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Graphene nanoflakes as efficient ionizing matrix for MALDI MS based lipodomics of cancer cells and cancer stem cells

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Abstract

This study demonstrates that graphene nanoflakes can be efficiently served as a successful interference free MALDI-MS matrix, the method is referred as graphene assisted laser desorption/ionization mass spectrometry (GALDI MS), for lipidomic analysis of cancer cells and cancer stem cells. The *in house* synthesized graphene nanoflakes exhibited transparent flake like shape with characteristic crumpled silk waves in TEM and typical absorption characteristics upon UV-Vis (λ_{max} = 270 nm) and FTIR spectroscopic analysis. Graphene nanoflakes have been tested as a sole matrix and co-matrix with traditional MALDI MS matrices for the lipid extracts obtained from normal breast, cancer and cancer stem cells with four different spotting

methods. In all the cases, graphene nanoflakes displayed a noise free and good quality mass spectrum. This study reveals the possibility that lipid could self-assemble as a multilayered structure on the graphene nanoflake platform through electrostatic interactions between graphene nanoflakes and lipid head groups and thus results in noise free spectra during GALDI- MS based lipidomics.

Keywords: graphene nanoflakes, cancer, cancer stem cell, mass spectrometry

Introduction

Cancer diagnosis has been attracted a huge attention nowadays towards finding out its cause and providing measures for treatment. Cancer cells result owing to uncontrolled and altered cell cycle programs. These cells could invade normal tissues or organs and lead to the development of malignant neoplasms over a period of time. Cancer stem cells (CSCs) have properties that are similar to stem cells, which are capable of self-renewal and differentiation[1]. It's not only regarded as a key to tumor formation, but also is related with cancer metastasis and recurrence.[2-4] Since CSCs play an important role in tumor initiation, many researchers aim towards geneomic[5] and proteomic[6-7] profiling in order to find out potential biomarkers and for developing targeted therapies[8-9].

Mass spectrometry (MS) is a very powerful analytical tool known for its rapidity,

simplicity and ease of handling. The Nobel Prize in Chemistry in 2002 was issued to Koichi Tanaka (MALDI-MS) and John B. Fenn (ESI-MS) for developing soft ionization techniques[10]. Matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) is a laser desorption/ionization MS developed in 1985 by Franz Hillenkamp (using organic acids as matrix) and Kochi Tanaka (used Co NPs as the ionization matrix)[10] Both techniques have been applied to biomolecule analysis and paved a doorway for interdisciplinary research involving, proteomics, lipidomics, glycomics, metabolomics and even genomics. However, in MALDI-MS analysis, matrix interference is a serious limitation when analyzing low molecular weight biomolecules below 500Da. To solve this problem, matrix free techniques have been invented[10]. With the introduction of nanomaterials into mass spectometry, the detection limit, sensitivity and the ability to analyze real world (complicated) samples have been significantly improved..In 2004, graphene was synthesized and established in a short time as a novel material and also intensely explored for various applications starting from modern electronics/bioelectronics to biological science such as disease diagnosis, antiviral, antibacterial, biosensing, cancer cell imaging, targeting and photothermal therapy, drug delivery and tissue engineering..[11] Graphene belongs to carbon nanomaterial with one to a few atomic layers.. Thus, it offers ultra-high surface area (2630 m^2g^{-1}) and delocalized electron properties owing to carbon-sp^2

hybridization bringing about π-π interaction with aromatic compounds or drugs. It was applied as a matrix for detection of molecules with low molecular weight metals and other analytes[12-14]. Recently, G has been proposed as an effective and self-organized differentiation of human neural stem cells (hNSCs) into neurons using the pulsed laser stimulation[15]. In association with Au NPs, graphene have also been used for the discrimination of cancer cells and cancer stem cells from human breast normal cells[16].

In the current study, for the first time, we have used graphene nanoflakes as a successful interference free matrix for lipidomic analysis of cancer cells and cancer stem cells using MALDI-MS. We call this technique as GALDI-MS. The ideal sample spotting method using graphene nanoflakes as matrices was optimized. We attempted the use of graphene nanoflakes as matrices and also as co-matrices for the analysis of cancer and cancer stem cell lipids during MALDI-MS analysis.

Materials and methods

Chemicals

$KMnO_4$ was purchased from Riedel-de Haën (Seelze, Germany). Natural graphite (-20+84 mesh, Sulfuric acid, nitric acid and hydrogen peroxide were purchased from Alfa Aeser (Great Britain). Methanol (HPLC grade) and potassium permanganate were purchased from Merck. Co (USA). All chemicals were used as received without

further purification. The de-ionized water obtained from a Milli-Q Plus water purification system (Millipore, Bedford, MA, USA) was used for all the experiments carried out.

Instruments

The Fourier transform infrared (FT-IR) spectra of G nanoflakes were recorded on a FT-IR spectrometer (Spectrum 100, Perkin Elmer, USA). The morphology was determined using a transmission electron microscope (TEM, Phillip CM200, Switzerland) at an accelerating voltage of 200 kV, and scanning electron microscopy (CM200, Philips) and elemental analysis was carried out using energy dispersive X-ray (EDX).

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) analysis was performed using a time-of-flight mass spectrometer (Microflex, Daltonics Bruker, Bremen, Germany) equipped with a 1.25 m flight tube by employing the reflection positive (RP)/negative ion mode (RN) (N_2 laser, 337 nm, pulse 3 ns) and accelerating potential in the source was maintained at +20 kV. All MALDI-MS spectra were obtained using an average of 200 laser shots. The laser power was adjusted to slightly above the ionization threshold of the cell lipid lysate to obtain significant resolution.

Preparation of graphene nanoflakes

From graphite precursor, graphene oxide (GO) was prepared and subsequently from

GO aqueous graphene was prepared. The oxidation/reduction of natural graphite to graphene was performed using the modified Hummers method [17]and the related procedures are described below.

Preparation of graphene

Natural graphite (1 g) was dispersed in 25 mL of deionized water and acid mixtures of nitric acid (10 mL, 69-72%) and sulfuric acid (15.0 mL, 96.0%) was added in acid-bath. Then, potassium permanganate (3 g, ≥99%) was added in stepwise and the mixture was stirred for 2h then the temperature was rised to 35 °C for 1h. The temperature was kept < 0 °C using an ice-bath. After removing the ice-bath, the hydrogen peroxide (30-32%, 15 mL) was added drop wise to remove the excess of permanganate (till bubbles stopped). After magnetic stirring for 2h, distilled water (200 mL) was poured slowly into the mixture to obtain a dark brown colloidal suspension. After further stirring for another 30 min, the dispersion was filtered, and then washed several times with a 5 wt% HCl solution to remove metal ions. The synthesized material (Graphene oxide, GO) was dried and kept for the next step i.e reduction to G. Colloidal solution of graphene oxide (GO, ~0.5 mg/mL, 25 mL) was prepared by ultrasonication (Sunway Scientific Corporation, LC30H) for 30 mins, followed by centrifugation at 4000 rpm for 1h. The aqueous solution of hydrazine (5 μL, 35 wt% NH_2-NH_2 in water) was added after which ammonium hydroxide solution

(35 µL, 28-30 wt%, J.T.Baker) was added into the flask. The mixture was subjected to magnetic stirring in an oil-bath at 100 ºC for 2 h to reduce GO to graphene nanoflakes. The synthesized graphene nanoflakes was characterized using Transmission electron microscope (TEM), SEM, EDX FT-IR, and UV-vis spectroscopy.

Cell cultures

Breast cancer stem cells ($HMLER^{sh\ Ecadherin}$), Breast cancer cells (control $HMLER^{sh\ Control}$) and Breast normal cells ($HMLE^{sh\ Control}$) were received from Dr. Robert Weinberg (MIT) as a generous gift. The cells were grown in MEGM medium and DMEM (1:1) supplemented with 10% FBS, insulin and hydrocortisone with 1% antibiotic mixture. The cells were grown at 37 ºC in a humidified incubator and maintained under 5% CO_2. The microscopic observations were made using an inverted imaging microscope (ESPA Inc, Taiwan). After the cell reached 80% confluence, the cells were trypnized in order to split the cells. The trypsinization was carried out for 3 min using 2.5% trypsin solution and followed by neutralization with 20% DMEM. The cells were washed with PBS solution three times and enumerated using a Haemocytometer (Marenfeld, Germany). Breast normal, cancer and cancer stem cells at the concentration of 1×10^6 cells were used for the lipid extractions.

Extraction of lipids from cell cultures

The lipid extraction from human breast normal, cancer and cancer stem cells was carried out by following the method described by Folch et al.[18]. Typically, to the cell suspension (200 µL in PBS), 600 µL chloroform: methanol (2:1, v/v) was added and

votexed well for 10 min. The lipid containing bottom layer was collected after phase separation in vials and stored at 4 ^{o}C until further use.

MALDI MS analysis of lipid samples obtained from human breast normal, cancer and cancer stem cells.

For the MALDI MS analysis of cellular lipids, 4 different sample preparation methods with graphene nanoflakes were **applied as below:** (1) dried droplet method, (2) Thin layer method, (3) Sandwich method and (4) Sonication method. Graphene nanoflake was used as the matrix in all the methods.

(1) Dried droplet method

In this method, graphene nanoflakes were dispersed in ethanol by mild sonication for 10 min. The matrix (graphene nanoflakes) was added to lipid (analyte) in the ratio 1:1 (v/v) and spotted on MALDI-MS target plate and air dried before MALDI-MS analysis.

(2) Thin layer method

Graphene flakes were dispersed in ethanol by sonication for 10 min. A thin layer of graphene flakes (matrix) were laid on the MALDI-MS target plate and allowed to air dry before spotting the sample. Then equal quantity of the lipid sample was spotted onto the dried matrix layer and again dried prior to MALDI-MS analysis.

(3) Sandwich method[19]

The thin layer sample preparation method was additionally overlaid with a layer of graphene nanoflake matrix, so as to make a sample layer sandwiched between 2 layers of graphene nanoflakes (matrix). After drying the spot, MALDI-MS analysis was carried out.

(4) Sonication method[14]

10 μL graphene nanoflake (matrix) was incubated with 200 μL lipid sample and sonicated for 10 mins. The mixture was then, centrifuged at 18,000 rpm for 10 mins. The pellet was re-suspended in 10 μL 50% ethanol ((v/v) in dH_2O). 5 μL of solution was spotted on to the target plate and dried completely before MALDI-MS analysis.

MALDI-MS detection

All the mass spectra were obtained from a MALDI-TOF MS (Microflex, Daltonics Bruker, Bremen Germany) equipped with a nitrogen laser (337 nm) and the accelerating voltages were acquired at +20 kV. All the results were performed under similar conditions in reflection positive/negative ion mode with laser energy 63.2 μJ and 200 laser shots.

Results and Discussions

Characterization of graphene

Graphene nanoflasks were prepared via harsh oxidation of graphite using strong oxidizing agents i.e potassium permanganate in acidic acid solution (HNO_3, H_2SO_4). The role of the strong oxidizing agent is to break the Van der Waals forces among the graphene layer of graphite. The morphology and the elemental composition of the *In house* synthesized graphene material was characterized using electron microscopy (TEM and SEM) and the reaction progress was monitored by following the function groups changes using FTIR and their absorption using UV-vis spectroscopy as shown in Figure 1 (A-C). As can be seen in the TEM image (Figure 1A), graphene has a nearly transparent flake like shape with characteristic crumpled silk waves and it is obvious in the high resolution TEM image (inset of Fig.1A). Due to the conjugated π-bonds, graphene exhibits a maximum UV absorption at about 295 nm (Figure 1B). Furthermore, it also absorbs UV wavelength in the range of 250-350 nm which could enable it to serve as a matrix for LDI-MS analysis on a typical MALDI instrument equipped with a N_2 laser (337 nm)[12]. The maxium absorption at 270 nm is well matched with the reported data [14]. This observation reveal the potential of the prepared material to serve as surface substrate for MALDI MS measurement i.e surface assisted laser desorption/ionization mass spectrometry (SALDI-MS). Figure 1C displays the FTIR spectra of G and GO. The intense peaks at 3450 and 1250 cm^{-1} are attributed to the stretching of the O-H band of C-O. The band at 1650 cm^{-1} is

associated with stretching of the C=O bond of the carboxyl groups. The FTIR spectrum (Figure. 1C) confirms the two step graphene (oxidation-reduction) process due to the absence of the carbonyl group (C=O) [20]. The morphology and the chemical composition of the in house synthesized G nanoflakes were confirmed by SEM (Fig.1D). SEM micrograph represents the nanofalsks of G and EDX represent almost pure carbon with a tiny amount of oxygen remains from the reduction process. EDX analysis further reveals the complexness of the oxidation/reduction process.

Cancer and cancer stem cells lipids analysis by MALDI MS with traditional organic matrices

Before the lipid analysis from cancer cells and cancer stem cells by MALDI MS, the stemness of cancer stem cells was verified to confirm its authenticity. For the verification of stemness of cancer stem cells, we had grown the breast cancer cells and breast cancer stem cells in a non-adherent condition. The mamosphere, a unique property of breast cancer stem cells, was observed with breast cancer stem cells and thus we confirm the stemness of stem cells.

The overall scheme for the lipidomic analysis of breast normal, cancer and cancer stem cells with graphene nanoflakes as the matrix and co-matrix is shown in Fig S1.

For matrix optimization, we attempted to use well known standard matrices such as 2,5-dihydroxybenzoic acid (DHB) and 9-aminoacradine (9-AA) for lipid analysis in MALDI-MS[21] with the ideal detection modes of reflection positive/negative ion mode. In Figure 2, we observed that both the matrices were suitable for different detection mode for lipidomic analysis of cancer and cancer stem cells. While, the 9-AA was promising as the matrix in the negative ion mode (Fig. 2A), as shown by the more distinct lipid pattern and less matrix interference (Fig. S1A) compared with DHB in the reflectron negative mode (Fig. 2B). However, in the reflection / positive mode, the DHB matrix performed significantly better (Fig. S1B). In our studies, the results showed that the range of detection was within 1,500 Da, and within this area the peaks from the normal cell, cancer cell and cancer stem cell lipids were observed clearly (Fig. 2B), in contrast to the 9-AA in reflection positive mode (Fig. S1B). Thus, for this study we used the 9-AA matrix to analyze the cell line lipid samples in reflection negative mode; for the positive detection mode, we chose DHB as the matrix. Data revealed also showed the presence of intensive interference of peaks from both conventional matrices (i.e DHB and 9-AA) that supress some of the lipid peaks due to high ionization ability of those compounds. Thus, some of the lipid peaks had been overpovered by the conventional matrices and no longer detected in the conventional MALDI MS analysis. For instance, less polar lipids are suppressed

in the presence of crude lipid mixture that is rich in phosphatidylcholines (PCs)[22].

Graphene nanoflakes as co-matrix and matrix for lipidomics of cancer and cancer stem cells

Due to the specific characteristics of G nanoflake such as its excellent UV absorption at 200-350 nm (λ_{max} = 270 nm), it was speculated to absorb the N_2 laser (337 nm) in this range and could perform energy transfer to the surrounding molecules and cause better ionization upon laser attack during MALDI-MS. Because the large surface areas of G nanoflakes, it can also work as surface for SALDI-MS. Therefore, we have probed the possibility of using graphene nanoflakes as either co-matrix or novel matrix for the detection of the cell line lipids. Furthermore, G nanoflakes are believed to display a hydrophobic surface, which is similar to the property displayed by lipids. Hence, it was assumed that G nanoflakes could also show some affinity towards lipids in chloroform and facilitate better lipid detection either as co-matrix or a sole matrix, (even in the absence of traditional MALDI MS matrices) during MALDI- MS analysis.

Cancer and cancer stem cell lipids had been analyzed initially by the following combinations such as (i) combination of 9-AA/graphene nanoflakes (Fig. 4A) and (ii) DHB/graphene nanoflakes (Fig. 4B), where G nanoflakes were employed as the co-matrix combined with the conventional MALDI matrices. As co-matrix, G

nanoflakes showed the better enrichment of MALDI MS peaks from the lipid extract obtained from cancer and cancer stem cell lipids (Fig. 3A and B) when compared with the traditional organic matrices (Fig 2A and 2B). Furthermore, it also suppresses the interferences of the conventional matrices.

Based on the efficiency of G nanoflakes as the co-matrix in MALDI-MS analysis of lipids, it was also attempted to exploit it as a sole novel MALDI-MS matrix for the lipid analysis. Thus, for the lipid analysis from cancer and cancer stem cells, four different sample preparations methods, with G nanoflakes as matrix, had been employed as follows, (1) dried droplet method (Fig. 4A, B (b)), (2) thin layer method (Fig 4A, B (c)), (3) sandwich method (Fig. 4A, B (d)), and (4) sonication based method (Fig 4A, B (e)). The results showed that irrespective of the method used, G nanoflakes worked as efficient matrix under both positive/negative ion detection modes in MALDI-MS. The method used did not have any specific positive contribution to the results obtained; this showed that the G nanoflakes application as matrix was not specific to the spotting method. However, through our studies, we have observed that in positive mode, more lipid peaks and more signals were obtained compared to that of negative mode. Therefore, the subsequent MALDI- MS of lipid analysis were conducted with G nanoflakes as a matrix in the positive MALDI-MS mode. In this approach, G nanoflakes replaced the traditional matrix in MALDI-MS

and therefore we describe the entire MALDI-MS analysis as Graphene Assisted Laser Desorption Ionization Mass Spectrometry (GALDI-MS).

Graphene nanoflakes assisted LDI-MS (GALDI MS) cancer lipidomics profiling for the discrimination of normal, cancer, and cancer stem cells.

Discrimination of the human normal,-, cancer and cancer stem cells is an urgent need for early cancer diagnosis and treatment. Thus, lipids have long been considered as one of the cancer biomarkers due to their differential expression on various cell types and thus, these cells (normal, cancer, and cancer stem cells) have different lipid contents. Mass spectrometry is already known as a powerful tool for its rapidity and sensitivity in the molecular analysis, Thus, the significance of this tool is more evidenced in discrimination of different molecules appeared in human normal, cancer and cancer stem cells. Lipid membranes also serves as biomarkers for cancer early diagnosis by using various nanoplatforms [23].The results showed that G nanoflakes matrix could lead to more resolved lipid peaks in the RP mode (Fig 4A, 4B, 4C) than the RN mode (Fig S3A, S3B, S3C) and some of peaks the spectra could be identified based on available literature (Table. 1) Especially, the peak at *m/z* 184 is reported to belong to a well reported biomarker phosphatidylcholines (PCs), believed to show up in the diseased cells. Kang et al., report that these phosphatidylcholines were over expressed in the breast cancer cells[24]. It is important to note that PCs are sensitive to

conventional matrices and undergo decomposition due to the acidity of those compounds. However, it was detected by positive ion mode using the present method. Schiller et al. reviewed the reported achievements in MALDI-TOF mass spectrometric analysis of lipids [25]. The peak observed at *m/z* 880 Da could possibly be cerebroside [Cer d18:0/26:0h+Na]$^+$, [Cer d18:1/25:0h+K]$^+$ that matched very well with the literature [22b].

In comparison with the other techniques, differentiation of the cells based on mass spectrometry is simple, fast, sensitive, high throughput and cheaper. Recently, Lu et.al[26] used nanoplatform based microRNAs as promising biomarkers for the diagnosis and prognosis of early-stage cancer (Table 2). Although, this technique works well, it is time dependent. Akhavan and Ghaderi[15] reported G for pulse laser simulation for differentiation of human neural stem cells (hNSCs). However, it require the cancer cell to culture on the graphene films for three days and allowed to differentiate for hNSCs into neurons, under pulsed laser stimulation more than three weeks. Pallua et.al [27] reported a new biomarker to improve the recognition of the prostate cancer detection and characterization of individual tumors based on the proteomics profiling. Imaging analysis of cancer, non-malignant benign epithelium and stromal areas of 15 prostatectomy specimens in a test and 10 in a validation set identified characteristic *m/z* peaks for each tissue type, e.g. *m/z* 10775 for benign

epithelial, *m/z* 6284 and *m/z* 6657.5 for cancer and *m/z* 4965 for stromal tissue. They extract the proteins of the prostate cancer tissue by nano-HPLC and subjected to MALDI TOF/TOF analysis.Finally, efforts were also made for the identification of the lipid peaks using graphene nanoflakes assisted laser desorption ionization mass spectrometry (GALDI-MS). GALDI-MS comparative spectra for the lipids obtained from the breast normal (Fig 5 (b)), cancer (Fig 5 (c)), and cancer stem cells (Fig 5(d)) are shown in Figure 5. Previously, most of cancer cell differentiation relied on proteomics based analysis to pick out specific cancer related proteins or biomarkers in order to differentiate normal and cancer cells. In our studies, we made some attempts to extract or obtain additional biochemical information based on the differences in the lipid profile using the GALDI-MS approach. Our results showed that most of the peaks from cell lipids were similar; however, some displayed differential expression. For instance, in the spectra of breast normal cell lipid (Fig.5 (b)), the peaks of *m/z* at 184.3, and 224.6 could be assigned as $[C_5H_{15}NPO_4]^+$ and $[C_8H_{19}NPO_4]^+$ ions of phosphocholine headgroup; the peaks of *m/z* at 551.7 could be assigned as $[C_{35}H_{67}NPO_4]^+$ ion of dipalmitoyl[28]; the peaks of *m/z* at 279.9, 307.4 could be assigned as $[M+Na]^+$ ion of n-hexadecanoic acid (C_{16}) and n-octadecanoic acid (C_{18})[14]; the peaks of *m/z* at 409.6 could be assigned as $[M+Na]^+$ ion of cholesterol[12]. These are all present in the case of the cancer cell lipids (Fig. 5(c)) and the cancer stem cells

(Fig. 5(d)) too.

Mechanism for graphene nanoflakes as MALDI-MS matrix

Graphene nanoflakes was utilized as the MALDI matrix to detect low-mass molecules, such as amino acids, polyamines, peptides, steroids, nucleosides, nucleotides and metallodrugs [12-14,16, 29-30] . Graphene nanoflakes provided a clear background compared to the organic matrices. Although various mechanisms for the laser desertion/ionization (LDI) have been proposed, it is still unclear so far. One of these attempts is the UV absorption mechanism. According to this mechanism, matrix might absorb the UV radiation, enabling to promote desorption and ionization of analytes in the gas phase. UV absorption (Fig.1B) of G nanoflakes display a continuous absorption with maximum value at 270 nm. G nanoflake (Fig.1B) has absorption at 337 nm which coincide which with the laser wavelength of MALDI, thus it can assist desorption/ionization of lipid. Furthermore, the large surface area of G nanoflakes can play a significant role during these processes, hence it is called as "surface assisted laser desorption/ionization (SALDI-MS)".The advantages of G nanoflakes over than other nanomaterials which can assist desorption/ionization is that G nanoflakes have double sides which can provide high capacity to load more lipid biomolecules (Fig. 6). These adsorptions are spontaneous due to the affinities among G nanoflakes and lipid molecules.

Speculation for the affinity of graphene nanoflakes to cancer cell lipid moieties

Graphene nanoflakes are multifunctional materials. It can interact with analytes via π-π, hydrophobic electrostatic interaction. These non-covalent interactions do not change the main properties of G [20]. Lipid can interact with G nanoflakes via electrostatic interactions between G platform and lipid head groups. Thus, lipid could self-assemble as a multilayered structure on the G nanoflakes platform[31]. Moreover, Liu et. al reported self-assembly of phospholipids on nonoxidative G surfaces via hydrophobic interactions[32] (Fig. 6). Recently, one-step methods of G preparation were proposed through lipid exfoliation in the low boiling point solvent chloroform. Molecular dynamics simulations suggest that the higher stability of exfoliated G nanosheets under the presence of lecithin (lipid) may be due to the formation of reverse micelles and their attachment to graphene[33]. Therefore, lipids probably undergo self-assembly on G nanosheet by different processes such as electrostatic and hydrophobic interactions. G nanoflakes with such a kind of lipid attachment could be desorbed/ionized completely upon laser strike during MALDI-MS process, and therefore assist the ionization/desorption to enhance the MALDI-MS signals (Fig. 6). Frost et al. investigated the interactions between graphene oxide and lipid membranes using both supported lipid membranes and supported liposomes [34]. They discovered

that GO induced rupture of preadsorbed liposomes and they undergo assembly process that form multilayer structure. They claimed that the electrostatic interactions were the driving process of these assembles over other interactions.

Conclusion

For the first time, the application of graphene (nanoflakes)-assisted laser desorption/ionization mass spectrometry (GALDI-MS) for lipidomics profiling of cancer and cancer stem cells had been demonstrated. The uses of G nanoflakes as the lipid matrix and as the co-matrix for the analysis of cancer cell and cancer stem cell lipids have been successfully reported.

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Figure captions

Fig. 1. Characterization of *in-house* synthesized graphene nanoflakes (a) TEM image of graphene nanoflakes; (b) UV/Vis absorption spectrum of graphene nanoflakes; (c) FT-IR spectrum of graphene nanoflakes.

Fig. 2. MALDI-MS spectral analysis of human breast normal, cancer and cancer stem cell lipids using traditional matrices (A) 9AA in RN mode (B) DHB in RP mode. (a) matrix control ; (b) normal cell ; (c) cancer cell ; (d) cancer stem cell.

Fig. 3. MALDI-MS spectral analysis of human breast normal, cancer and cancer stem cell lipids using graphene nanoflakes as co-matrix with (A) 9AA in RN mode (B) DHB in RP mode. (a) matrix control ; (b) normal cell; (c) cancer cell; (d) cancer stem cell.

Fig. 4. MALDI-MS spectral analysis of human breast normal (A), cancer (B) and

cancer stem cell (C) lipids using graphene nanoflakes as matrix with 4 different sample preparation methods. (a) matrix control; (b) method-1; (c) method-2; (d) method-3; (e) method-4.

Fig. 5. GALDI MS based differentiation of human breast cells. (a) graphene nanoflakes control spectrum; (b) GALDI MS spectrum of normal cells; (c) GALDI MS spectrum of cancer cells (d) GALDI MS spectrum of cancer stem cells.

Fig. 6. Schematic representation of self-assembly of lipids on the graphene nanoflakes prior to desorbs/ionize after laser shot during graphene assisted laser desorption/ionization-mass spectrometry.

Supporting information

Fig S1. Schematic showing lipidomic analysis of lipid extracts from human breast cancer and cancer stem cells using graphene nanoflakes based 4 different sample preparation methods .

Fig. S2. MALDI-MS spectral analysis of human breast normal, cancer and cancer stem cell lipids using traditional matrices (A) 9AA in RP mode (B) DHB in RN mode. (a) matrix control; (b) normal cell; (c) cancer cell; (d) cancer stem cell

Fig. S3. MALDI-MS spectral analysis of human breast normal (A), cancer (B) and cancer stem cell (C) lipids using graphene nanoflakes as matrix with 4 different

sample preparation methods. (a) matrix control; (b) method-1; (c) method-2; (d) method-3; (e) method-4. All MALDI-MS spectra detected by RN mode.

Table -1

Tentative identification of lipid biomarkers expressed in Cancer and cancer stem cells by GALDI MS

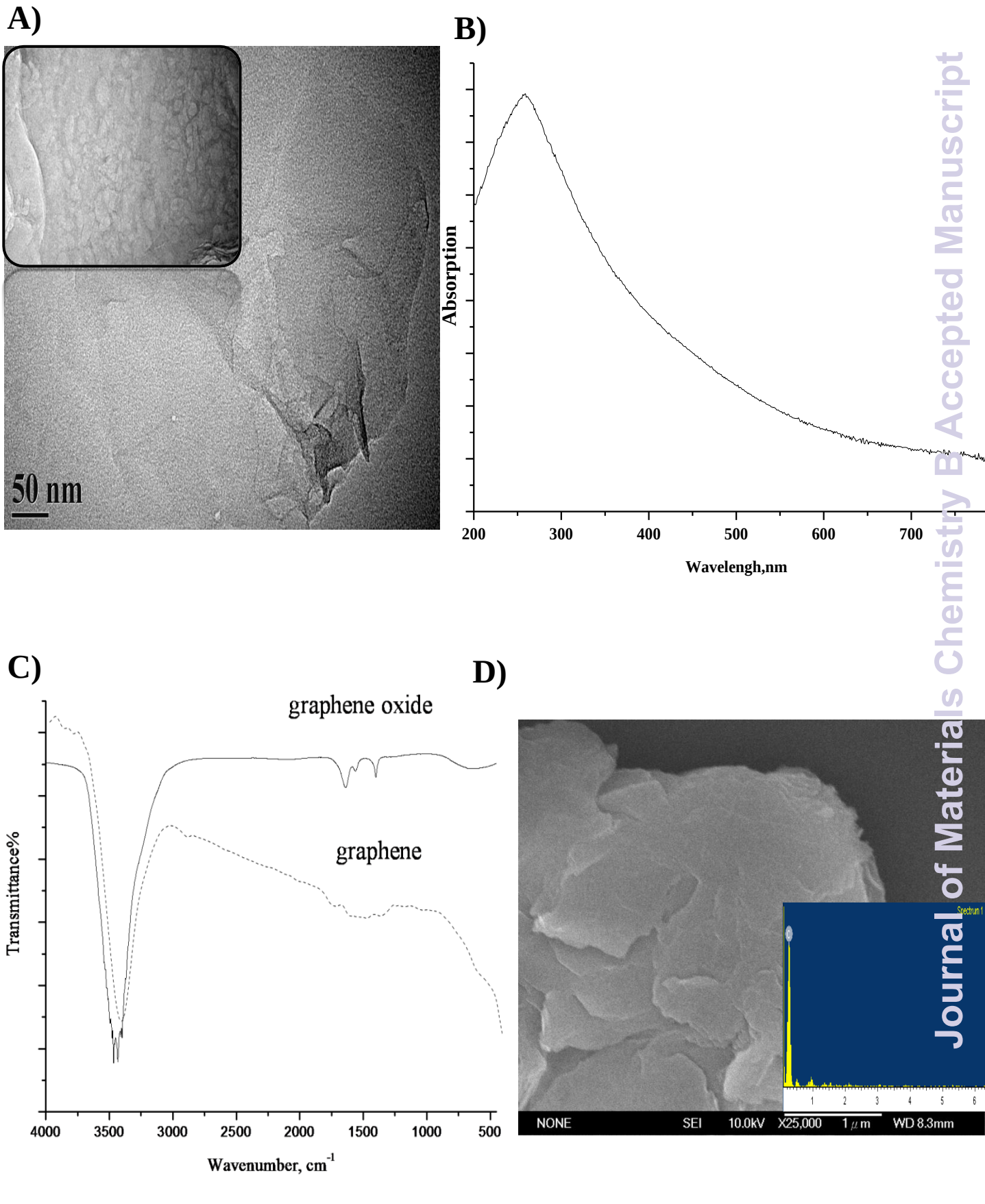
No	molecular weight (*m/z*)	Molecules	Ionic species of molecule
1	86.10	DPPC	DPPC, phosphocholine headgroup $[C_5H_{12}N]^+$
2	184.07	DPPC	DPPC, phosphocholine headgroup $[C_5H_{15}NPO_4]^+$
3	224.11	DPPC	DPPC, phosphocholine headgroup $[C_8H_{19}NPO_4]^+$
4	551.50	DPPE and DPPC	dipalmitoyl $[C_{35}H_{67}NPO_4]^+$
5	880	Cerebroside	$[Cer\ d18:0/26:0h + Na]^+$, $[Cer\ d18:1/25:0h + K]^+$

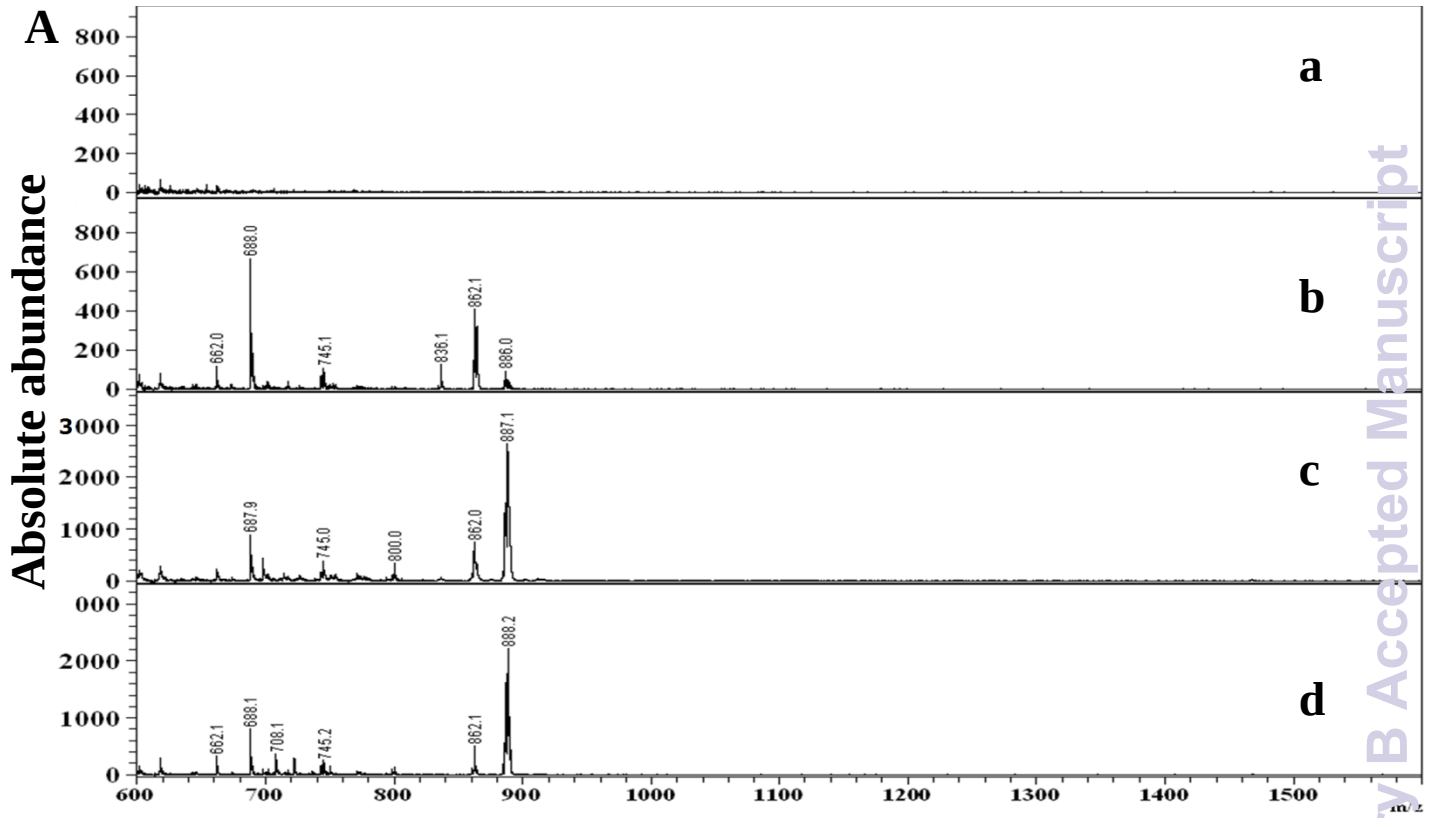
Table 2 comparison among the present technique that use to discriminate among the different cancer cells

Cells	**Technique**	**Materials**	**Mechanism**	**Assay time (mins)**	**LOD**	**Ref**
Breast cancer cells	Raman spectroscopy	Au@G	Vibrational changes of the cells content	<1	1 zmole	16
Human cancers	field-effect transistor	CMOS	Change of the electric comductvivity	< 1	nd	26
Breast cancer cell	SALDI-MS	G	Molecular weight of lipids	2-5		Here
prostate cancer	MALDI	Sinapinic acid	Molecular weight of proteins	nd	nd	27

Nd, not avaliable

Fig.1





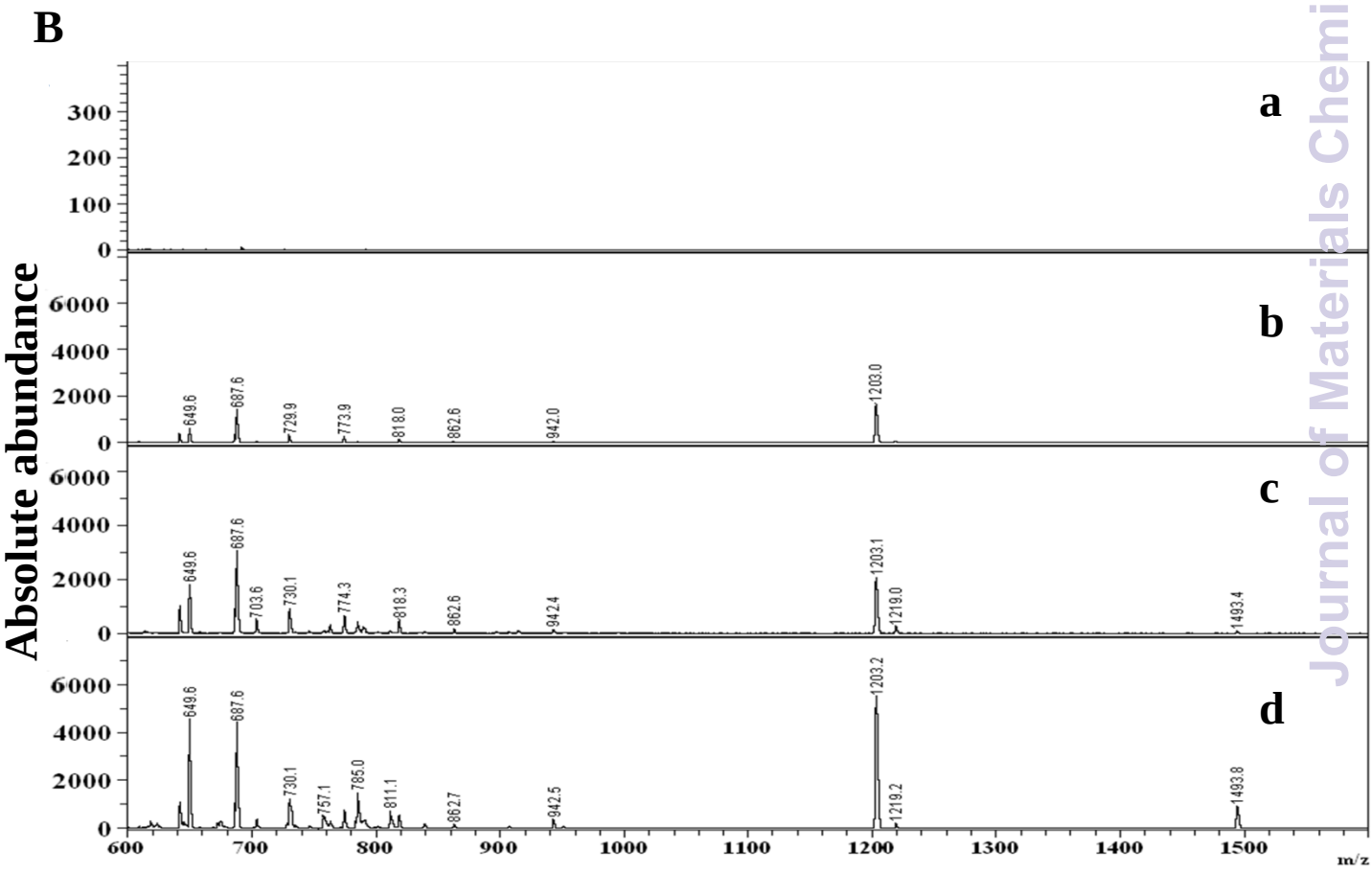
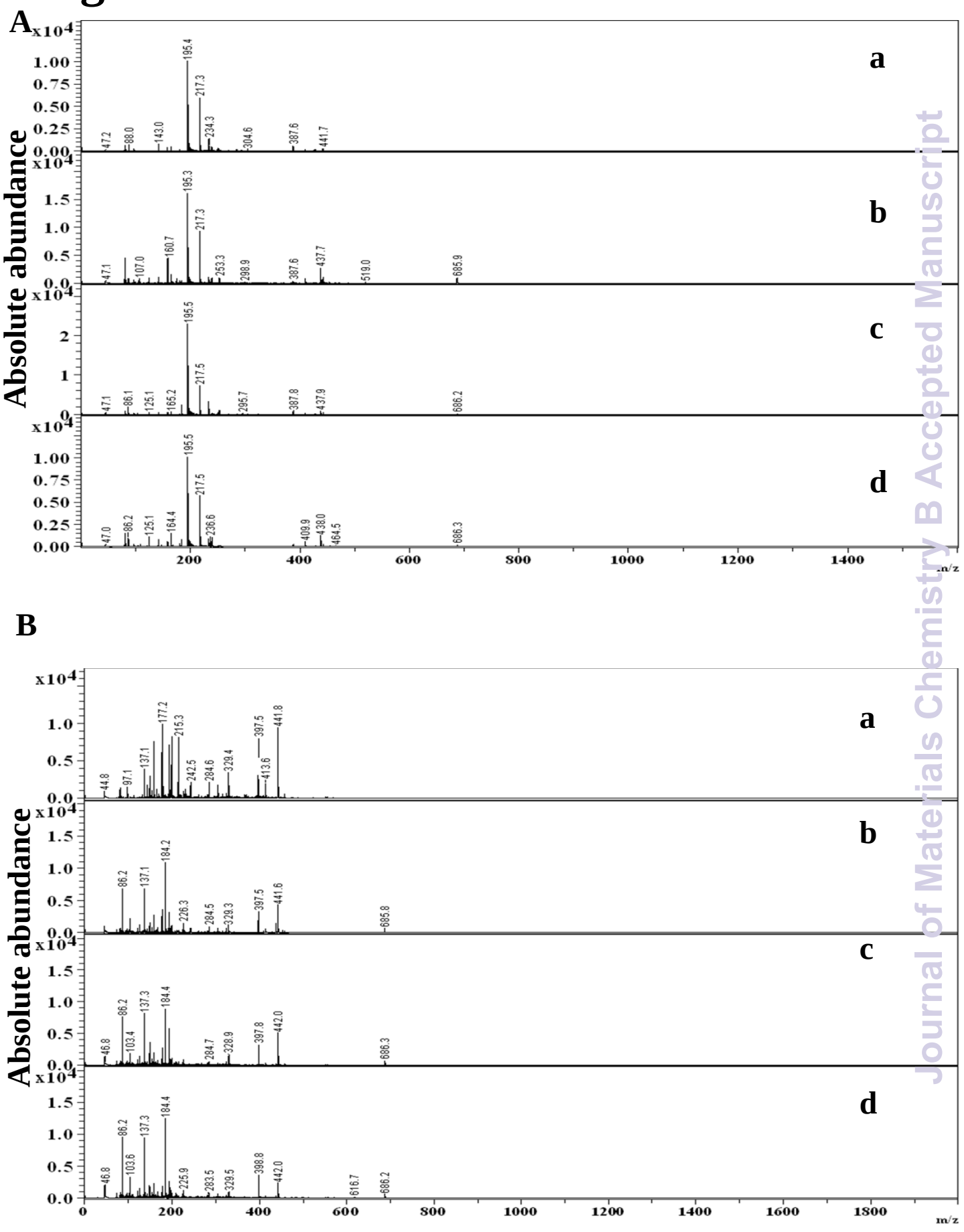


Fig.3



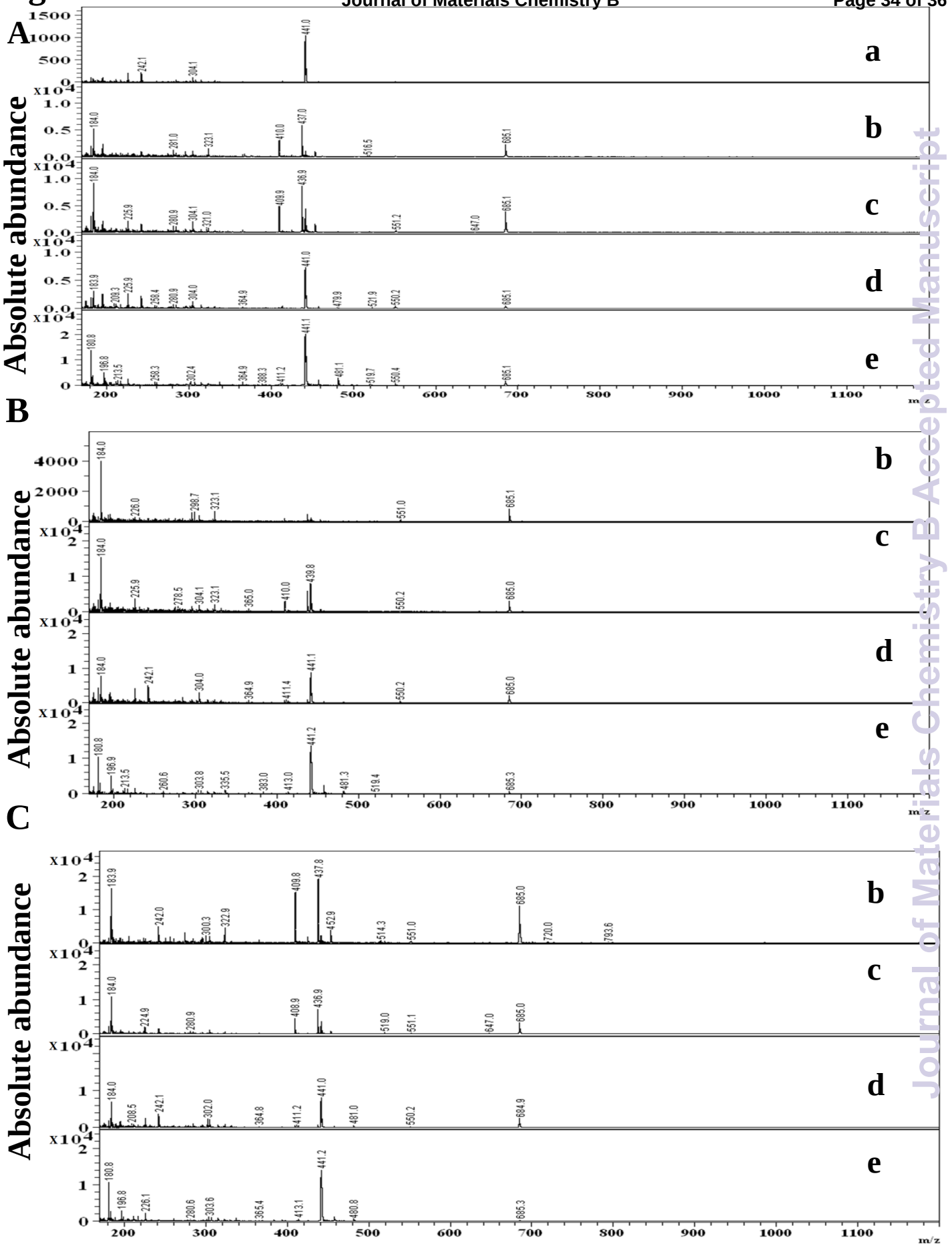


Fig.5