-CNDs) = 2.0 $\text{ mg ml}^{-1}$; $C_{\text{CND}}$ (S-CNDs) = 2.8 mg ml$^{-1}$) we obtain a molar concentration of $c_{\text{CND}}$ = 340 $\text{ mg ml}^{-1}$ for R-CNDs and $c_{\text{CND}}$ = 180 $\mu\text{g ml}^{-1}$ for S-CNDs. Because of the limited accuracy of CND diameter determination, also these concentrations must be considered as a rough estimate. In the TEM micrographs of S-CNDs we find $(N_{\text{CND}}) = (N_{\text{CND}})$ $< 3014 \text{ CNDs mm}^{-2}$ on average (Supplementary Table 2). For R-CNDs we find $(N_{\text{CND}}) = 1920 \text{ CNDs mm}^{-2}$ on average on the micrographs. The accuracy of the numbers is limited by the contrast, depending on the micrograph. The standard deviation $\sigma$ for both average numbers are very high (Supplementary Table 2), with the mean value of both standard deviation being 0.68. This mean value for $\Delta N_{\text{CND}}(N_{\text{CND}})$ would correspond to the uncertainty in concentration determination $\Delta N_{\text{CND}} = 0.08$, underlining that the concentration determination with TEM is not feasible. Assuming a homogeneous coating of the whole TEM grid (area = $7.3 \times 10^{6}$ µm$^2$) with these CNDs, $d_{\text{ex}} = 2.4 \times 10^{-6}$–2.8 x $10^{-6}$ CNDs in the dried 2 µL that were drop-casted onto the grids. This would correspond to $c_{\text{CND}} < 10$–20 $\mu\text{m}$ solutions. As expected, this value is several orders of magnitude off the theoretical value, underlining that it is not feasible to obtain a meaningful CND concentration based on TEM analysis.

Concentration determination by using the nanoparticle tracking analysis (NTA). Nanoparticle tracking analysis (NTA) was performed with a NanoSight LM10 (Malvern Panalytical) operated with a 405 nm laser. R-CND solution were diluted to $c_{\text{CND}} = 18 \mu\text{m}$ (see the respective section in "Methods") and S-CNDs to $c_{\text{CND}} = 5.5 \mu\text{m}$. As can be observed in Supplementary Fig. 5, only large aggregates were tracked by the system for both samples. The main population of CNDs is too small and scatters too weakly to be discernible with this technique. The CND concentrations (which are in fact aggregate concentrations) determined with NTA were $c_{\text{CND}} = 3.4 \times 10^{6}$ CNDs ml$^{-1}$ for R-CNDs and $c_{\text{CND}} = 1.0 \times 10^{5}$ CNDs ml$^{-1}$ for S-CNDs. This CND concentrations correspond to the diluted range. Considering the sample preparation of the CNDs we can not detect the number of aggregates in the CND solutions seems negligible. Note that presence of aggregates to a large extent can be ruled out by the FCS measurements shown in the respective section in "Methods".

Other physicochemical characterization data. Additional standard characterization of the CNDs is provided in the form of Fourier-transform Infrared (FT-IR) spectra (KBr), shown in Supplementary Fig. 2, electronic circular dichroism (ECD) spectra shown in Supplementary Fig. 1 and X-ray photoemission spectroscopy (XPS) shown in Supplementary Fig. 3. FT-IR spectra were recorded on a Perkin Elmer 2000 spectrometer. ECD spectra were measured on a Jasco J-815. XPS spectra were measured on a SPECS Sage HR 100 spectrometer.

Influence of cell culture medium on the properties of the CNDs. While the characterization in the respective section about CND properties in "Methods" was carried out in water, uptake experiments of the CNDs took place in cell culture.
medium. Thus, it needed to be tested how the presence of cell culture medium affects the properties of the CNDs. For this, 400 µL of CND solutions (C_{CND} = 200 µg mL^{-1}) were mixed with the same volume of either (i) distilled water (as the negative control), (ii) RPMI (Roswell Park Memorial Institute) 1640 medium without phenol red (Thermofisher, USA) supplemented with 10% fetal bovine serum (FBS, Biochrom, UK), and (iii) RPMI 1640 medium without phenol red without serum. Thus, the final CND concentration was C_{CND} = 100 µg mL^{-1}. After different incubation times of t = 0, 8, and 48 h, and 3 and the CND solutions were characterized in a UV-Kuevette, ZH 8.5 mm Deckel (Sarstedt, Germany) with UV-Vis absorption spectroscopy and with fluorescence spectroscopy (λ_{ex} = 405 nm). In addition, the hydrodynamic diameters d_{h} of the CNDs in the different media were measured with dynamic light scattering (DLS), Malvern NANO ZS, England48. In Supplementary Figs. 8, 9 and 32 the absorption and fluorescence spectra of the three different types of CNDs are shown. In all three cases there is a slight fluorescence increase of the different types of CNDs after incubation in particular in serum containing RPMI 1640 medium. At this increase is similar, fluorescence intensities of the three different types of CNDs (R-CNDs, S-CNDs, and N-CNDs) can be also directly compared when the CNDs are exposed to cell culture medium.

The hydrodynamic diameters d_{h} of the CNDs after incubation with the different medium were measured after different time points by DLS. Data are shown in Supplementary Fig. 33. Due to the very small size of the CNDs and in the case of RPMI 1640 medium supplemented with 10% FBS due to the presence of proteins of similar size as the CNDs, the DLS values are unreliable and are not further interpreted in this study. In fact, the hydrodynamic diameter as detected in the plain medium without proteins (yellow bars; water and RPMI 1640 medium without serum) most likely correspond to dust. The increase hydrodynamic diameters as detected in the serum-supplemented medium (yellow bars, RPMI 1640 medium with FBS) originate from serum proteins. Presence of or absence of the CNDs (red and blue versus yellow bars) does not change the results, demonstrates that it is not the CNDs which are detected here with DLS, which is due to their tiny size. Hydrodynamic radii r_{h} = d_{h} \times 2^{1/3} of the CNDs were instead measured with fluorescence correlation spectroscopy (FCS, see the respective section in "Methods"), where only the CNDs and CND–protein complexes, but not the free proteins provide signal.

**Protein adsorption on CNDs.** To explore whether the chiral surface of CNDs has an impact on protein adsorption, the interaction of the CNDs with different proteins, human serum albumin (HSA, CAS No. 70024-90-7, Sigma Aldrich, Germany), transferrin human (Tf, CAS No. 11096-37-0, Sigma-Aldrich, Germany), alpha-2-macroglobulin (a2M, CAS No. 91564-72-6, Sigma-Aldrich, Germany), were investigated with fluorescence correlation spectroscopy (FCS)48–49. Measurements were carried out in a Confocal Light Scanning Microscope (CLSM) (LSM 880, Zeiss, Germany) with a Zeiss Plan-Apochromat x40/1.0 Water DIC (WD: 85 µm) objective integrated PBS set-up (Zeiss). FCS studies were performed with two solvents either in filtered Milli-Q water or in phosphate buffer saline (PBS, Gibco, Invitrogen, Belgium). For measurements, proteins at different concentration were mixed with CNDs in either PBS or water, leading to a final variable protein concentration c_{p} (P = HSA, Tf, a2M) and a fixed CND concentration, C_{CND} = 10 µg mL^{-1} for batch #1 and 50 µg mL^{-1} for batch #2 and #3. Before measurements all samples were incubated for 15 min and were then loaded to 35 mm petri dishes with glass bottom (Cat.No: 81218-200, ibidi, Germany). For each data point three independent measurements were carried out. In Supplementary Fig. 35 the data for S-CNDs (batch #1) as exposed to different concentrations of HSA in PBS are presented. The corresponding diffusion times t_{D} and diffusion coefficients D as determined from the fit as provided in Supplementary Table 11. From the diffusion coefficients D the corresponding hydrodynamic radii were calculated according to the Stokes–Einstein equation:

\[ r_{h} = \frac{k_{B} T}{6 \pi \eta D} \]  

(5)

k_{B} = 1.38 \times 10^{-23} \text{ J K}^{-1} is the Boltzmann constant, \( T = 298.15 \text{ K} \) room temperature, and \( \eta \) is the solution viscosity. The solution viscosity was assumed to depend linearly on the protein concentration according to:

\[ \eta = \eta_{0} (1 + C) \]  

(6)

The protein mass concentration C_{P} relates to the molar mass concentration C_{p} of the protein. Hereby the following values were used: HSA: M_{W}(HSA) = 66.5 kDa, \( \eta = 4.2 \text{ cm}^{2} \text{ s}^{-1} \) g^{-1}; Tf: M_{W}(Tf) = 80 kDa, \( \eta = 4.4 \text{ cm}^{2} \text{ s}^{-1} \) g^{-1}; a2M: M_{W}(a2M) = 725 kDa40. In Supplementary Table 11 the conversion of diffusion coefficients D into hydrodynamic radii r_{h} is demonstrated. The resulting hydrodynamic radii r_{h} versus protein concentrations c_{p} are listed of the different types and batches of CNDs as recorded in water in Supplementary Fig. 36 and as recorded in PBS in Supplementary Fig. 10. In the following, all further discussion will be carried out on the results obtained in PBS. The data recorded in PBS with HSA show a saturation of the hydrodynamic radius, e.g. at high protein concentration the CND surface is completely saturated with proteins and thus the hydrodynamic radius does not increase further with raising concentrations. The behavior can be fitted with the Hill model42. For this, the (n_{H}c_{p}) curves shown in Supplementary Fig. 10 were fitted with the following equation:

\[ V_{HSA} = \frac{V_{HSA}^{max} n_{H} c_{p} / K_{H}}{1 + \frac{V_{HSA}^{max} n_{H} c_{p} / K_{H}}{1 + C_{p}}} \]  

(7)

V_{HSA} is the volume of one HSA molecule. Assuming a triangular prism shape with 8.4 nm side length and 3.2 nm height we used V_{HSA} = 96 nm^{3}49. V_{CND} is the volume of one CND, considering the CNDs as spheres, and the radius r_{0} obtained from the FCS of the control sample, no protein:

\[ V_{HSA} = \frac{4}{3} \pi r_{0}^{3} \]  

(8)

r_{0} is an experimentally determined value and is enlisted in Supplementary Table 5. The fit function had the following free fit parameters: \( V_{HSA}, K_{H}, n_{H} \) and \( n_{H} \) is the value for the fit for the hydrodynamic radius of the CNDs with no adsorbed proteins, i.e. \( r_{0}(\text{control}, K_{H}) \) is a fit parameter, \( r_{0}(\text{control}) \) is an experimentally determined value, \( V_{HSA}^{max} \) is the number of HSA molecules bound per CND in saturation (i.e. \( c_{p} \approx C_{p}^{max} \), \( K_{H} \) is the dissociation coefficient, and \( n_{H} \) is the Hill coefficient42. The resulting fit values from the curves shown in Supplementary Fig. 10 are presented in Supplementary Table 5.

**Cell culture techniques.** Two cell lines were used in this study: THP-1 monocytes and HeLa cells. The human monocytic leukemia cell line THP-1 (ATCC® TIB-202®) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). THP-1 cells were cultured in suspension in RPMI 1640 medium.
macrophages and DMEM in the case of HeLa cells). Afterwards, the fluorescence spectra of each well were collected from 570 to 620 nm with an excitation of 560 nm as described above. Subsequently, Matlab software was used for data analysis based on the fluorescence intensity at 590 nm, which was considered proportional to the number of living cells. The viability V represents the fluorescence intensity of cells treated with CNDs normalized to the fluorescence intensity of untreated control cells. All experimental conditions were recorded for further experiments. After stimulation with CNDs for 38 and 40, both cell lines maintained high viability at different exposure doses of CNDs ranging from $C_{CND} = 0.488 \mu g \cdot mL^{-1}$ to 1000 $\mu g \cdot mL^{-1}$ for different incubation times (24 and 48 h) in culture medium supplemented with or without 10% FBS. These results are consistent with the other biocompatibility tests regarding CNDs.75

Time- and dose-dependent uptake studies based on flow cytometry

Time-dependent uptake of CNDs by HeLa cells. Uptake of the different CNDs by HeLa cells was investigated. Firstly, HeLa cells were seeded at a density of 40,000 cells well$^{-1}$ with 10% FBS contained DMEM medium of volume $V_{medium} = 1$ mL per well in 24-well plates (Sartstedt, Germany) with 1.9 cm$^2$ seeding area per well. On the next day, the medium in each well was removed and then CNDs diluted in DMEM medium supplemented with 10% or 0% FBS ($V_{medium} = 0.5$ mL) were added to the HeLa cells for specific time points (1, 3, 6, 24 or 48 h) at a concentration of $C_{CND} = 400 \mu g \cdot mL^{-1}$. After the exposure time, cells were washed three times with 0.5 mL cold PBS, detached by addition of 0.05% trypsin-EDTA, isolated by centrifugation at 300 x g for 5 min, and finally re-suspended in 0.3 mL cold PBS for flow cytometer analysis (BD LSRFortessa™, BD Biosciences, US). The CND fluorescence signal $I$ within each cell was collected with the flow cytometer at a 450/50 nm bandpass filter upon 405 nm excitation, 10,000 gated cells were collected and analyzed for each sample. Then, the FlowJo software was used to analyze the flow cytometry data. The recorded mean CND fluorescence per cell $I$ was then background-corrected by subtracting the fluorescence of control cells which had not been exposed to CNDs, leading to the background-corrected mean CND fluorescence per cell $I_R$.
Time-dependent uptake studies based on confocal microscopy. Uptake of CNDs by THP-1 derived macrophages was also quantified by confocal laser scanning microscopy (CLSM). L510 THP-1 monocytes were seeded at a density of 75,000 cells per well in complete RPMI 1640 medium with the volume \(V_{\text{medium}} = 0.3 \text{ mL}\) per well in a μ-Slide 8 Wells (ibidi GmbH, Germany) with 1 cm² growth area per well, and were differentiated into THP-1 derived macrophages within 3 days (see the respective section in “Methods”). On the fourth day, the medium was removed, and CND solution (\(V_{\text{medium}} = 0.3 \text{ mL}\)) was added to cells at a concentration of \(C_{\text{CNDs}} = 400 \mu\text{g mL}^{-1}\) in RPMI 1640 medium supplemented with 10% or 0% FBS subsequently. At specific time points the cells were harvested for fluorescence microscopy experiments (Supplementary Fig. 12 and Supplementary Table 8). In addition, fluorescence imaging was performed with a 405 nm laser as the excitation source and a 420 nm long pass filter for recording the fluorescence emission. Representative images are shown in Supplementary Figs. 52 and 53. After obtaining the CLSM images at each time point, quantitative analysis of cellular CND uptake was performed by utilizing a combination of software for open-source software in ImageJ. In a first step, the images as obtained from the confocal microscopy software were converted to TIFF format utilizing Matlab software. In a second step, Adobe Photoshop CS6 was used to manually denote the perimeter of the cells. In a third step, the sum of the fluorescence intensities of all pixels belonging to a cell was calculated by the image analysis software CellProfiler v2.2.0, and converted to the mean fluorescence per cell by dividing the summed up pixel intensities by the number of fluorescent cells. The intensity values were then corrected by the fluorescence difference between the different CND sample according to Supplementary Table 4:

\[
I_{\text{CNDs}}(n) = \frac{I(n) - I_{\text{nontreated}}(n)}{I_{\text{control}}(n) - I_{\text{nontreated}}(n)}
\]

\[
I_{\text{control}}(n) = \frac{I(n) - I_{\text{nontreated}}(n)}{I_{\text{control}}(n) - I_{\text{nontreated}}(n)}
\]

Colocalization of CNDs with intracellular organelles

Colocalization studies of mitochondria or lysosome and CNDs. Colocalization studies of CNDs with cell organelles, i.e., mitochondria and lysosomes, were carried out for THP-1 derived macrophages and for HeLa cells using Confocal Laser Scanning Microscopy (CLSM) (LSM 510, Zeiss, Germany) with a Plan-Apochromat ×63/1.40 Oil DIC M27 objective. Firstly, THP-1 monocytes were seeded at a density of 75,000 cells per well with complete RPMI 1640 medium with the volume \(V_{\text{medium}} = 0.3 \text{ mL}\) supplemented with PMA at a concentration of 150 nM in μ-Slide 8 Wells (ibidi GmbH, Germany) with 1 cm² growth area per well. After 72 h incubation time in a cell culture incubator, the THP-1 monocytes had been differentiated into THP-1 derived macrophages. In the case of HeLa cells, 12,000 THP-1 derived macrophages and HeLa cells, respectively. After 24 or 48 h incubation time, mitochondria and lysosomes were labeled with corresponding staining reagents as described in the following.

Immunostaining procedures. For mitochondrial staining, MitoTracker® Deep Red FM (Catalog No: M22426, Thermofisher Scientific) was used to specifically label the mitochondria. Briefly, cells were washed three times with 200 μL PBS and then 200 μL of pre-warmed (37 °C) MitoTracker® Deep Red FM in complete RPMI 1640 medium at a concentration of 400 nM was added and cells were further incubated in the incubator for 30 min at 37 °C. Afterwards, the staining solution was replaced with fresh pre-warmed RPMI 1640 medium without phenol red (Catalog No: 11835030, Thermofisher Scientific) and cells were observed using CLSM. A laser diode emitting at 405 nm and a bandpass emission filter LP 420–480 nm were used to visualize the CNDs. A helium–neon laser of 633 nm and long pass filter LP 650 nm were used for recording the fluorescence of MitoTracker® Deep Red FM. For lysosome staining, LysoTracker® Green DND-26 (Catalog No: L7526, Thermofisher Scientific) was selected as the lysosomal marker. Cells were washed three times with 200 μL PBS and then 200 μL of pre-warmed (37 °C) LysoTracker® Green DND-26 at a concentration of 1 μM in complete RPMI 1640 medium was added and cells were further incubated at 37 °C in 5% CO₂ for 30 min prior to imaging with CLSM. The excitation laser and emission collection setups were the same for the CNDs as that used in the colocalization studies of mitochondria and CNDs. An argon laser of 488 nm together with the BP 505–530 nm bandpass filter was used for observing the fluorescence of LysoTracker® Green DND-26.

Calculation of Manders’ coefficients from the colocalization data. Based on the CLSM images, colocalization was quantified by quantitatively calculating Manders’ (ii) and (iii) coefficients, and for that purpose the overlap G and blue fluorescent pixels from two different fluorescence channels ranging from 0 to 1. To achieve this purpose, Matlab and CellProfiler v2.2.0 were used to calculate Manders’ coefficients. Briefly, the Matlab software was firstly used to subtract the background from the 8-bit grayscale TIFF images. Secondly, CellProfiler v2.2.0 was used to identify pixels belonging to cells. Then the colocalization of CNDs and mitochondria or lysosomes for all pixels corresponding to cells was calculated. Thirdly, the below given equations were used to calculate Manders’ coefficients using Matlab. Hereby \(m_1\) is the percentage of blue fluorescent pixels (i.e. CNDs) that overlapped with red or green fluorescent pixels (i.e. mitochondria or lysosomes) which overlap with blue fluorescent pixels (i.e. CNDs).

\[
m_1 = \frac{\sum I(R)I(CND) - \sum I(R)I(CND)_{nonoverlapping}}{\sum I(R)I(CND)_{overlapping}}
\]

\[
m_2 = \frac{\sum I(B)I(CND) - \sum I(B)I(CND)_{nonoverlapping}}{\sum I(B)I(CND)_{overlapping}}
\]

Studies about the uptake pathway

Distinguishing CNDs adherent to the cell membrane from endocytosed CNDs. Flow cytometry based on standard fluorophores cannot distinguish trivially between CNDs adherent only on the outer cell membrane from actually endocytosed CNDs. Both scenarios however can be distinguished using confocal microscopy. To confirm that the majority of fluorescence signal from CNDs is internalized (i.e. CNDs which originate from internalized CNDs) and not the overall fluorescence which is overlapped with CNDs which overlapped with red or green fluorescent pixels (i.e. mitochondria or lysosomes) which overlap with blue fluorescent pixels (i.e. CNDs).

Uptake of CNDs by THP-1 derived macrophages under the presence of inhibitors. The endocytosis pathway of CNDs can be investigated by evaluating the inhibition of certain internalization pathways by pharmacological/chemical inhibitors as described in [57]. In our case, we investigated the cellular uptake pathway of CNDs using the following chemical inhibitors: (i) Nocodazole (an inhibitor of endocytosis of lager nanoparticles [56,57], CAS No:31430-18-9, Sigma-Aldrich, Germany), 10 μM. (ii) Cilengitide (an inhibitor of αvβ3 integrin, 200 μL) (an inhibitor of αvβ5 integrin, 200 μL, InvivoGen, France), and (iii) Amiloride (an inhibitor of micropinocytosis [58,59], CAS No: 17440-83-4, Sigma-Aldrich, Germany), and (iv) Chlormoprazine (an inhibitor of micropinocytosis [58,59], CAS No: 17440-83-4, Sigma-Aldrich, Germany).
of clathrin-associated endocytosis\textsuperscript{88,91}. CAS No.69-09-0, Sigma-Aldrich, Germany). While the inhibitors were applied, still cytotoxicity experiments for the above inhibition were conducted. In order to ensure that the concentration of the inhibitors was not toxic. For experiments on the first day, THP-1 monocytes were seeded at a density of 34,000 cells per well with complete RPMI 1640 medium (\(V_{\text{medium}} = 0.136 \text{ mL}\)) in 96-well plates with 0.34 cm\(^2\) growth area per well and were differentiated into THP-1 derived macrophages. On the fourth day, the supernatant was removed and the cells treated with three types of CNDs were taken up by the HeLa cells largely via a phagocytosis pathway, while N-alkyl-deoxyiminosugar CNDs is an energy consumed process. Besides, the three types of CNDs were taken up by the HeLa cells at the used concentration ranges. To ensure that there is no reduction of cell viability of the THP-1 derived macrophages after the uptake inhibitors at desired concentrations (Supplementary Table 17). The cells treated with inhibitors (or as positive control without inhibitors) were incubated at 37 °C for 1 h. As negative control cell which have not been treated with inhibitor were cultured at 4 °C for 1 h. After this incubation interval, the three types of CNDs were removed and then each inhibitor diluted in RPMI 1640 medium containing 10% or 0% FBS was added to the cells for 7 or 25 h. The exposure concentrations of each inhibitor are summarized in Supplementary Table 16. After the incubation time of 7 h or 25 h, the cell viability test was performed. All the protocols described in the respective section in "Methods". As shown in Supplementary Fig. 69, the four chemical inhibitors were non-toxic to THP-1 derived macrophages at the used concentration ranges. To ensure that there is also not reduction of cell viability of the THP-1 derived macrophages after exposing to CNDs pre-incubated with the cellular uptake inhibitors, also for this scenario a viability assay was carried out. Briefly, THP-1 monocytes were differentiated into THP-1 derived macrophages and then each inhibitor diluted in RPMI 1640 medium containing 10% or 0% FBS was further incubated at the normal conditions for another 6 h before the viability measurements. As shown in Supplementary Figs. 70 and 71, the exposure of the above inhibitors together with the CNDs did not reduce viability of the THP-1 derived macrophages. Thereafter, the cellular uptake pathway of CNDs in THP-1 derived macrophages was investigated. The experiment was conducted as follows. On the first day, THP-1 monocytes were seeded at a density of 100,000 cells per well with 10% FBS containing RPMI 1640 medium (\(V_{\text{medium}} = 0.4 \text{ mL}\)) in 48-well plates (Sartstted, Germany) with 1 cm\(^2\) growth area per well. After 3 days, the cells were differentiated into THP-1 derived macrophages. Afterwards, the previous cell culture medium was replaced with fresh RPMI 1640 medium containing 10% or 0% FBS, supplemented optionally with Nocodazole, Bafilomycin A1, Amiloride, or Chlorpromazine. The concentrations used for each inhibitor are described in Supplementary Table 17. Afterwards, cells treated with the inhibitors were incubated at 37 °C. Cells cultured at 37 °C without exposure to inhibitors were used as positive controls. Cells cultured at 4 °C (i.e. conditions where there is reduced endocytosis) without exposure to inhibitors were used as negative controls. After 1 h incubation time, the three types of CNDs were directly added at a final concentration of 2.00 μg mL\(^{-1}\) and 4.00 μg mL\(^{-1}\) and the plates were further incubated at the original conditions for another 6 h before the viability measurements. All experiments done with CNDs were repeated at least three times. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data supporting the findings of this study are either provided in the article and its Supplementary Information or available from the corresponding authors upon request.

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References

1. Brooks, W. H., Guida, W. C. & Daniel, K. G. The significance of chirality in drug design and development. Curr. Top. Med. Chem. 11, 760–770 (2011).
2. Caner, H., Groner, E., Levy, L. & Agranat, I. Trends in the development of chiral drugs. Drug Discov. Today 9, 105–110 (2004).
3. Hutt, A. J. & Tan, S. C. Drug chirality and its clinical significance. Drugs 52, 1–12 (1996).
4. Yuan, L. et al. Chiral polymer modified nanoparticles selectively induce autophagy of cancer cells for tumor ablation. J. Nanobiotechnol. 16, 55 (2018).
5. Smith, S. W. Chiral toxicology: it’s the same thing… only different. Toxicol. Sci. 110, 4–30 (2009).
6. Nguyen, L. A., He, H. & Pham-Huy, C. Chiral drugs: an overview. Int. J. Biomed. Sci. 2, 85–100 (2006).
7. De Frenz, M. et al. Exploring the effect of chirality on the therapeutic potential of N-alkyl-deoxyiminosugars: anti-inflammatory response to Pseudomonas aeruginosa infections for application in CF lung disease. Eur. J. Med. Chem. 175, 63–71 (2019).
8. Ma, W. et al. Chiral inorganic nanostructures. Chem. Rev. 117, 8041–8093 (2017).
9. Zhao, J. et al. Chiral graphene quantum dot photoluminescence: opportunity and challenge. J. Mater. Chem. C 10, 1744–1755 (2016).
10. Sun, M. et al. Intracellular localization of nanoparticle dimers by chirality reversal. Nat. Commun. 8, 1847 (2017).
11. Gao, X., Han, B., Yang, X. & Tang, Z. Perspective of chiral colloidal semiconductor nanocrystals: opportunity and challenge. J. Am. Chem. Soc. 141, 13708–13707 (2019).
12. Zhao, X., Zang, S.-Q. & Chen, X. Stereospecific interactions between chiral inorganic nanomaterials and biological systems. Chem. Soc. Rev. 49, 2481–2503 (2020).
13. Zafar, M. S. & Ragusa, A. Chirality at the nanoparticle surface: functionalization and applications. Appl. Sci. 10, 5357 (2020).
14. Zhao, X. et al. Tuning the interactions between chiral plasmonic films and living cells. Nat. Commun. 8, 2007 (2017).
15. Tang, H., Li, Q., Yan, W. & Jiang, X. Reversing the chirality of surface ligands can improve the biosafety and pharmacokinetics of cationic gold nanoclusters. Angew. Chem. Int. Ed. 60, 13829–13834 (2021).
16. Lundqvist, M. et al. Nanoparticle size and surface properties determine the conformation of serum albumin: possible implications for biological impacts. Proc. Natl. Acad. Sci. USA 105, 14265–14270 (2008).
17. Wang, X. et al. Chiral surface of nanoparticles determines the orientation of adsorbed transferrin and its interaction with receptors. ACS Nano 11, 4606–4616 (2017).
18. Wang, X. et al. Probing adsorption behaviors of BSA onto chiral surfaces of nanoparticles. Small 14, e1703982 (2018).
19. Deng, J., Zheng, H. & Gao, C. Influence of protein adsorption on the cellular uptake of AuNPs conjugated with chiral oligomers. *Mater. Chem. Front.* 1, 5584–5591 (2017).

20. Srivatsan, A. et al. Effect of chirality on cellular uptake, imaging and photodynamic therapy of photosensitizers derived from chlorophyll-a. *Bioorgam. Med. Chem.* 23, 3603–3617 (2015).

21. Deng, J., Wu, S., Yao, M. & Gao, C. Surface-anchored poly(acrylic)-L(D)-valine) with enhanced chiral-selective effect on cellular uptake of gold nanoparticles. *Sci. Rep.* 6, 31595 (2016).

22. Li, Y. et al. Chiral evolution of carbon dots and the tuning of laccase activity. *Adv. Ther.* 1, 1800006 (2018).

23. Hu, L. et al. Chiral control of carbon dots via surface modification for tuning the enzymatic activity of glucose oxidase. *ACS Appl. Mater. Inter.* 13, 5877–5886 (2021).

24. Hu, L. et al. Chiral nanoprobes for targeting and long-term imaging of the Golgi apparatus. *Chem. Sci.* 8, 6829–6835 (2017).

25. Zhang, M. et al. Chiral hydrothermal synthesis of chiral carbon dots and their effects on mung bean plant growth. *Nanoscale* 10, 12734–12742 (2018).

26. Zhang, M. et al. Chiral evolution of carbon dots and the tuning of laccase activity. *Nanoscale* 10, 2333–2340 (2018).

27. Zhang, M. et al. Malтase decorated by chiral carbon dots with inhibited enzyme activity for glucose level control. Small 15, 1901512 (2019).

28. Zhang, M. et al. Chiral control of carbon dots via surface modification for tuning the enzymatic activity of glucose oxidase. *ACS Appl. Mater. Inter.* 13, 5877–5886 (2021).

29. Li, S. et al. Highly fluorescent chiral N-S-doped carbon dots from cysteine: affecting cellular energy metabolism. *Angew. Chem. Int. Ed.* 57, 2377–2382 (2018).

30. Zeng, M. et al. Lysozyme-derived carbon dots for chiral inhibition of prion protein fibril assembly. *Adv. Ther.* 1, 1800006 (2018).

31. Zhang, L. et al. Chiral evolution of carbon dots and the tuning of laccase activity. *Nanoscale* 10, 2333–2340 (2018).

32. Zhang, M. et al. Malтase decorated by chiral carbon dots with inhibited enzyme activity for glucose level control. Small 15, 1901512 (2019).

33. Dordevic, L., Arcudi, F. & Prato, M. Preparation, functionalization and characterization of engineered carbon nanodots. *Adv. Funct. Mater.* 30, 2000999 (2020).

34. Felu, N. et al. Nanoparticle dosage—a nontrivial task of utmost importance for quantitative safety research. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* 8, 479–492 (2016).

35. Arcudi, F., Dordovic, L. & Prato, M. Design, synthesis, and functionalization strategies of tailored carbon nanodots. *Acc. Chem. Res.* 52, 2070–2079 (2019).

36. Dordovic, L., Arcudi, F. & Prato, M. Preparation, functionalization and characterization of engineered carbon nanodots. *Nat. Protoc.* 14, 2931–2953 (2019).

37. Arcudi, F., Dordovic, L. & Prato, M. Synthesis, separation, and characterization of small and highly fluorescent nitrogen-doped carbon nanodots. *Angew. Chem. Int. Ed.* 55, 2107–2112 (2016).

38. Dordovic, L. et al. Design principles of chiral carbon nanodots help convey chirality from molecular to nanoscale level. *Nat. Commun.* 9, 3442 (2018).

39. Prato, M. & Feliu, N. Synthesis, separation, and characterization of inorganic colloidal nanoparticles. *Beilstein J. Nanotechnol.* 10, 5807–5871 (2019).

40. Prato, M. & Feliu, N. Quantum dots for bioimaging and diagnostics. *Angew. Chem. Int. Ed.* 58, 1–6 (2019).

41. Prato, M. & Feliu, N. Protein corona formation around nanoparticles—from the past to the future. *Annu. Rev. Immunol.* 38, 395–429 (2020).

42. Hühn, J. et al. Dissociation coefficients of protein adsorption to nanoparticles: what have we learned thus far? *Beilstein J. Nanotech.* 5, 1477–1499 (2014).

43. Baalousha, M., Prasad, A. & Lead, J. R. Quantitative measurement of the nanoparticle size and number concentration from liquid suspensions by atomic force microscopy. *Environ. Sci. Process. Impacts* 16, 1338–1347 (2014).

44. Hühn, J. et al. Standardized protocols for the synthesis, phase transfer, and characterization of inorganic colloidal nanoparticles. *Chem. Mater.* 29, 399–461 (2017).

45. Maffre, P., Nienhaus, K., Amin, F., Parak, W. J. & Nienhaus, G. U. Characterization of protein adsorption on FePt nanoparticles using dual-focus fluorescence correlation spectroscopy. *Beilstein J. Nanotechnol.* 2, 375–383 (2011).

46. Hühn, D. et al. Polymer-coated nanoparticles interacting with proteins and cells: focusing on the sign of the net charge. *ACS Nano* 7, 3253–3263 (2013).

47. Mahmoudi, M. et al. Temperature: the “ignored” factor at the nanobio interface. *ACS Nano* 7, 6555–6562 (2013).

48. Maffre, P. et al. Effects of surface functionalization on the adsorption of human serum albumin onto nanoparticles—a fluorescence correlation spectroscopy study. *Beilstein J. Nanotechnol.* 5, 2036–2047 (2014).

49. Pelaz, B. et al. Surface functionalization of nanoparticles with polyethylene glycol: effects on protein adsorption and cellular uptake. *ACS Nano* 9, 6996–7008 (2015).

50. Calbertson, C. T., Jacobson, S. C. & Baranyi, J. R. Diffusion coefficients of nanoparticles in microfluidic devices. *Talanta* 96, 365–373 (2012).

51. Caballero-Díaz, E. et al. The toxicity of silver nanoparticles depends on their uptake by cells and thus on their surface chemistry. *Part. Part. Syst. Char.* 30, 1079–1085 (2013).

52. Sri, S., Kumar, R., Panda, A. K. & Solanki, P. R. Highly biocompatible, fluorescent, and Zwitterionic carbon dots as a novel approach for biosensing applications in cancerous cells. *ACS Appl. Mater. Int.* 10, 37835–37845 (2018).

53. Doshi, N. & Mitragotri, S. Macrophages recognize size and shape of their targets. *PLoS ONE* 5, e10051 (2010).

54. Aderem, A. & Underhill, D. M. Mechanisms of phagocytosis in macrophages. *Annu. Rev. Immunol.* 17, 593–623 (1999).

55. Carpenter, A. et al. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* 7, R100 (2006).

56. Hsieh, C. W., Chu, C. H., Lee, H. M. & Yuan Yang, W. Triggering mitophagy with far-red fluorescent photosensitizers. *Sci. Rep.* 5, 10376 (2015).

57. Zhu, D. et al. Intracellularly disintegratable polynucleotides for efficient gene delivery. *Adv. Funct. Mater.* 27, 160863 (2017).

58. Yan, H. et al. Facile synthesis of semi-library of low charge density cationic polymers from poly(alkylene maleate) for efficient local gene delivery. *Biomaterials* 178, 559–569 (2018).

59. Liu, X. et al. Fusogenic reactive oxygen species triggered charge-reversal vector for effective gene delivery. *Adv. Mater.* 28, 1743–1752 (2016).

60. Sanders, M. E. M., Verbeek, F. J. & Aten, J. A. Measurement of colocalization of objects in dual-color confocal images. *J. Microsc.* 169, 373–382 (1993).

61. Zhu, D. et al. Detailed investigation on how the protein corona modulates the physicochemical properties and gene delivery of polyethyleneimine (PEI) polypelexes. *Biomater. Sci.* 6, 1800–1817 (2018).

62. Roy, S., Elbaz, N. M., Parak, W. J. & Felu, N. Biodegradable alginic polyelectrolyte capsules as plausible biocompatible delivery carriers. *ACS Appl. Bio. Mater.* 2, 3245–3256 (2019).
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Author contributions

H.Y. and M.C. designed and performed the experiments. M.P., W.J.P. and N.F. conceived and coordinated the project. W.J.P. wrote the manuscript with input from all the authors. L.D., F.A. and D.Z. oversaw the research and contributed to the experimental design. S.M. contributed with the FCS measurements. F.S. contributed with the TEM and NTA analyses. M.P., W.J.P. and N.F. oversaw the research and secured the funding.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Maurizio Prato, Wolfgang J. Parak or Neus Feliu.

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