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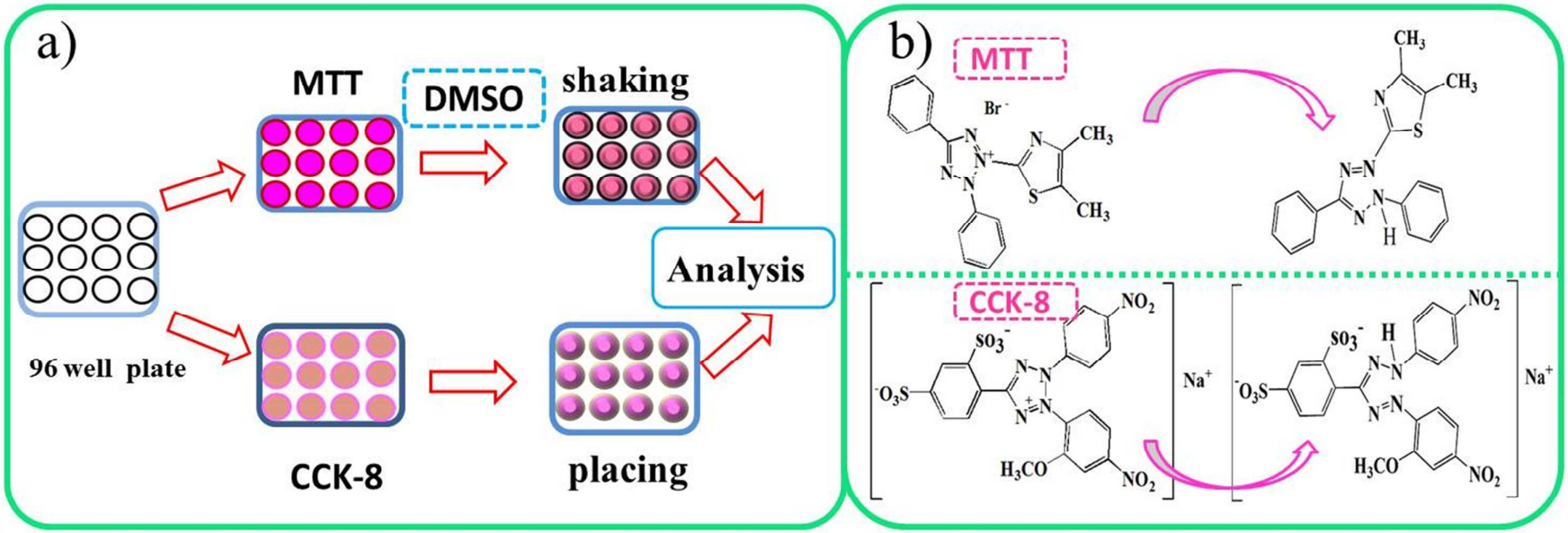
Abstract

Cellular toxicity test is a key step in assessing the graphene toxicity for its biomedical applications. In this study, we investigated the cytotoxicity of graphene with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) and water-soluble tetrazolium-8-[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tet-razolium] monosodium salt (CCK-8) assay on HepG2 cell line and Chang liver cell line. The cell viability data obtained by using MTT and CCk-8 assay showed inconsistent. Graphene induced adsorption, optical interferences, as well as electron transfer can prevent to appropriate evaluate graphene toxicity. Our findings demonstrated the importance of careful interpreting of obtained data from classical *in vitro* assays on assessment of graphene cytotoxicity.



Flow Chart

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PAPER

Limitations of MTT and CCK-8 assay for evaluation of graphene cytotoxicity

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Guozheng Jiao,[a] Xiong He,[a] Xin Li,*[ab] Junqiang Qiu,[c] Hongying Xu,[c] Ning Zhang,[c] and Shumin Liu*[c]

Cellular toxicity test is a key step in assessing the graphene toxicity for its biomedical applications. In this study, we investigated the cytotoxicity of graphene with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) and tetrazolium-8-[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tet-razolium] monosodium salt (CCK-8) assay on HepG2 cell line and Chang liver cell line. The cell viability data obtained by using MTT and CCK-8 assay showed inconsistent. Graphene induced adsorption, optical interferences, as well as electron transfer can prevent to appropriate evaluate graphene toxicity. Our findings demonstrated the importance of careful interpreting of obtained data from classical *in vitro* assays on assessment of graphene cytotoxicity.

Introduction

Graphene(G), two-dimensional sp^2 carbon nanomaterial, has attracted considerable attention because of its extraordinary electronic, optical, mechanical, as well as chemical properties,[1] and its derivatives, have demonstrated great promise in biological and biomedical applications, such as biosensors,[2-7] antibacterial materials,[8-10] and tissue engineering scaffolds.[11, 12] In particular, they have emerged as promising drug delivery systems.[13-15] Dai's group has shown that graphene oxide can be used for loading anticancer drug with high efficiency.[16, 17] Recently, our works also demonstrated that multifunctional graphene possess a superior capability of binding anticancer drug with high loading capacity.[18, 19] However, the cytotoxicity of graphene is still the health risk for people, including the users and the producers. Hence, the measures that analyses the toxicity effect of graphene have been considerable focused with great efforts.

A number of classical *in vitro* assays, such as, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide(MTT), Lactic dehydrogenase(LDH), tetrazolium-8-[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tet-razolium] monosodium salt(CCK-8), reactive oxygen species (ROS), are widely used for toxicity assessment of graphene-based materials. Unfortunately, several studies demonstrated contradictory results because toxicity and non-toxicity of graphene were simultaneously reported.[20, 21] For example, graphene oxide (GO) at low concentrations (20~50 μg.mL^{-1}) was found to have dose-dependent toxicity to mammalian cells,[22] or human fibroblast cells.[23] On the contrary, GO showed highly biocompatible[24] and non-toxic to A549 cells.[25] Furthermore, the two classical assay methods, MTT and CCK-8, exhibited inconsistent testing results about the cytotoxic effects of graphene oxide and graphene toward the A549 cells[26] or human skin fibroblast cells.[27] Despite availableness of common *in vitro* assays for molecular toxicology, these approaches are not suited to assess the nanotoxicity because the interference between nanomaterial and assay agentia. For example, the MTT reagent has been found to be disturbed by mesoporous silicon microparticles,[28, 29] carbonaceous particles,[30, 31] carbon nanotubes.[32-35] To date, there are only limited studies investigating the interferences induced by graphene with assay agentia. Hurt and Co-workers reported[36] the graphene-induced adsorptive and optical artifacts with *in vitro* assays by using dichlorofluorescein (DCF) as a molecule probe. Regarding the possible interference caused by nanomaterials, considerable attentions should be paid to.

In the present work, we compared with the results of MTT and CCK-8 assay for determination of graphene cytotoxicity. MTT and CCK-8 assay are the most common *in vitro* nanotoxicity assessment so that they were simultaneously employed in the current study. We found that adsorption, optical interferences, as well as electron transfer can prevent to appropriate evaluate graphene toxicity. Our results suggested the importance of careful consideration of obtained data from MTT or CCK-8 assay in the presence of graphene related materials.

Results and discussion

[a] Department of Chemistry, Harbin Institute of Technology, Harbin 150090, China E-mail:lixin@hit.edu.cn.
[b] State Key Lab of Urban Water Resource and Environment, Harbin Institute of Technology, Harbin 150090,
[c] Institute of Traditional Chinese Medicine, Key Laboratory of Chinese Materia Medica, Ministry of Education, Heilongjiang University of Chinese Medicine, Harbin 150040, China E-mail:keji-liu@163.com

† Electronic Supplementary Information (ESI) available: Additional experimental section, figure of he charge distribution and density of states of MTT and G
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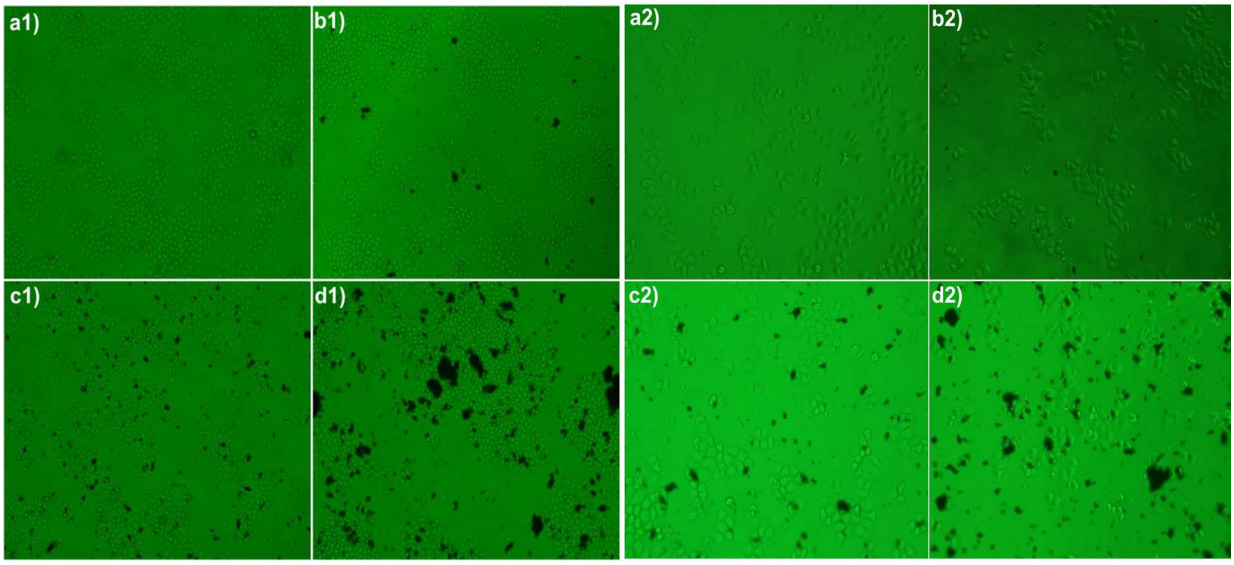

Fig. 1 The cellular morphology images of Chang liver cell and HepG2. Control group (a1, a2), Low dose group (b1, b2) 2.5 μg·mL^{-1}, Middle dose group (c1, c2) 10 μg·mL^{-1}, High dose group (d1, d2) 100 μg·mL^{-1}. Chang liver cell (a1, b1, c1, d1) amplification is 10×40. HepG2 (a2, b2, c2, d2) amplification is 20×40.

Optical observation

Optical microscopy was employed to observe the morphological changes of cell lines under treatment of graphene for 24 h (Fig. 1). As shown in Fig. 1b1, 1c1and 1d1, Chang liver cells were closely sticking the wall, and the attached traces were distinct. In scope of vision, no suspending and dead cells were found. Compared with the cell growing status of control group (in Fig. 1a1), that of the treated groups have not displayed the significant discrepancy. It showed that graphene with the concentration of 2.5 μg·mL^{-1} ~ 100 μg·mL^{-1} was little or no toxic to Chang liver cell after 24 h incubation. As for HepG2, similar experimental results were obtained (Fig. 1a2, 1b2, 1c2, and 1d2).

Measuring the cell viability with MTT and CCK-8 assay

To investigate the cytotoxicity of graphene, MTT and CCK-8 assays were performed to analyse the cell viability of Chang liver cell line and hepG2 cell line (in Fig. 2). We can find that the cell viability of HepG2 and Chang liver cell were all kept over 90% with the concentration of graphene from 2.5 μg·mL^{-1} to 100 μg·mL^{-1} by MTT measured (Fig. 2a1 and Fig. 2b1). However, the cell viability of CCK-8 assay displayed the prominent differences compared with that of MTT measured (in green Dashed Box). As for HepG2 (Fig. 2a2), the concentration of graphene ranged from 20 μg·mL^{-1} ~ 100 μg·mL^{-1}, the cell viability of CCK-8 assay displayed that the graphene killed the cells over 32% much more than that of MTT measure. As for Chang liver cell (Fig. 2b2), the graphene concentration ranged from 80 μg·mL^{-1} to 100 μg·mL^{-1}, similarly, the cell viability of CCK-8 assay uncovered that the graphene killed the cells over 25% much more than that of MTT measure. Clearly, there are contradictory results and no obviously correlation between the MTT and CCK-8 assay.

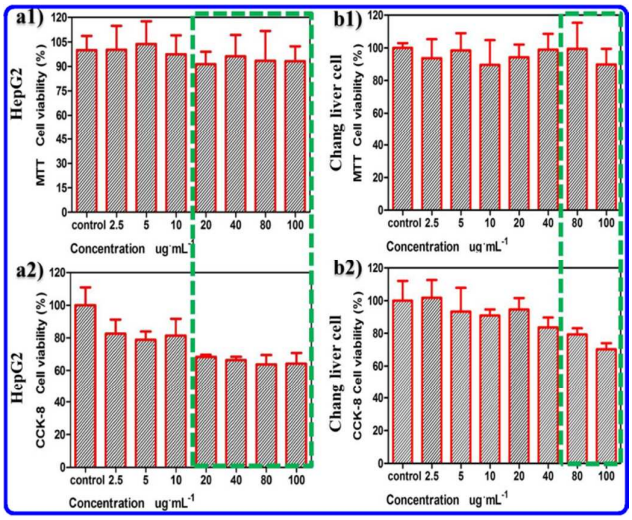

Fig. 2 The cellular viability assayed by MTT (up) and CCK-8 (down). HepG2 (a1, a2), Chang liver cell (b1, b2), the green dashed box contains the shining different analysis histogram. Threshold value was set low 80%.

Adsorption interference

Adsorption interference was described by previous studies maybe a major source of spurious results.[36, 37] In order to investigate the adsorption of graphene, cell-free adsorptive experiments were performed. In Fig. 3, we can find that the absorption intensity ratio of treated groups ***a*** slowly reduced to 93% while the MTT contacted graphene for 2 h (Fig. 3a). The absorption intensity ratio of treated groups ***b*** prominently reduced to 73% while CCK-8 exposed of graphene for 2 h (Fig.3b).The graphene adsorption intensity to MTT/

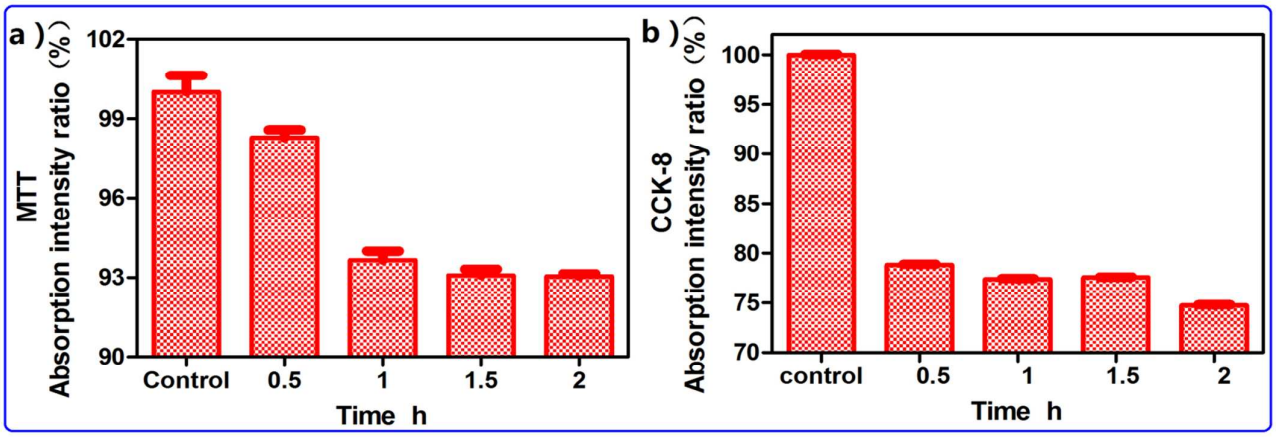

Fig. 3 The adsorption intensity ratio of graphene to MTT/CCK-8 solution; Control group ***a*** (PBS, MTT), Control group ***b*** (PBS, CCK-8). Treated group ***a*** (PBS, MTT and G), treated group ***b*** (PBS, CCK-8 and G). Treated groups were subjected to the interval period of 0.5 h, 1 h, 1.5 h, and 2 h for assay.

CCK-8 is a relationship to the time of contact, that is, the graphene adsorption quantity to MTT/CCK-8 is directly proportional to duration of contact, displaying the time-respond. Moreover, the graphene adsorption intensity for CCK-8 was much sharply fallen than MTT along with the contact time. Like carbon nanotube and activated carbon, graphene may adsorb dye through π-π and electrostatic interaction adsorption.[38, 39] Graphene induced adsorption may lead to the reagent less opportunity to arrive at the plate bottom to contact cells, which may cause to the formazan quantity of MTT/CCK-8 sharply decrease.

Apparently, the quantity of CCK-8 molecule was absorbed by graphene much more than that of MTT. It may involve in two factors. The first reason is that the π-π conjugated system of CCK-8 molecule much stronger than that of MTT. Because the molecule of CCK-8 is consisted of three benzene rings and one five-membered heterocycle, on the contrary, that of MTT only contains two benzene rings and two five-membered heterocycles, namely, the more the π-π conjugation system is, the stronger the adsorption property (is).[38, 39] The second reason is that the substituent groups of benzene ring has strongly influence the adsorption,[40] compared with the methyl group of benzene ring, the nitro group of benzene ring can improve the adsorption capacity much higher, the assistant adsorption role of substituent groups of MTT molecule is lower than that of CCK-8.

Electron transfer

As previously reported that, the graphene can suppress the fluorescence effect of some fluorescence dye molecules,[41-43] one of the reasons involves in electron transfer, that is, the electron transfer from dye molecule to the graphene. Although the reagents of MTT and CCK-8 are not regarded as the fluorescence reagent, owing to having the positive electron on the MTT/CCK-8 molecule, it is similar to some fluorescence dye molecules, the graphene having the π-π system, we inferred that they may also produce the electron transfer phenomenon to disturb the dye molecule touching enzymes.

In the following study, the Dmol3 code was carried out to calculate, and integrated the density functional theory (DFT), local density approximation (LDA), and PWC functional to perform calculations of electron transfer from MTT/CCK-8 to graphene. The optimal structure-activity of the molecules (CCK-8/MTT) of mutual effect on graphene was acquired (Fig. 4a and 4b). By virtue of the Fermi energy level with the adjacent to charge distribution and density of states (DOS) overlap, we can find that between the CCK-8 and graphene displayed orbital overlap and electrons coupling (Fig. 5a and 5b), therefore, we concluded that between CCK-8 molecules and the graphene had taken place electron transfer. But, as for MTT

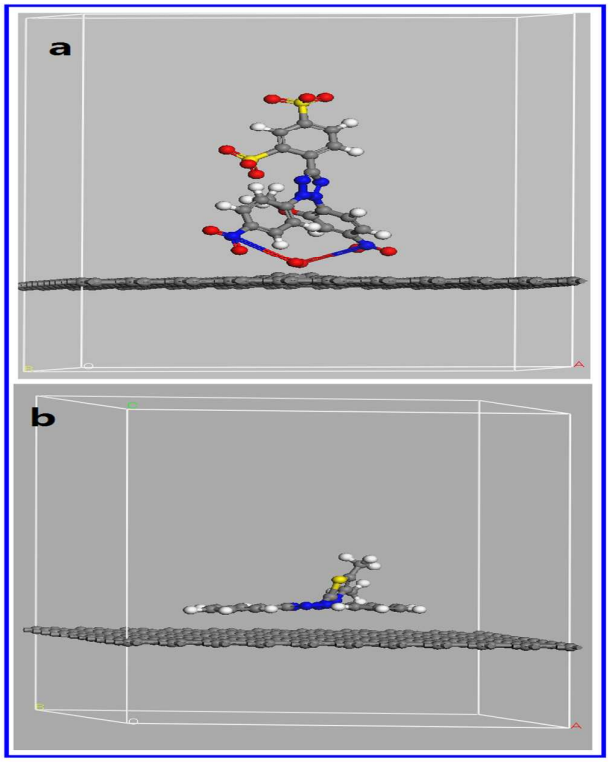

Fig. 4 The plan form of optimum structure of CCK-8 (a) and MTT (b)

molecules, the condition of orbital overlap and electrons coupling was lower than that of CCK-8 (Fig. S1.a†, and S1.b†). Compared with the intensity of electron transfer of CCK-8, the intensity of electron transfer from MTT to graphene is not pronounced. We inferred that maybe the unique spatial structure of substituent groups on benzene ring of CCK-8 molecule and its stronger π-π conjugation system play an important role in electron transfer. In this respect, the CCK-8 molecules were disturbed by graphene much more significantly than MTT molecules. So the activities of MTT/CCK-8 molecules were disturbed.

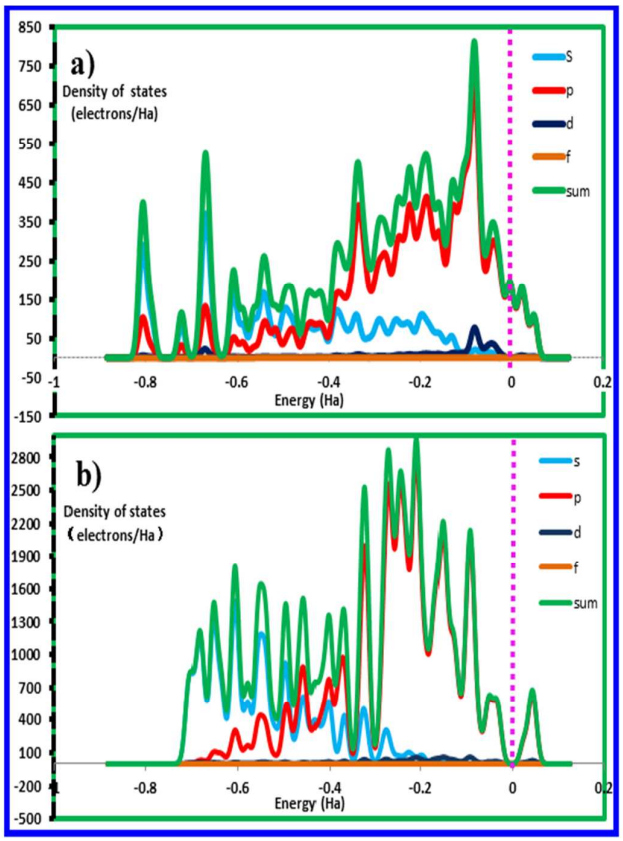

Fig. 5 The charge distribution and density of states of CCK-8 (a) and graphene (b)

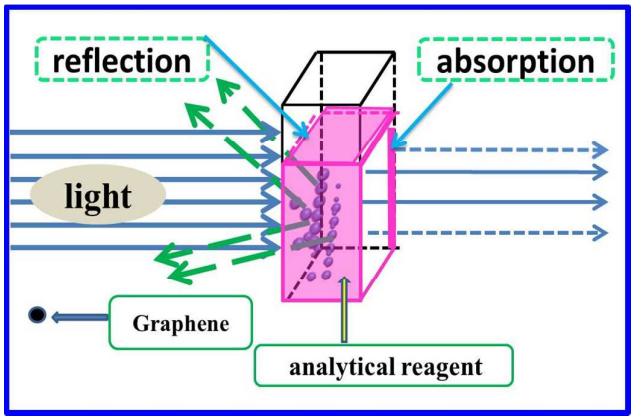

Fig. 6 The illustration of optical effect

Optical effect

The optical properties of graphene may be another interference for *in vitro* assay, including absorption and reflection effect (Fig. 6). Owing to the monolayer graphene having prominent light absorption property, while white light transmit through the single monolayer graphene, light intensity was weakened 2.3%, only 97.7% was able to travel through. Moreover, the absorption is increased with the graphene layers thickness, namely, adding each one layer of graphene, the absorption intensity was augmented 2.3%.[44,45] In previously reported, the single monolayer graphene can only reflect 0.1% less than the visible incident light, however, the reflection property is increased to 2% while the graphene are added to 10 layers.[45-47] Thus, the optical properties of graphene may cause detecting light signals loss *in vitro* assay.

Conclusions

In summary, our presented results in this work demonstrated that graphene can interfere with MTT and CCK-8 reagents and generated artifacts for the *in vitro* evaluation of graphene toxicity. Similar phenomena may occur in other common *in vitro* assays, such as 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2 H-tetrazolium hydroxide(XTT), Neutral red, Trypan blue, Commassie Blue, Alamar Blue, Hematoxylin and Eosin, Actin-Tracker Green, Propidium iodide (PI), Hoechst(33342, 33258) and others dye reagents. Clearly, further works are needed to screen and identify methods for assessment of graphene toxicity.

Acknowledgements

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