NEW APPROACH FOR BIOLOGICAL SYNTHESIS OF REDUCED GRAPHENE OXIDE

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Abstract

Graphene production is currently investigated due to its exceptional properties. This two-dimensional carbon nanomaterial has created great interest because of the potential applications in different fields. Graphene synthesis from chemical reduction of graphene oxide involves harmful reactants. This work shows the reduction capability of different isolated bacteria and a microbial consortium producing reduced graphene oxide efficiently. A battery of both gram-negative and gram-positive strains as well as a microbial consortium from natural environment were tested in graphene oxide reduction at simple operating conditions. Several techniques were used in reduced graphene oxide characterization, such as UV-Vis and Raman spectroscopies, thermogravimetric analyses or XRD measurements. Results showed biological reduction of GO by all microorganisms, under aerobic conditions and at 20-25 ºC. TGA analyses indicated that only weak weight losses of 2.5 % both at 200ºC and above 300ºC, were achieved for Shewanella baltica strain and Tinto river consortium related to the presence of oxygen functional groups, indicating the GO reduction. We propose a bacterial reduction strategy that involves dependent mechanisms of cellular metabolism and production of extracellular redox components, in addition to other mechanism, which does not imply the active participation of the cell. The method described in the present work is comparable to other biological as well as physicochemical processes and environmentally friendly taking advantage of natural resources for graphene synthesis.

Keywords

Reduced graphene oxide; eco-friendly process; biological reduction; microbial consortium, extreme habitat.

1. Introduction

Graphene is a carbon nanomaterial with one-atom thickness and two-dimensional structure forming a layer with similar appearance to a honeycomb with sp^2 bonded carbon atoms [1]. This material is remarkably versatile because its exceptional electronic, mechanical and thermal properties [2], making graphene potentially useful in a large range of research fields such as biotechnology, medicine, or electronics [3]. However, graphene production systems are the main concern that limits its use. In fact, graphene has been mainly obtained by reduction of graphene oxide (GO) through chemical, electrochemical or thermal methods. Although these processes are secure and scalable, the use of highly polluting reagents are involved, generating environmental and economic problems [4–6]. Currently, some studies direct their efforts towards the development of more environmental friendly techniques using natural reducing agents such as vitamin C [7], glucose [8,9] or plant extracts from leaves, roots or fruits [10]. Furthermore, methods based on biological GO reduction by using bacterial strains belonging to different genera like *Desulfovibrio* [11] *Geobacter* [12,13], *Shewanella* [14], *Staphylococcus* aureus [15] or *Azotobacter chroococcum* [16] are also considered as promising environmental alternatives. These microbial strains have in common that use insoluble substrates, such as metals or GO, as final electron acceptors producing their reduction [17,18]. Although, in some cases, toxic effects have been described for bacterial strain, as a consequence of the presence of graphene or GO, the effect can be modulated or even annulled using appropriate protocols [15,19]. Therefore, three possible mechanisms of extracellular electron transfer (EET) could be distinguished in GO reduction process, i.e., (i) direct electron transfer from the cell to the material surface, (ii) transfer through cellular structures and (iii) electron transfer through electroactive metabolites with redox capacity [20].

Biological reduction processes are notably influenced by both the nature of the bacteria species and environmental factors that affect bacterial metabolism such as temperature, presence of oxygen and carbon source. For example, a reduction of 60% in oxygenated functional groups from GO structure was observed by using *Escherichia coli* under anaerobic conditions and presence of glucose as carbon source [21]. In addition, *E. coli* showed capability to produce reduced graphene oxide (RGO) under aerobic conditions without any carbon source at 37ºC, its optimal growth temperature [22]. Moreover, *Shewanella* strains, another facultative anaerobic bacteria, have also reduced GO under both presence [14] and absence [23,24] of oxygen, reporting comparable results to other physicochemical reduction processes.

However, taking into account the wide range of metabolic characteristics provided by bacterial diversity, biological reduction processes should not be restricted to strains studied until the moment. On the other hand, results related to optimal conditions for biological reduction processes, are still sparse and contradictory [6,14,22,23]. In this work, we propose studies focused on new microorganisms with GO reducing capacity that also provide more information about the biological process, in order to promote the scientific, technological and industrial development of graphene. To this end, we develop a biological eco-friendly GO reduction processes by using new bacterial strains and a consortium that have not been used before for the reduction of GO. In addition, no chemical reducing agents, nutrients or substrates for bacteria have been added in our process using mild temperature conditions. Therefore, this procedure for the biological synthesis of RGO further improves the industrial viability of the process.

There is a lack of knowledge about bacterial mechanisms involved in GO reduction process. Most of the studies have been focused on *Shewanella* and *Escherichia coli* strains as bacterial models for GO reduction [14,22]. Therefore, the main goal of the

current study is to expand the spectrum of microorganisms with potential GO reduction capacity, ranging from single strains such as *Enterobacter cloacae* (gram-negative) and *Bacillus* (gram-positive) to a microbial consortium from a natural extreme habitat (Tinto river). For the characterization of RGO, several techniques, such as UV-Vis and Raman spectroscopies, thermogravimetric analyses or XRD analysis, were used.

2. Materials and methods

2.1. Graphene oxide

GO was purchased as an aqueous suspension (4.0 mg/mL) from Graphenea (San Sebastián, Spain). Prior to use, the mixture was sonicated for 40 min in a UP400S ultrasonic device (Hielscher Ultrasonics. Teltow, Germany) at maximum amplitude to facilitate the dispersion of the GO sheets.

2.2. Preparation of biomass

Different bacterial strains and consortium were used in GO reduction experiments. *E. cloacae* were isolated from a petroleum polluted soil [25]. *Bacillus sp.*, *E. coli* and *S. baltica* CECT323, were purchased from the Spanish Collection of Type Cultures (CECT. Valencia, Spain). The bacterial consortium RTc.15 was extracted from Tinto River (Huelva, Spain). All microorganisms were cultured in 50 mL of Luria Bertani medium (tryptone 10 g/L, yeast extract 5 g/L and NaCl 5 g/L) and incubated at room temperature and 150 rpm in an orbital shaker (New Brunswick Scientific. Edison, NJ, USA) under dark conditions until turbidity was observed. Bacterial growth was monitored by absorbance at 600 nm using a Cary-500 NIR/UV/Vis spectrophotometer (Varian. Palo Alto, CA, USA). Biomass was collected at the end of the exponential growth phase by centrifugation at 1500 rpm using a Digicen 20 centrifuge (Ortoalresa. Madrid, Spain). Then, the biomass pellet was washed with phosphate buffer saline

(PBS) (Panreac. Barcelona, Spain) and the procedure was repeated three times to remove any trace from the original nutrient medium.

2.3. Biological synthesis of reduced graphene oxide

The bacterial GO reduction process was developed under presence of oxygen and without carbon and nutrient sources to simplify the operating conditions of the process. The GO reduction assays were performed in 30 mL of GO dispersion in deionized water (0.4 mg/ml) inoculated with bacterial biomass (30 mg/mL). Replicates were incubated in an orbital shaker at 150 rpm during 72 h at 20-25ºC under aerobic conditions. Samples were collected at 48 and 72 h and stored at 4ºC until their characterization, in order to study the reduction extent.

2.4. Analytical methods of characterization

For RGO characterization, the collected samples were dispersed in a sonicator during 10 min and then, centrifuged twice at 1500 rpm for 15 min to separate cells. From the supernatant containing GO/RGO suspension, UV-Vis spectra were obtained using a Jasco V-630 spectrophotometer (Jasco. Madrid, Spain). Structural changes and stability of the samples were analysed by thermogravimetry using a TGA/DSC 3+ equipment (Mettler-Toledo. Columbus, OH, USA). Raman spectroscopy analyses were performed using a LabRam HR instrument (Horiba Scientific. Tokyo, Japan) with CCD detector, 632.8 nm He-Ne laser source and confocal microscope. In addition, X-ray diffraction (XRD) patterns were registered by an X'Pert MPD powder diffractometer (Philips. Amsterdam, The Netherlads) to further characterize the crystal structure of both GO and RGO. In these cases, samples collected in aqueous phase were previously dried at 65° C during 24 h. All analyses were carried out using GO/RGO samples at 48 and 72 h.

3. Results and discussion

Fig. 1 shows UV-Vis spectra of GO and RGO aqueous dispersions after testing reduction experiments by the different bacterial strains and consortium at 48 and 72 h. GO presented two absorption peaks centred at wavelength around 230 and 300 nm, respectively (Fig. 1). First peak is attributed to $\pi \rightarrow \pi^*$ transitions of aromatic C-C bonds and the shoulder at about 300 nm corresponds to the $n \rightarrow \pi^*$ transitions of C=O. After reduction experiments with different bacteria and consortium, the peak at 230 nm disappear and the absorption maximum gradually red-shifts up to 270 nm, approximately. These results prove an increase in the electronic conjugation, typical in graphitic and graphene-like structures, has been established [26]. In addition, the intensity of the shoulder at ~300 nm decreases due to carbonyl groups elimination in GO [27]. Clear evidence for this reduction was observed with *E. coli* and *S. baltica* (Figs. 1C and 1E, respectively). The above-described red-shift was also slightly present in RGO by *E. cloacae* (Fig. 1D). Nevertheless, *Bacillus sp.* (Figs. 1B) showed a maximum absorption peak at 230 nm, very similar to that of GO and with lower intensity at ~300 nm. Biologically RGO by *E. cloacae* (Fig. 1D) presented a maximum shift near 270 nm and no shoulder at ~300 nm was observed after 72 h. In agreement with previous reports [28], the absorption in the whole spectral range increased with time, except for *S. baltica* (Fig. 1E), suggesting progressing reduction of GO.

Digital pictures of aqueous dispersion solutions were taken for *E. coli* experiments before reduction and after 48 and 72 h of reaction time (Fig. 2). The lighter color of GO (bottle 1) quickly darkened in the presence of the microbial strains and gradually changes becoming black as observed in bottles 2 and 3, corresponding to 48 and 72 h of biological reduction treatment. The color of the suspension change is a sign of GO reduction degree as increasing the RGO absorbance [7,14].

Raman spectroscopy is a technique widely used in the determination of the structure and the presence of disorders or defects in graphitic materials [29]. Results of Raman measurements are presented in Fig. 3, which shows the spectra of GO and RGO after testing biological reduction with the different strains used. It is well known that Raman spectrum of graphite shows a remarkable band at 1580 cm^{-1} (G-band) due to the presence of sp^2 carbon atoms [30]. In the case of GO, the spectrum is characterized by two main broad bands usually observed at ~1340 cm^{-1} (D-band) and ~1590 cm^{-1} (G-band) (Fig. 3). The presence of D-band was assigned to the defects originated in the structure due to the extensive oxidation in GO with oxygen functionalities, indicating the distortion of the basal plane sp^2 domains [31].

RGO samples presented the same pattern of G and D bands with higher intensity as compared to Raman spectrum of GO. Furthermore, a G band shift was observed in RGO spectra compared to GO, suggesting a transition to graphene mediated by the bacterial strains [8,14]. In addition, Table 1 summarizes the intensity ratio between these two bands (I_D/I_G) calculated for both GO and RGO for all different microorganisms and consortium used in reduction experiments.

A progressive increase in the I_D/I_G of RGO samples was observed for 48 and 72 h compared to GO. Nevertheless, Raman spectra of GO treated with *S. baltica* and Tinto river consortium were especially remarkable due to a decrease in the ratio I_D/I_G with time, from 48 h to 72 h. According to the literature, I_D/I_G increases with the structural disorder of graphite [22,32]. Our results indicated that there was an increase of this value in all RGO cases at 48 h compared to GO which confirmed the reduction of oxygenated functional groups and the creation of structure defects [28]. Moreover, I_D/I_G values continue raising at 72 h except for *S. baltica* strain and Tinto river consortium where a decrease of the intensity ratio was observed, indicating less deformation of sp^2

domains in the structure [31]. As a result, in agreement with bibliography [33], the RGO product obtained after biological reduction of GO with *S. baltica* strain and Tinto river consortium presented a framework similar to graphitic crystalline structure.

Chemical transformations achieved by biological reduction of GO were again confirmed by thermogravimetric analysis (TGA) (Fig. 4). Original GO showed a small weight loss (~5 wt%) below 100ºC, corresponding to the removal of physically adsorbed water. Then, a larger weight loss of around 25 wt% at 200ºC was attributed to the pyrolysis of the less stable oxygenated functional groups, such as carboxylic and ketone groups [34,35]. Finally, a third weaker, but significant, weight loss observed in GO above 300ºC which amounts to ~20% of total weight loss was assigned to the removal of more stable functional groups [36].

TGA of RGO samples after reduction, at 48 h and 72 h, with all biological agents showed a very different behavior to that found for GO (Fig. 4). Weaker weight losses (lower than ~2.5 wt%) were observed in RGO curves below 100ºC compared to GO. Taking into account that all samples where pre-treated in the same way before TGA measurements, there was more water absorbed in GO samples, suggesting that RGO exhibited less oxygen-containing functional groups acting as hydrophilic adsorption sites for polar water molecules. The degree of GO reduction was also measured by a second mass loss at 200ºC. It was observed that, a weight loss of around 5 wt% was achieved for *Bacillus sp.* and *E. cloacae*. A slightly smaller loss (~3 wt%) was measured for *E. coli* (Fig. 3C), similar to the mass loss (~2 wt%) found for *S. baltica* and Tinto river consortium (Fig. 4). Thereby, in all cases clearly lower weight losses were achieved at this temperature for RGO after biological treatment compared to that measured for GO (25%). As a result, the labile oxygen functional groups in graphene oxide were remarkably removed by biological reduction especially after treatments with

Tinto River consortium and *S. baltica*, since higher thermal stability was achieved. Finally, the third weaker weight loss above 300ºC observed in RGO plots assigned to the removal of more stable functional groups was around ~2-5%. Therefore, RGO showed higher thermal stability as compared to GO where the mass loss observed was around 20 wt% within the same temperature range.

With the aim of evaluate the structural information of the reduced nanomaterial obtained, XRD patters of GO and RGO were determined and compared. As shown in Fig. 5, GO exhibits its characteristic broad peak at 10.3° that corresponds to an interlayer d-spacing of ~8.4 Å [37,38] mainly due to the presence of oxygenated functional groups in GO as well as water molecules located within the interlayer space of the hydrophilic GO [39]. However, this peak disappeared from the XRD pattern of RGO samples after the bacterial reduction processes. This change is indicative of GO reduction due to the removal of the those oxygen-containing functional groups [8,37,40]. After biological or chemical reduction of GO, other authors reported a broad peak within the range 23-27° [38,41,42]. This reflection is close to the characteristic well-defined peak of graphite at 26.4º (d-spacing 0.33 nm), corresponding to the distance between the stacked layers. Therefore, the absence in our patterns of this peak observed by other authors in samples of reduced GO could indicate the predominant presence of single RGO sheets.

In this work it is shown, for the first time, that *Bacillus* sp. and *E. cloacae* strains as well as the microbial consortium from Tinto river, are able to reduce GO under aerobic conditions. These results, therefore, open the range of bacterial strains capable of reducing graphene oxide beyond the strains described so far [22,23] and notably, it is shown that bacterial strains physiologically very different and taxonomically little related, can be used for the biological production of graphene.

However, little is known about this biological process and the bibliography is contradictory to describe the biological mechanisms involved, as well as the optimal conditions [14,21–23]. However, these are related and dependent factors. There are few works [14,23] that address this issue and most are focused on *Shewanella* as model microorganism. These works suggest that the process is mediated by inner/outer membrane protein systems and cytochromes typical of this genera and/or other mechanism through self-secreted electron shuttles [24]. Nevertheless, we have proved that the nature of GO reducing microorganisms can be very diverse, and therefore the mechanisms and cell structures differs involved in the process. We propose a possible bacterial reduction strategy (Fig. 6) in which, in addition the mechanisms similar to those above described [14,23,24], some cellular components with reduction power could contribute to GO reduction, when they are released to media by cellular lysis. However, further studies are necessaries to decipher the mechanisms involved in the reduction process depending on the strain and to determine the best conditions for an effective process.

4. CONCLUSIONS

Our original results demonstrate the biological reduction of graphene oxide by a method using different bacterial strains and a microbial consortium isolated from Tinto River as biological reducing agents. Our work presents a novel and environmentally friendly alternative system to produce graphene under minimum logistic and economic requirements. These significant results highly contribute to increase the lack of studies based on biological processes obtaining graphene. Among the broad microbial diversity, we describe new bacterial strains and natural consortia capable to synthetize RGO, beyond what has been described so far. Furthermore, we are developing new studies in

order to provide greater knowledge about the microbial mechanisms involved in the process, as well as improved conditions in order to optimize the biological process.

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Table 1. Intensity ratio of bands D and G (I_D/I_G) measured in Raman spectra of GO (A) and RGO, after 48 h and 72 h, by *Bacillus* sp. (B), *E. coli* (C), *E. cloacae* (D), *S. baltica* (E) and Tinto river consortium (F).

		RGO									
	A	B		C		D		E		F	
		48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h
I_D/I_G	1.09	1.08	1.20	1.13	1.13	1.15	1.17	1.30	0.99	1.11	1.05

Caption of figures:

Fig. 1. UV-Vis spectra of GO (A) and RGO after biological treatment with *Bacillus* sp. (B), *E. coli* (C), *E. cloacae* (D), *S. baltica* (E) and Tinto river consortium (F). Dashed and solid line spectra correspond to 48 and 72 h, respectively.

Fig. 2. Biological reduction of graphene oxide by *E. coli*. Illustration of aqueous dispersions of GO (bottle 1) and suspension of RGO after biological reduction treatment with at 48 and 72 h (bottles 2 and 3, respectively).

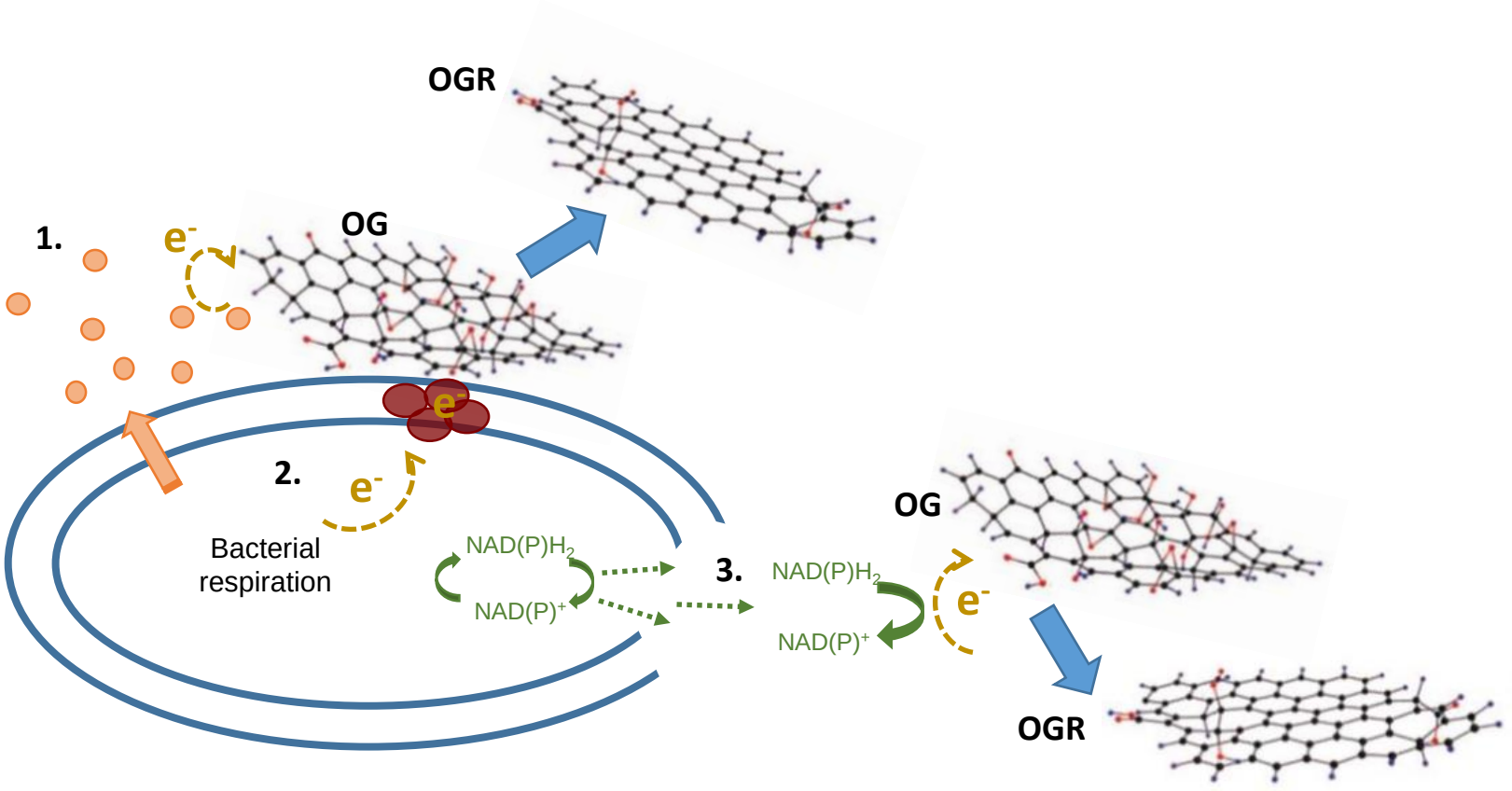
Fig. 3. Raman spectra of GO (A) and RGO after microbial treatment with *Bacillus* sp. (B), *E. coli* (C), *E. cloacae* (D), *S. baltica* (E) and Tinto river consortium (F). Dashed and solid line spectra correspond to 48 and 72 h, respectively.

Fig. 4. Normalized TGA plots for: GO (i) and RGO after biological reduction at 72 h with *Bacillus sp.* (B), *E. coli* (C), *E. cloacae* (D), *S. baltica* (E) and Tinto river consortium (F). Full scale (A) and zoom in on y-axis (B) to appreciate the differences between TGA plots of RGO produced by different biological treatments.

Fig. 5. XRD patterns of GO (A) and RGO after microbial treatment with *Bacillus* sp. (B), *E. coli* (C), *E. cloacae* (D), *S. baltica* (E) and Tinto river consortium (F) at 72 h.

Fig. 6. Bacterial reduction strategy mediated by the following three mechanisms: (1), OG reduction by self-secreted electron shuttles; (2), GO reduction by direct contact cell-GO and electron transfer mediated by inner/outer membrane proteins complex similar to *Shewanella*; (3), extracellular reduction by redox intracellular components released by lysis.

Graphical Abstract



Figure

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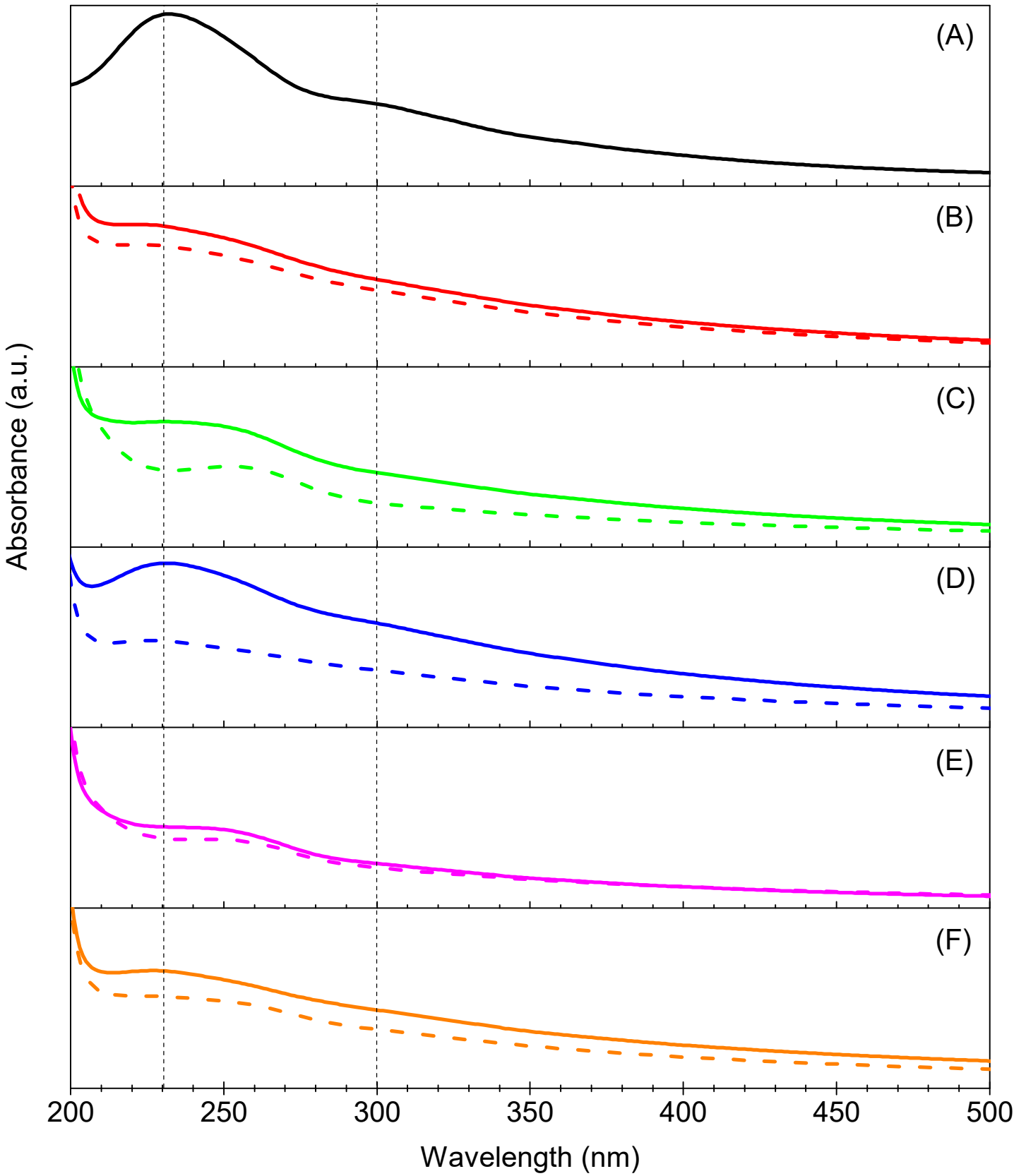

Figure 2

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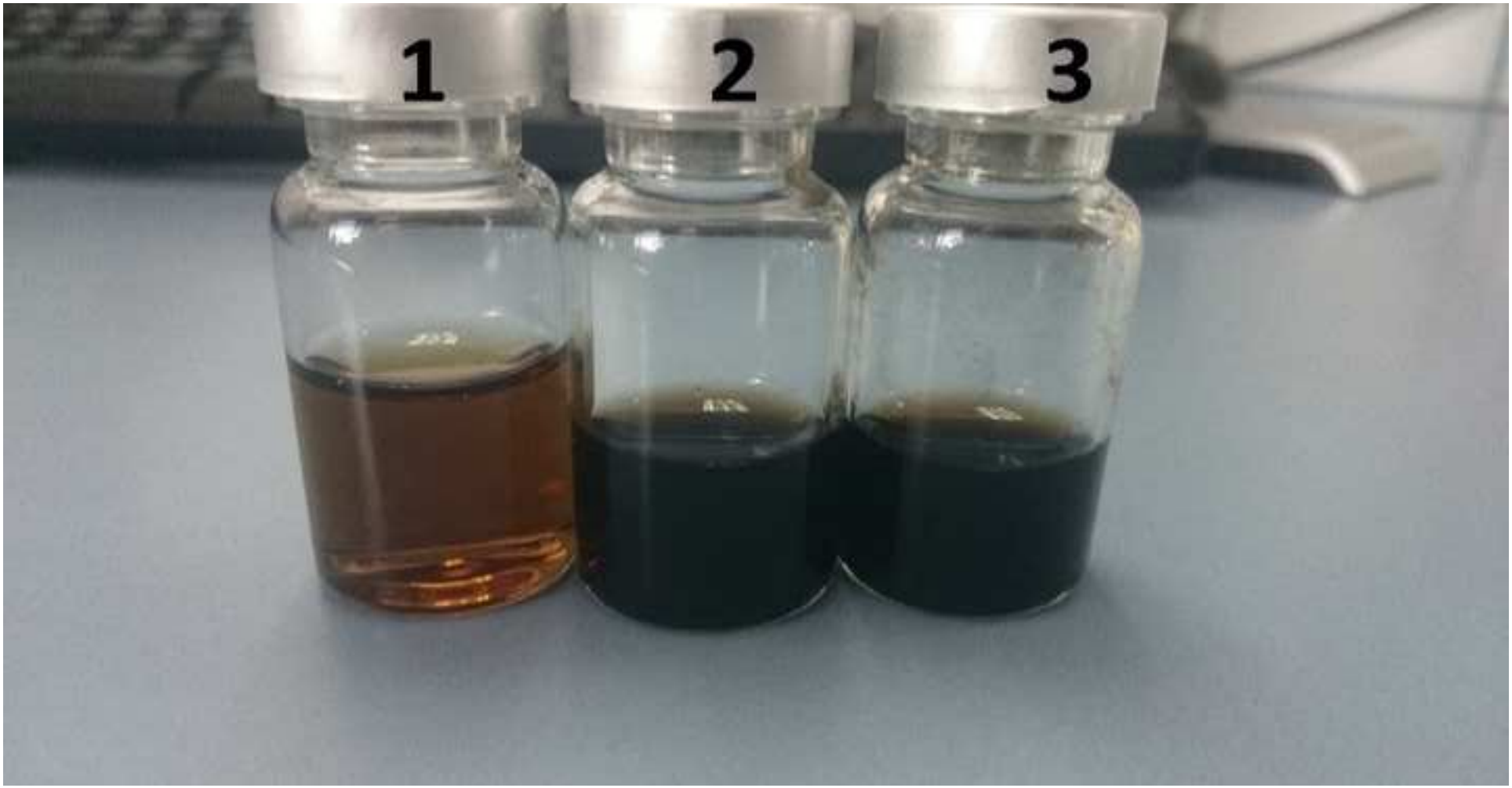

Figure 3

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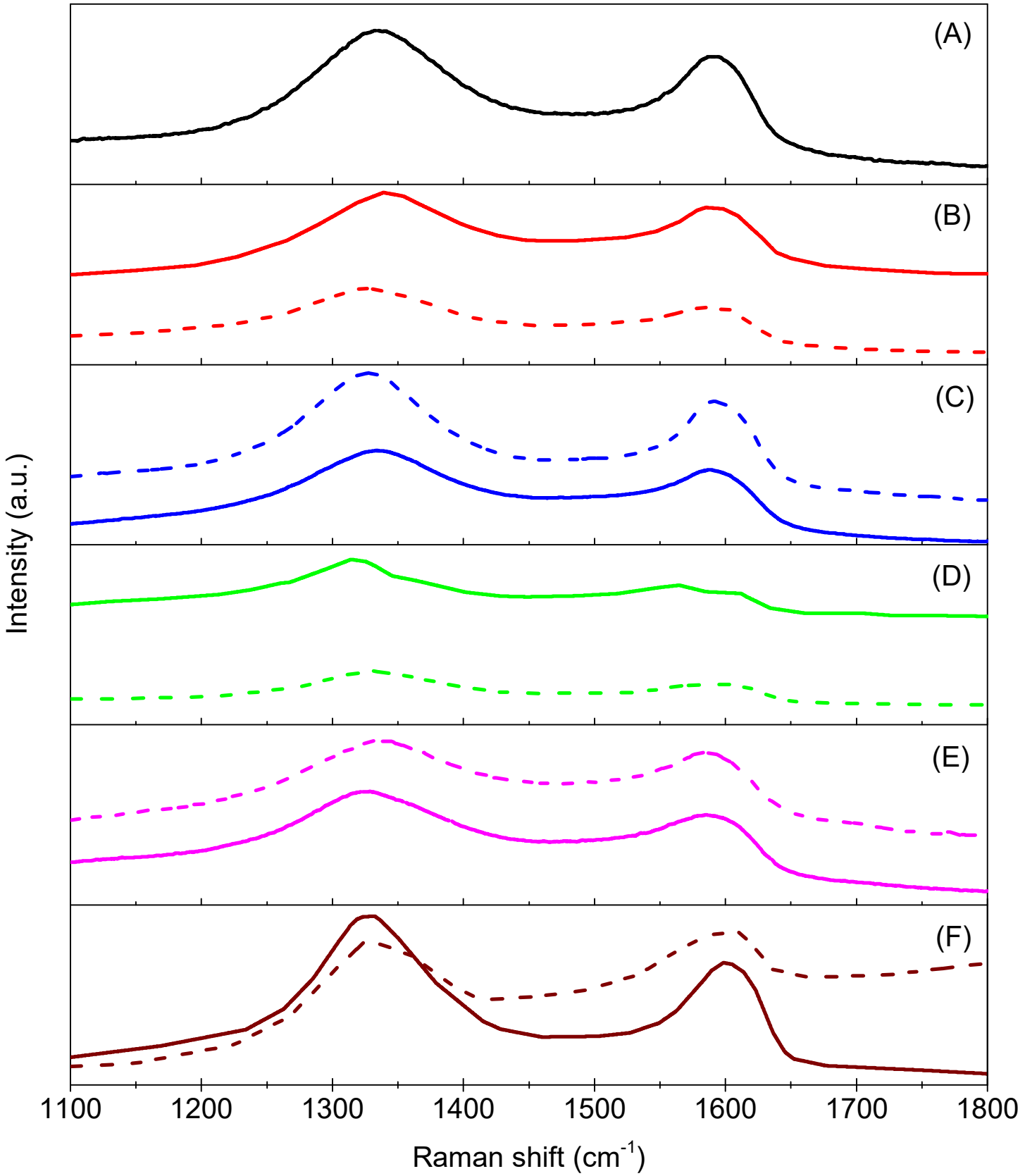

Figure 4

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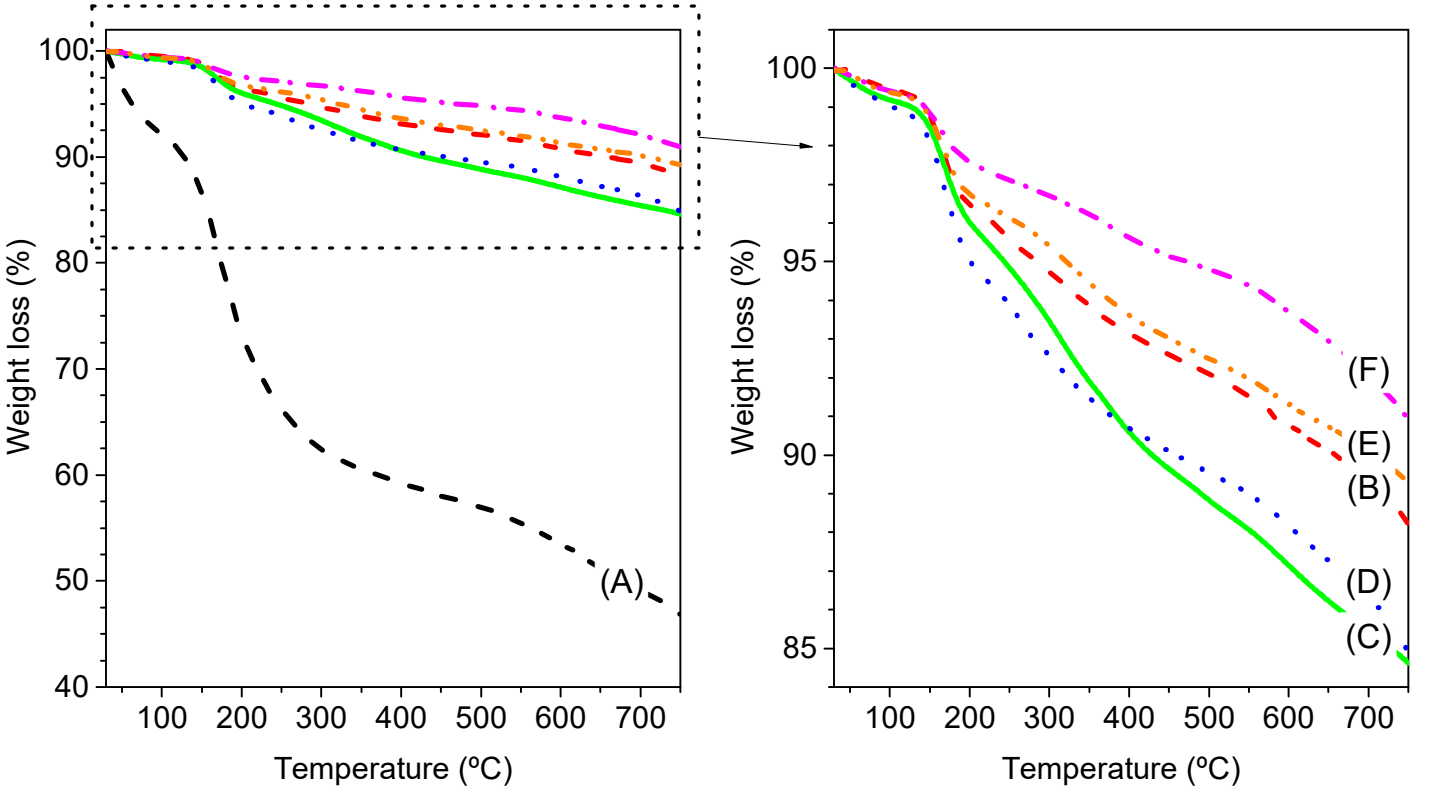

Figure 5

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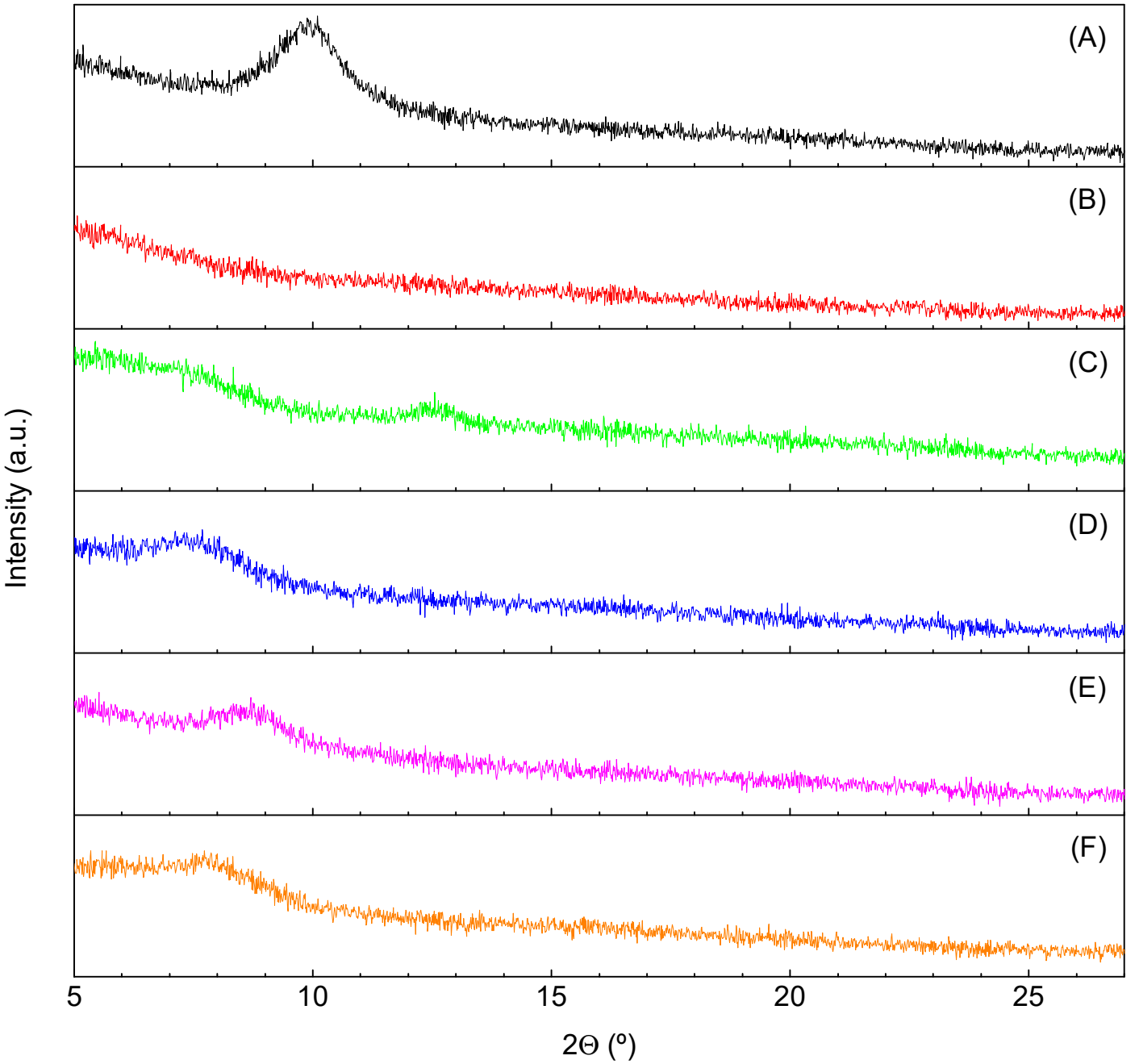

Figure 6

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