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RESEARCH ARTICLE

Large-Area Graphene Films as Target Surfaces for Highly Reproducible Matrix-Assisted Laser Desorption Ionization Suitable for Quantitative Mass Spectrometry

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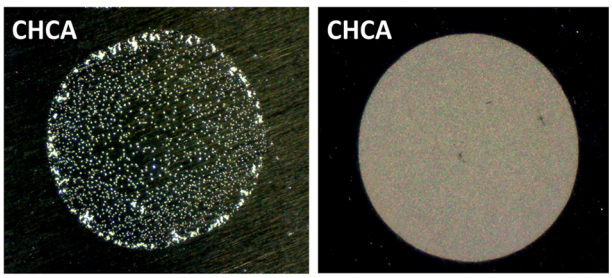

Abstract. Due to the known sweet-spot issues that intrinsically arise from inhomogeneous formation of matrix-analyte crystals utilized as samples in matrix-assisted laser desorption ionization (MALDI) mass spectrometry, its reproducibility and thus its applications for quantification have been somewhat limited. In this paper, we report a simple strategy to improve the uniformity of matrix-analyte crystal spots, which we realized by adapting large-area graphene films, i.e., non-inert, interacting surfaces, as target surfaces. In this example, the graphitic surfaces of the graphene films interact with excess matrix molecules during the sample drying process, which induces spontaneous formation of optically uniform MALDI sample crystal layers on the film surfaces. Further, mass spectrometric imaging reveals that the visible uniformity is indeed accompanied by reproducible MALDI ionization over an entire sample spot, which greatly suppresses the appearance of sweet spots. The results of this study confirm that the proposed method achieves good linear responses of ion intensity to the analyte concentration ($R^2 > 0.99$) with small relative standard deviations ($\sigma < 10\%$), which is a range applicable for quantitative measurements using MALDI mass spectrometry.

Keywords: Large-area graphene films, MALDI mass spectrometry, Sample preparation, Quantification

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Introduction

For decades, matrix-assisted laser desorption ionization (MALDI) has been established as a versatile method for intact ionization of thermally labile molecules, for which broad applications involve mass spectrometry (MS) of large biomolecules, such as peptides, glycans, and lipids, as well as synthetic polymers [1]. In recent years, MALDI MS has been further highlighted for new bio-clinical research opportunities with imaging mass spectrometry (IMS), which allows label-free chemical mapping of biomolecules on the surfaces of specimens such as single cells, tissues, and microarrays [2]. In the MALDI method enabling intact desorption ionization of large molecules, co-crystallization of analytes with the excess matrix molecules, such as α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB), is essential. However, using matrix-analyte crystals as MALDI samples also results in certain drawbacks; for example, tiny yet inhomogeneous sample crystals create noticeable position-to-position variations in ionization efficiency, the so-called sweet-spot issues, even in the same sample spot. This makes the MALDI method not generally amenable to quantification, which is still a subject of further research [3–5].

In the early days, various methods to improve the homogeneity of matrix crystals were utilized; examples include vacuum drying, fast-solvent evaporation, electrospray deposition, and vacuum sublimation of the matrix, as well as multilayer methods [6–9], some of which are still used as

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good lab practice to maintain MALDI reliability. Later, to avoid the use of such solid crystals, various room-temperature ionic liquid (RTIL) matrixes were also examined. RTILs for MALDI were formed as acid-base ion pairs with extremely low vapor pressures of acidic matrixes, such as CHCA and DHB, and organic bases including 1-methylimidazole, which exhibited good linearity suitable for quantification [10, 11]. Furthermore, to avoid using the matrix itself, researchers extensively explored LDI methods that utilized various nanostructured surfaces [12], such as nanostructured Si [13], ZnO-nanowire surfaces [14], and Au nanoparticle-implemented surfaces [15] as well as special flat surfaces that exhibited LDI capability, such as calcinated films on gold [16] and amorphous Si [17] as matrix-free target surfaces. Unfortunately, despite these extensive investigations, the proposed methods were still not robust enough to fully replace the conventional MALDI method utilizing common organic matrixes.

In fact, the most recent endeavors to achieve MALDI reproducibility to a quantitative level have still focused on sample preparation methods, where sophisticated instruments are often employed. Examples include a new interface that couples liquid chromatography with MALDI IMS by employing an automated matrix sprayer produced uniform matrix layers suitable for quantification [18] and excitation of evaporating MALDI sample drops using AC-electrowetting with specially patterned on-plate electrodes that led to formation of smaller and more homogeneous MALDI sample crystals [19]. Furthermore, self-aliquoting plates combined with a specially designed sliding device, which divided a sample drop of microliters into 20 tiny replicates, offered sufficient statistical sampling for accurate quantification even without the use of internal standards [20].

Graphene, a two-dimensional allotrope of carbon, is a single layer of carbon atoms arranged in a hexagonal lattice. Due to the intriguing material properties resulting from structural confinement as well as its wide applications, including composites, nanoelectromechanical systems, and various sensing devices, graphene has been the special material of extensive research in recent years [21, 22]. With its unique structural properties, graphene offers atomically thin interfaces when employed in molecule-on-substrate systems, suppressing direct interaction between adsorbed molecules and underlying substrates [22]. In the fields related to MS, flakes and nanoparticles of graphene and graphene oxides have been demonstrated as efficient matrix alternatives or additives for MALDI MS and IMS [23–30]. Recently, production of large-area and high-quality graphene films became facile with the chemical vapor deposition (CVD) method, and the large-area graphene films on Cu foils are also commercially available [31, 32].

Herein, we report a simple strategy to induce spontaneous formation of highly uniform matrix-analyte crystals by exploiting large-area graphene films as attractive target surfaces, which leads to highly reproducible MALDI ionization that is suitable for quantitative measurements.

Experimental Methods

Chemicals

Matrixes including CHCA (Part No.: 70990) and DHB (149357) were obtained from Sigma-Aldrich Korea (Suwon, Korea). Other standard reagents including [glu^1]-fibrinopeptide B (GluFib_B; F3261), angiotensin II (Angio_II; A9525), substance P (S6883), cyclosporine A (Cyclo_A; 30024), sirolimus (37094), human serum (H4522), and solvents (HPLC-grade) were also purchased from Sigma-Aldrich Korea. The standard peptide mixture (Peptide mixture II) was obtained from Protea Biosciences, Inc.

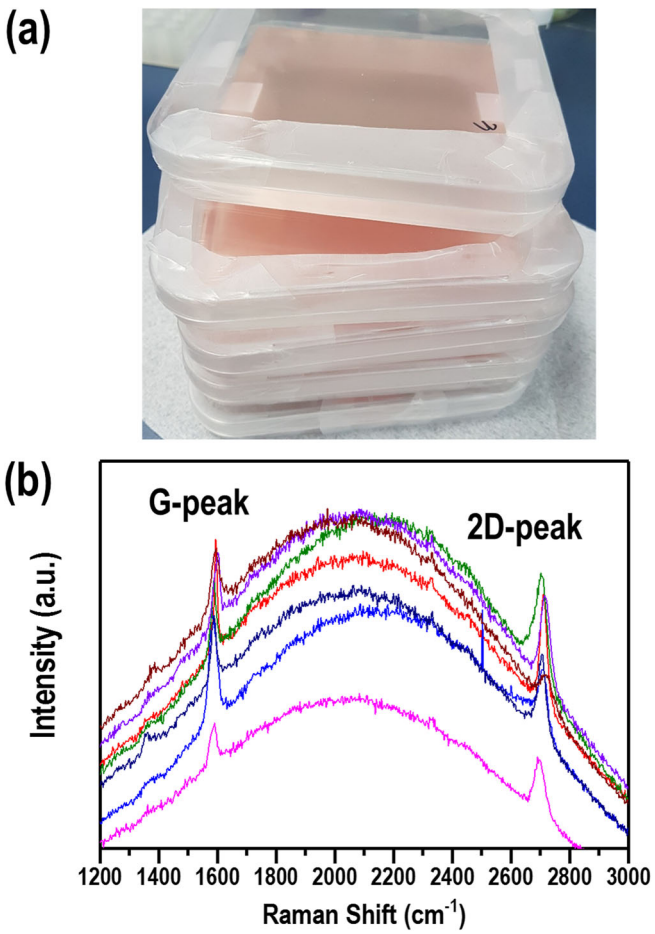

Figure 1. (**a**) Photo of commercially obtained large-area graphene films on Cu foils as received (10 × 10 cm sheets). (**b**) Raman spectra taken from various positions on the graphene films; the characteristic Raman peaks of pristine graphene, G and 2D peaks, were clearly observed from all positions. (baselines were not subtracted)

(Morgantown, WV, USA). 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC(18:0); 855775) and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (855675) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). The sheets (10 × 10 cm) of graphene films on Cu foils were commercially obtained from MCK Tech (Ansan, Korea) (Figure 1).

MALDI Experiments

For MALDI experiments using the graphene films, target surfaces were prepared by attaching the graphene foils to stainless steel (SUS) target plates using conductive carbon tapes. For IMS of CHCA sample spots, the matrix solution was prepared by dissolving CHCA in a solution

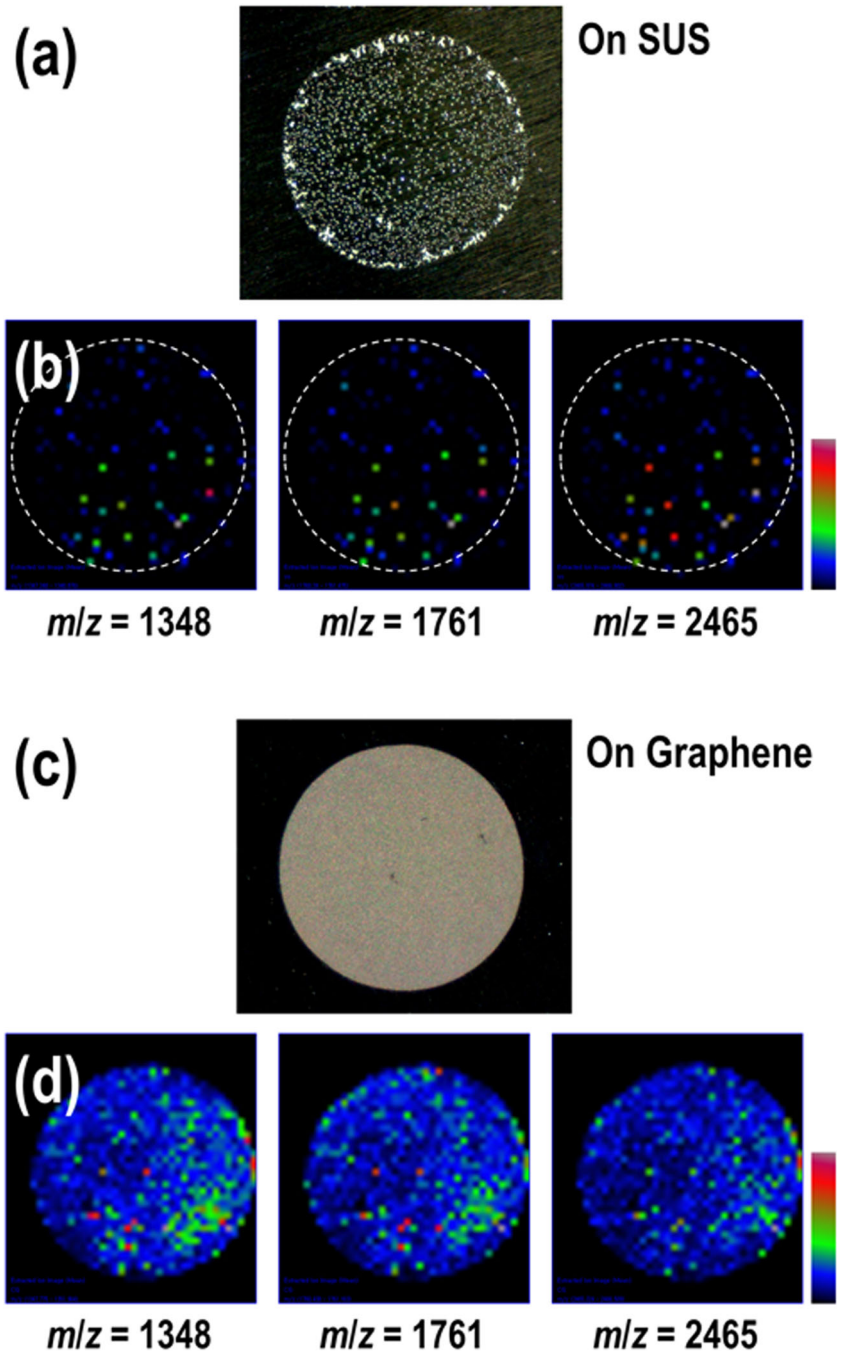

Figure 2. (**a**) Optical image and (**b**) mass images of a MALDI sample spot prepared on the SUS target plate for peptide mixtures using a CHCA matrix. (**c**) Optical image and (**d**) mass images of the sample spot prepared on the graphene films on Cu foils. Mass images are drawn for substance P (*m/z* = 1348), renin (1761), and ACTH 18-39 (2465)

of 50% acetonitrile (MeCN) with 0.1% trifluoroacetic acid (TFA) to a final concentration of 5 mg/mL. For analytes, an aqueous mixture of standard peptides (Peptide mixture II), which includes substance P (monoisotopic mass = 1346.7 Da), renin (1759.9), and ACTH 18-39 (2464.2), was prepared to contain about 20 pmol of each peptide in a 1-μL drop. It was then mixed with the same volume of matrix solution just prior to experiments. The drops loaded on target surfaces were allowed to dry in ambient conditions. For IMS of DHB sample spots, the matrix solution of DHB (20 mg/mL) was prepared in a solution (MeCN, 20 mM NaCl(aq) = 8:2), which was then mixed with the same amount of the analyte solution. A 1-μL drop of the sample solution loaded on the SUS and graphene surfaces was dried in a vacuum desiccator. NaCl was included in the matrix solution as a cationization agent. However, the cationization reagent was not used in MALDI MS of LPC(18:0) using a DHB matrix. The analytes for IMS, N-glycans, were released directly from human serum, of which detailed procedure can be found elsewhere [33]. In MALDI MS using a DHB matrix, commercial self-focusing MALDI plates (μfocus plate; ASTA Corp., Suwon, Korea) were utilized.

MALDI MS experiments were performed using an imaging MALDI-TOF mass spectrometry of reflectron type (IDSys IM; ASTA Corp., Suwon, Korea), which is equipped with a Nd:YLF laser (349 nm; 1 kHz). The ion acceleration energy was 19 keV, and 23 kV was applied to the reflectron. Using the specially designed laser optics module, the beam diameter of laser at the sample surface is adjustable, which can be set to be from 15 to 200 μm. In this study, mass spectrometric imaging was carried out at a spatial resolution of 50 μm in the positive ion mode. Typically, IMS data was acquired by accumulating ion signals for 100 consecutive laser shots (100 Hz) at each position. For data processing, imaging softwares, IDsys2.0 and MALDIVision (PREMIER Biosoft, Palo Alto, CA, USA), were utilized.

Results and Discussion

MALDI Using CHCA on the Graphene Films

In this study, commercially obtained large-area graphene films on Cu foils produced by the CVD method were utilized as a well-defined single layer of graphitic surfaces for MALDI target plates [32]. The obtained graphene surfaces were examined by Raman microscopy; the characteristic Raman peaks of pristine graphene, G and 2D peaks, were clearly observed from all measured positions (Figure 1).

First, MALDI MS on graphene films for a mixture of standard peptides using CHCA was examined and compared with the conventional way obtained on SUS target plates. Figure 2a, c displays the optical images obtained for the sample spots prepared on a SUS target plate and the graphene films on Cu foils, respectively. The sample solution deposited on the SUS target plate resulted in formation of many tiny crystals scattered randomly over the MALDI sample spot. Formation of crystal rims was also evident around the sample spot (Figure 2a). However, although the same sample solution was dropped in the same way, the sample spots formed on the graphene films appeared quite differently, exhibiting an optically dense and uniform crystal layer in the resulting sample spot (Figure 2c).

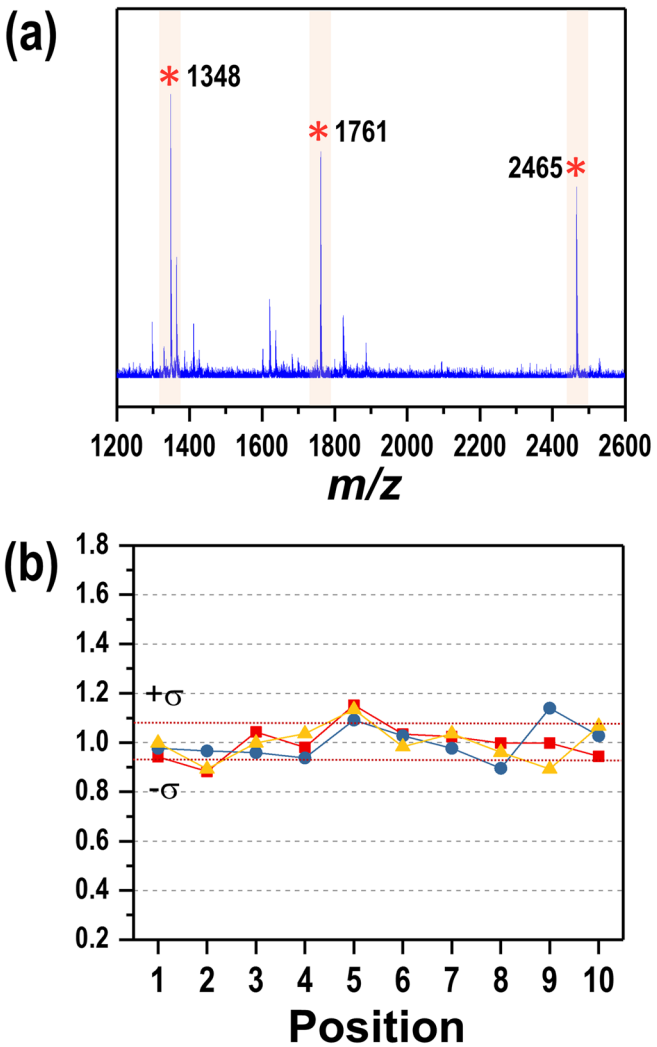

Figure 3. (**a**) A typical mass spectrum obtained in IMS of a CHCA sample spot prepared on the graphene films. (**b**) Intensity variations of three peptide ions obtained from ten different positions in the mass images (Fig. 2(d)). The variation is given as a ratio to the average value, and the σ values for three peptides were found to be about 8% (■ (substance P; m/z = 1348), ▲ (renin; 1761), ● (ACTH 18-39; 2465))

High-resolution IMS is a useful tool to investigate MALDI sample crystals [34]. To examine both sample spots more closely, we carried out mass spectrometric imaging using a MALDI imaging mass spectrometer with a spatial resolution of 50 μm. Mass images for the spatial distributions of embedded peptides in the sample spots are given in Figure 2b, d. As clear in Figure 2b, production of MALDI ions on the SUS target plate depended on their positions, which were strongly pronounced only from certain positions in the sample spot. This apparently reflected the nature of tiny crystals randomly scattered on the surface, which is in agreement with the observed optical image (Figure 2a). However, the mass spectrometric images for the sample spot formed on the graphene films showed generation of MALDI ions over the entire sample spot in a reproducible way, which in other words greatly

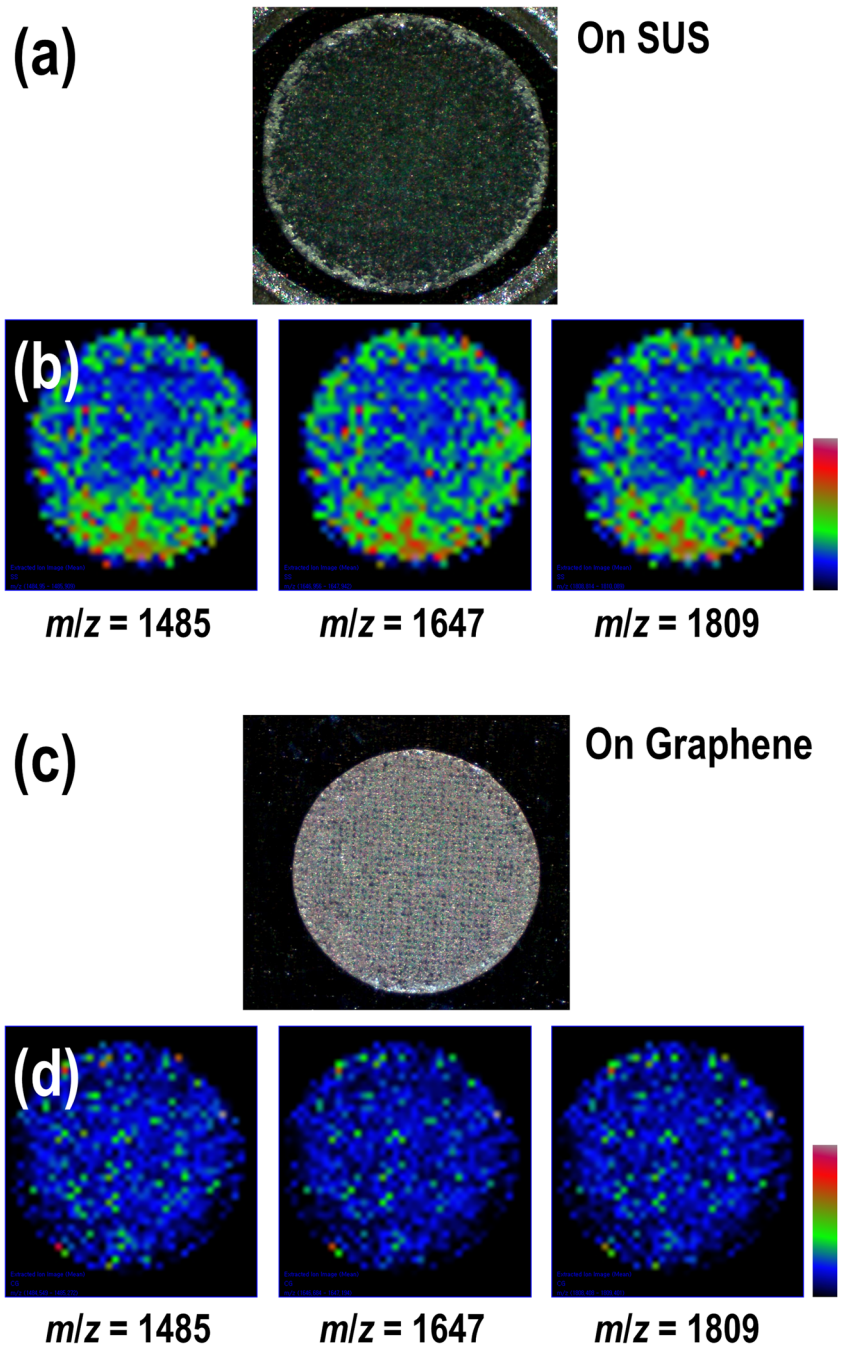

Figure 4. (**a**) Optical image and (**b**) mass images of a MALDI sample spot prepared on the SUS target plate, a commercial self-focusing plate, for N-glycans using a DHB matrix. (**c**) Optical image and (**d**) mass images of the sample spot prepared on the graphene films on Cu foils

minimized the sweet-spot issues (Figure 2d). The plot of intensities of MALDI ions produced from ten different positions of the sample spot prepared on the graphene films is also given in Figure 3b; the standard deviations (σ) for absolute intensities (without the use of internal standards) of three peptides were less than 8% of the respective average values.

MALDI Using DHB on the Graphene Films

As a case for experimental samples other than standard materials, we applied this method for MALDI MS using DHB of N-glycans that we released directly from human serum using the PNGase F enzyme. DHB is a versatile matrix but requires certain amount of practice to achieve reproducibility due to its propensity to forming large crystals. In this study, we employed an optimized protocol, which we found to be reliable and routinely used for MALDI MS monitoring of N-glycans [33].

The optical images for MALDI sample spots using DHB on the SUS target plates and the graphene films are given in Figure 4a, c, respectively. In virtue of the optimized protocols employing vacuum drying, the sample spots on the SUS target plate, a commercial self-focusing plate, appeared to be rather uniform but with clear development of rim structures around the edge of the spots (Figure 4a). As for the sample spot prepared on the graphene films, the crystal granules looked finer and denser than those on the SUS plate, and formation of the rim structure could be minimized by optimizing the DHB concentration and drying speed (Figure 4c). As shown in Figure 4b, despite the rather uniform appearance of crystal distribution on the SUS target plate, the intensity of MALDI ions greatly varied according to the ionization position, which showed strong ionization along the rim structure and also the lower part of the sample spot. As for the sample spot on the graphene, however, the mass images showed that generation of MALDI ions was rather uniform over the entire sample spot (Figure 4d). As noticeable in Figure 5b, the position-dependent fluctuation in ion intensity in a sample spot was not significant; the σ values for absolute intensities of three N-glycans were found to be about 11% of the respective averages.

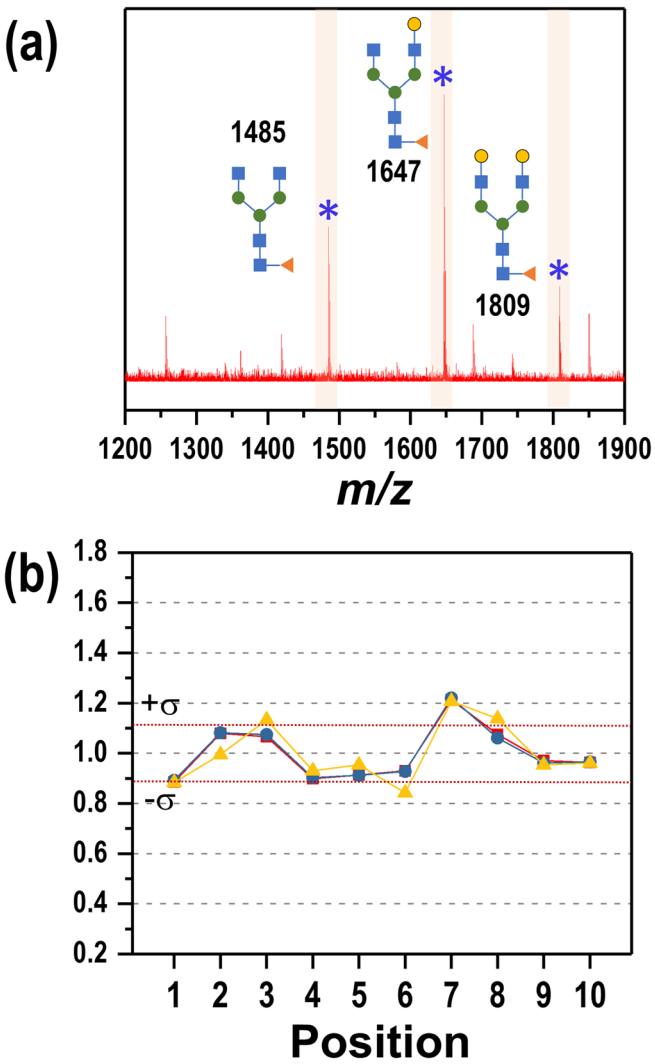

Figure 5. (**a**) A typical mass spectrum obtained in IMS of a DHB sample spot prepared on the graphene films. (**b**) Intensity variations of three N-glycans obtained from ten different positions in the mass images (Fig. 4(d)). The variation is given as a ratio to the average value, and the σ values for three N-glycans were found to be about 11%; (■ (m/z = 1485), ▲ (1647), ● (1809))

Calibration Curves

The observed reproducible ionization suggests an opportunity for quantification using MALDI MS. In this regard, dependence of the ion intensity on the analyte concentration in terms of calibration curves was further examined for the two target surfaces. Figure 6 shows typical results for the calibration curves (150 fmol–1.2 pmol) for standard peptides, GluFib_B and Angio_II, which were obtained for MALDI on SUS and graphene films. The measurements were carried out for five different spots and a fixed amount of substance P was added as an internal standard (600 fmol). The resulting average values and standard deviations (σ) for ion intensity obtained for each concentration are plotted in Figure 6. The CHCA matrix is known to possess a reasonably good reproducibility [3]. Although its sample spots consist of scattered crystals on the SUS target plates, the wider laser beam with a 200 μm diameter and accumulation of 500 shots over random positions in a spot effectively averaged out the visible heterogeneity of the sample crystals, which resulted in certain linear behavior with a square of correlation coefficient (R^2) of 0.982 for GluFib_B. Their σ values were measured in a range of about 10–26% of the respective average values (Figure 6a). The results were slightly worse for Angio_II, where certain deviation from linearity was noticeable in the graph with an R^2 value of 0.945 (Figure 6c). As for MALDI on the graphene films, however, the linearity was greatly improved so that the R^2 values were better than 0.99 for both peptides (Figure 6b, d). In addition, the σs were

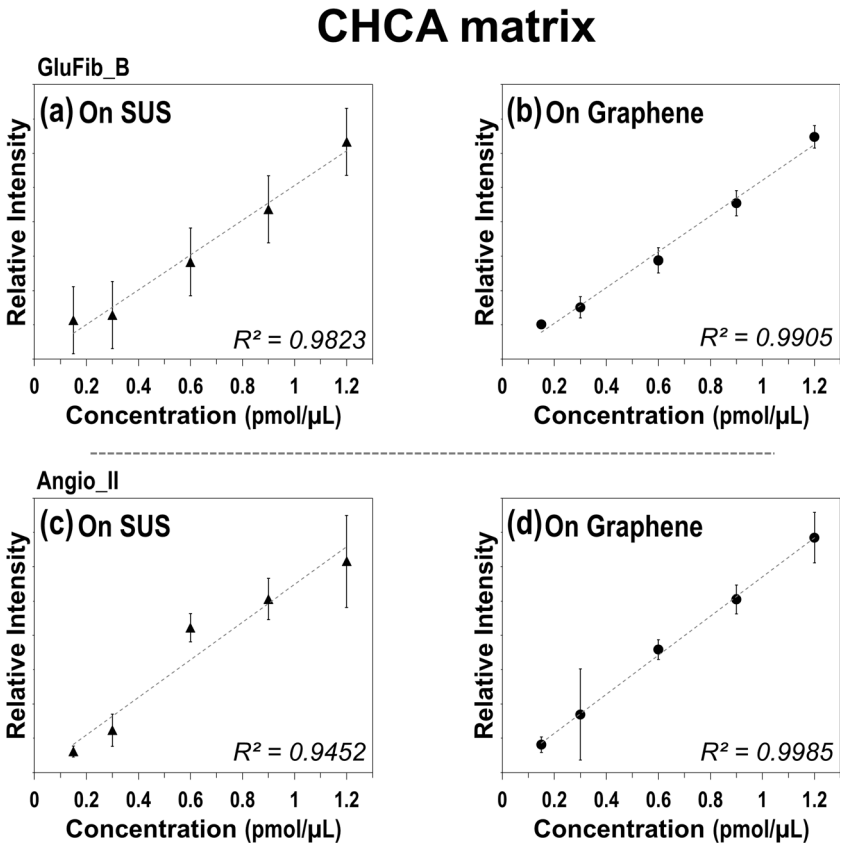

Figure 6. Calibration curves for MALDI MS using a CHCA matrix of GluFib_B on the SUS plate (**a**) and on the graphene films (**b**). Calibration curves for Angio_II on the SUS plate (**c**) and on the graphene films (**d**). The square of correlation coefficient values (R^2) is given in figures. Error bars indicate the standard deviations (σ)

measured typically to be as good as 3–10% of the respective average values, which are values considered to be suitable for quantitative applications using MALDI MS.

The calibration curves using a DHB matrix were also examined for a lipid (LPC(18:0)) and a drug (Cyclo_A) added with fixed amounts of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine and sirolimus as internal standards, respectively (Figure 7). In the results for LPC(18:0), MALDI on the graphene films showed a better linearity of $R^2 = 0.988$ (Figure 7b) than that on the SUS target plate of $R^2 = 0.958$ (Figure 7a), which exhibited relative σs of 1–4%, which were narrower than those obtained on the SUS plate (6–18%). In the experiments of lipids, σs of better than 10% were routinely achieved on the graphene films. As for Cyclo_A, an immunosuppressant, its quantification is clinically important in monitoring drug levels in transplant patients with a therapeutic window of 75–150 ng/ml (60–125 fmol/μl). As shown in Figure 7c, the calibration curve obtained for MALDI on the graphene films had a good linearity ($R^2 = 0.995$) in the low femtomole region (5–25 fmol/μl), which is much lower than the therapeutic window, while the σs were somewhat broader because the ion intensity was weak in this region close to the detection limit (~ 1 fmol). In this low concentration region of Cyclo_A, MALDI on the SUS plates was not reproducible at all under our experimental conditions. The immunosuppressant ions were detectable but did not show a sufficient reproducibility applicable for quantification.

Conclusions

We successfully demonstrated a simple strategy for inducing spontaneous uniform crystallization of MALDI sample spots using large-area graphene films as attractive target surfaces. It is probably driven by interaction of graphene surfaces with excessive matrix molecules during the sample drying process, where the infinitely periodic graphitic surfaces may serve as the seeds for observed homogeneous crystallization of the organic matrixes. Indeed, the comparison of calibration curves between MALDI on SUS target plates and on graphene films revealed that the use of induced uniform spots offered a significant improvement in linearity ($R^2 > 0.99$) and standard deviation ($\sigma < 10\%$), which is beneficial for MALDI applications for quantification.

Detailed understanding of crystallization process induced by the graphene surfaces and its possible effects on the actual MALDI process still needs further study. Nevertheless, this

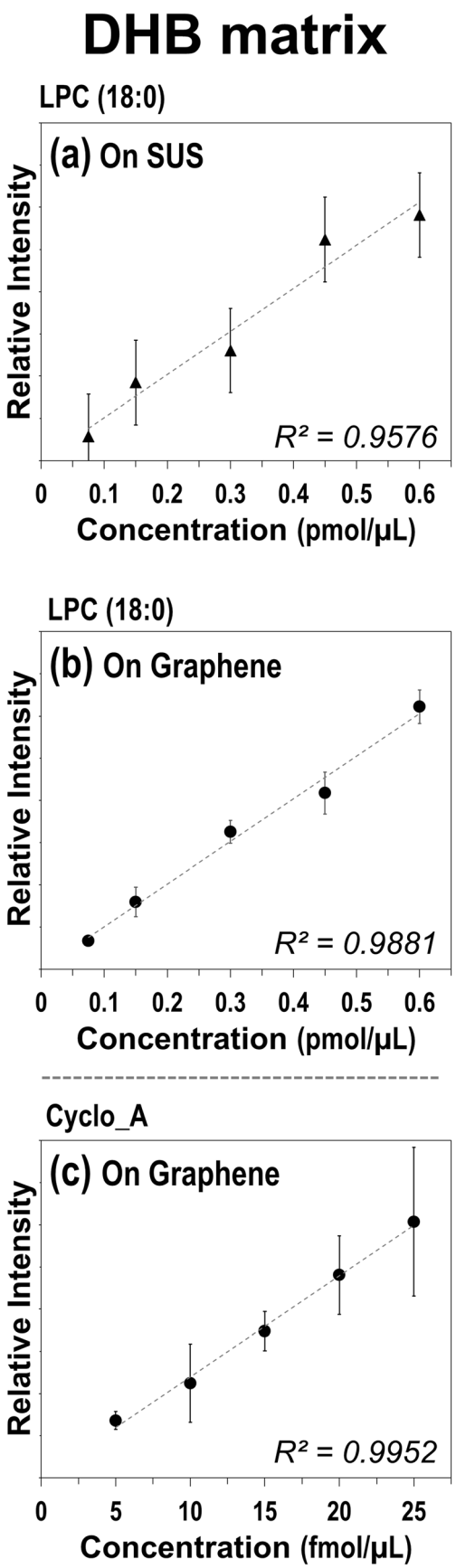

Figure 7. Calibration curves for MALDI MS using a DHB matrix of LPC(18:0) on the SUS plate (**a**) and on the graphene films (**b**). Calibration curve for Cyclo_A on the graphene films (**c**). The square of correlation coefficient values (R^2) is given in figures. Error bars indicate the standard deviations (σ)

study using graphene films clearly demonstrated that controlling the properties of target surfaces can be an important strategy in the development of sample preparation to achieve highly reproducible, and thus quantitative, MS using MALDI ionization.

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