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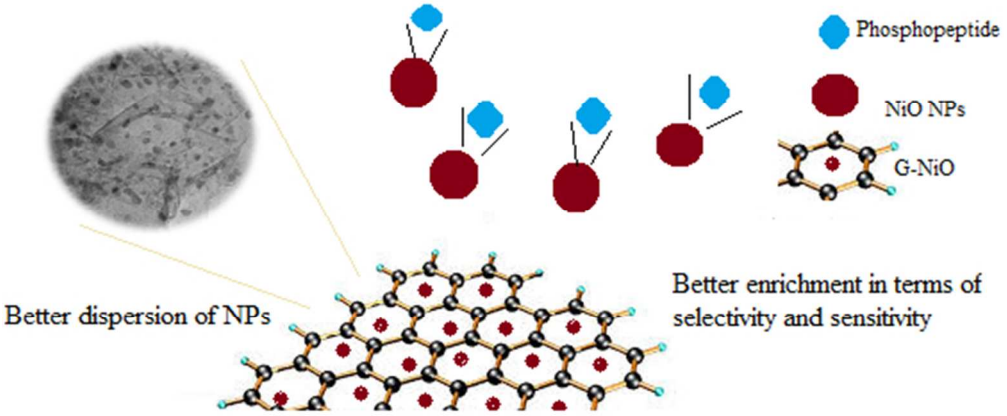

Graphical Abstract
148x63mm (96 x 96 DPI)

Enhanced Enrichment Performance of Nickel Oxide Nanoparticles via Fabrication of Nanocomposite with Graphene Template

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Abstract

Metal oxide based nanocomposites are applied in phosphoproteomics through enrichment by the surface hydroxyl groups of metal oxides with rare description of the role of metal. Using graphene as template and its modification with nickel oxide, a nanocomposite with increased surface area is fabricated and applied to phosphopeptides. Characterisation provides narrow size distribution of 15-20 nm, BET surface area 179.70 m^2/g and pore volume as 0.44 cm^3/g. Graphene possesses well distributed NiO nanoparticles showing selectivity up to 1000 folds of complexity whereas sensitivity down to 1 femtomol. G-NiO nanocomposite shows higher selectivity towards phosphopeptides as compared to TiO_2, ZrO_2 and NiO nanoparticles. The enrichment is made for biological samples like egg yolk, non-fat milk and human serum. Phosphopeptides having phosphorylations up to 6 phosphate groups, derived from phosvitin and lipovitellin are enriched from egg yolk digest. Phosphopeptides characteristic of casein variants are enriched from non-fat milk digest with recovery of α_S1 21.9%, α_S2 30% and β-casein 20%. Phosphorylated proteins are identified from human serum through the enrichment of phosphopeptides.

Introduction

The phosphoproteomics need the selective and sensitive enrichment strategies[1]. Several analytical tools including the mass spectrometric protocols have been introduced. The potential substitutes for the separation of phosphorylated species are reported[2]. Enrichment tools are based on immuno-affinity, binding through metal ions or ion-exchange interactions[3,4]. Bi-dentate ligand chemistry offered by the hydroxyl groups on metal oxides also helps their separation.

Sample complexity is reduced by the enrichment followed by MS detection. Low abundant phosphopeptides are suppressed in MS if detected without enrichment[5]. The enrichment methods are thus developed to achieve higher sensitivity and recovery[6]. These two factors are limited by the nature of molecules, materials, methods and tools of analysis. The loading and washing conditions enhance specificity at the cost of inferior sensitivity plus recovery[7]. Nano-based enrichment methods address specificity and sensitivity of peptides/proteins with PTMs like phosphorylation present in low-abundance[8,9]. On-going work focuses on the versatile nature of phosphorylation/de-phosphorylation process. The development of enrichment methods is made to find phosphorylations at different complexity levels[10].

In recent years, the phosphopeptides enrichment on nanocomposites and their identification with MS has led to the biomarker identifications[11]. Composites exhibit properties of each component when compared to individual metal oxides[12]. Nanocomposites are synthesized by combining the materials through physical interactions or covalent bonding[13]. They show higher surface to volume ratios resulting in large number of binding sites[14,15]. The high intensity signals of non-specifically bound entities restrain the ion signals of phosphopeptides[16]. Some contain mono/multiply phosphorylated species in which mono-phosphorylated peptides suppress the signal of multiple phosphorylated peptides[17,18].

Both of these problems have been addressed by nanocomposites having variety of chemistries and short analysis time. They are created by chemical modifications for the phosphopeptides enrichment[19]. Among carbon based materials, graphene is preferred as support because of the characteristics[20] and its nanocomposites with Fe_3O_4 and TiO_2 have been previously used for the phosphopeptides enrichment[21].

Metal oxide based nanocomposites work on the principle of Metal Oxide Affinity Chromatography (MOAC). Surface hydroxyl groups interact with phosphate groups on peptides/proteins and enrichment is achieved. The metals in MOx affect acidity of metal oxides as they are Lewis acids and may contribute to the difference in selectivity[22]. Further explanation is required for the role of metal in MOAC-based enrichment strategies as it may help to understand the difference in performance of metal oxides.

In this study, graphene nano-foam is derivatized to incorporate nickel oxide using Stober's method and applied for phosphopeptides enrichment. Comparison of synthesized nanocomposite with NiO and other metal oxides is made using β-casein digest. Selectivity and sensitivity measurements are also carried out. Biological fluids like egg yolk, non-fat milk and serum are analysed with MALDI-MS after enrichment with graphene-nickel oxide nanocomposite.

Results and Discussion

Nickel ions based affinity sorbents have been commercialized for the separation of His-tagged proteins whereas NiO NPs are reported for the phosphopeptides enrichment[23]. In G-NiO, there is graphene support with NiO on its surface having surface hydroxyl groups for binding with phosphate group of phosphorylated biomolecules. Type of metal and its coordination, change in properties with pH shift and distribution of particles on support material also contribute towards the enrichment efficiency. At lower pH, the Ni^{2+} coordination occurs through C-terminus and it can bind to acidic peptides by carboxylate group from glutammic acid or histidine anchoring binding site. Furthermore, at lower pH, amide deprotonation occurs and coordination is unfavoured[24], thus histidine rich peptides/proteins are not enriched by NiO.

Graphene has negatively charged surface because of the presence of carboxylic, hydroxyl and epoxy groups which bind nickel oxide through electrostatic interactions. The morphology of nano-porous graphene-NiO nanocomposite is studied by transmission electron microscopy (TEM). Nano-porous graphene foam has slightly creased wavy morphology which is observed in the TEM image before modification (Fig. 1a). After the modification, nano-porous graphene gets homogenously decorated with nickel oxide (Fig. 1b). The graphene strongly interacts and acts as dispersant in two-dimensional growth template for NiO. Fig. 2a illustrates the X-ray diffraction (XRD) pattern of graphene-NiO nanocomposite with three characteristic peaks of nickel oxide. The nanocomposite contains graphene with nano-pores filled with nickel oxide obtained because of thermal treatment of the mixture. The average size of nickel oxide nanoparticles on graphene nanofoam is approximately 15-20 nm. The thermal stability is determined by the thermo-gravimetric analysis (TGA). The nanocomposite is stable up to 400 °C with a drop in weight from 400-500 °C because of the burning of carbon (Fig. 2b).

Two factors contribute towards the change in surface area of the nanocomposite. First, overlapping of graphene fibres and second is the nickel oxide nanoparticles. The silica spheres cause mesopores in graphene foam, however after their removal , nanoporous graphene oxide is achieved. In nanoporous graphene foam without NiO nanoparticles (Fig. S1a), the pore sizes are distributed around 50 nm which is close to the particle diameter of silica sphere hard templates (Fig. S1b). Nanoporous graphene foam without NiO nanoparticles thus show higher BET surface area of 401.01 m^2/g and pore volume of 1.35 cm^3/g. The pores inside nanoporous graphene foam accommodate nickel nitrate and after the decomposition, NiO nanoparticles within the pores are placed. Therefore surface area of G-NiO composite is 179.70 m^2/g and pore volume 0.44 cm^3/g which is less than the nanoporous graphene foam (Fig. 2c & 2d).

Application to Phosphoproteomics - Phosphopeptides Enrichment (β-Casein)

Having metal oxide in nanocomposite, graphene-nickel oxide enriches phosphopeptides from standard protein digest. The derivatization of hydroxyl groups of graphene and its negatively charged surface is not suitable for enrichment of phosphopeptides as protein digest is applied to graphene nano-foam and derivatized graphene without NiO (Fig. S2). Being negatively charged surface, no phosphopeptide is detected in case of graphene nanofoam whereas after derivatization with orthosilicate, four phosphopeptides are detected because of the presence of silica on the graphene nanofoam. It has been reported that NiO exhibits weak ferromagnetism or super-paramagnetism depending on the NP size. This may cause agglomeration of nanoparticles. Therefore graphene nano-foam is used as support for the even distribution of NiO NPs. G-NiO enriches 12 phosphopeptides from β-casein digest. MS spectra are recorded for β-casein digest before and after enrichment (Fig. 3). Phosphopeptides are suppressed in the presence of abundant non-phosphopeptides and only one mono-phosphopeptide is observed at m/z 2061 before enrichment. After applying G-NiO to the β-

casein digest (20 μL/mg), 8 phosphopeptides characteristic of β-casein are observed out of which four are mono-phosphorylated and four multi-phosphorylated. The masses at 1104 (KFQ**S***EEQQQT), 2061 (FQ**S***EEQQQTEDELQDK), 2556 (FQ**S***EEQQQTEDELQDKIHPF) and 3054 (KIEKFQ**S***EEQQQTEDELQDKIHPF) correspond to mono-phosphopeptides whereas 3122 (RELEELNVPGEIVE**S***L**S***S***EESITRI), 3187 (RELEELNVPGEIVE**S***L**S***S***EESIT), 3477 (RELEELNVPGEIVE**S***L**S***S***EESITRINKK) and 3605 (RELEELNVPGEIVE**S***L**S***S***EESITRINKKI) are multi-phosphorylated peptides. Four phosphopeptides are also detected from α-casein because of its contamination in commercial β-casein. At m/z 1253 (TVDMME**S***TEVF), 1594 (TVDME**S***TEVFTK) are mono-phosphorylated and 1329 (EQL**S***T**S***EENSK), 1927 (KDIG**S***E**S***TEDQAMEDIKQ) are di-phosphorylated peptides from α-casein.

Comparison to Metal Oxides NPs

Although graphene-NiO has performed better than the reported NiO NPs, it is necessary to perform enrichment using other metal oxides. Commercial nanoparticles of titania, zirconia and nickel oxide are applied to β-casein digest. Metal oxides can act as Lewis acid or Lewis base depending on the buffer pH applied during activation and sample loading. Therefore the selectivity of metal oxide towards phosphate groups depends on the net charge on surface, affected by nature of metal present in MOx. The isoelectric point of NiO (9.9-10.8), ZrO_2 (4-11) and TiO_2 (3.9-8.2) shows that NiO resides as M-OH^{2+} specie for range of pH whereas in case of titania and zirconia, M-O^- specie predominates above pH 4 and causes loss of phosphopeptides during washing. Zirconia NPs show loss of multi-phosphopeptides (Fig. S3a) whereas mono-phosphopeptides are lost for titania NPs (Fig. S3b). This is characteristic for these two metal oxides and there is no clear explanation to it. NiO has wide pH range in

which it binds negatively charged species. It enriches number of mono and multi-phosphopeptides derived from β-casein however G-NiO enriches enhanced number of phosphopeptides also in higher mass range because of higher surface area of nanocomposite as compared to NiO NPs (Fig. S3c).

Estimation of Selectivity (Spiked β-Casein Digest in de-HeLa Cell Extract)

De-phosphorylated HeLa cell extract spiked with β-casein digest is used to obtain varying complexity. Five mixtures (1:100, 1:300, 1:500, 1:700 and 1:1000) are tested to determine the selectivity levels of G-NiO nanocomposite. The phosphorylated peptides from β-casein digest present in non-specific background of De-phosphorylated HeLa cell extract are enriched by G-NiO. The number of enriched phosphopeptides decreases as the complexity levels increase which is because of the saturation of binding sites with non-phosphopeptides present in abundance (Fig. 4). Although non-specifically bound species are washed away however their initial presence decreases the phosphopeptides binding. At the complexity level of 100, seven phosphopeptides are identified however at 1:1000 level, the number decreases to two.

Sensitivity - Femtomolar Concentration of β-Casein

Sensitivity study determines the ability of nanocomposite to enrich the lowest concentrations. Three dilutions in femtomole concentrations of β-Casein are made. At 5 femtomoles, three phosphopeptides are detected at the intensity level of 400. With increase in dilution to 3 femtomoles, number of phosphopeptides remains same however intensity drops. At 1 femtomole, phosphopeptides can still be distinguished from the noise (Fig. 5).

Enrichment of Phosvitin/Lipovitellin from Egg Yolk

Phosvitin is phosphoprotein in egg yolk which is highly phosphorylated because of successive serine sites. Egg yolk is lyophilized, solubilised in ammonium bicarbonate and digested to enrich the multi-phosphorylated peptides by G-NiO nanocomposite. The MS

spectrum is first recorded for egg yolk digest without enrichment. Only one phosphopeptide from lipovitellin is observed (Fig. 6a). Graphene without nickel oxide is also tested. Because of the negatively charged surface, no phosphopeptide binds and non-specific species are detected (Fig. 6b). G-NiO nanocomposite binds phosphopeptides with multi-phosphorylation level up to 6P (Fig. 6c) and the amino acid sequence for detected phosphopeptides is listed in Table 1.

Enrichment of Casein-Phosphopeptides from Milk

Milk is a source of all casein variants in which α- and β-casein are phosphorylated at serine sites. α-casein has two variants namely α-S1 and α-S2. One phosphopeptide is detected in case of the digest directly analyzed by MALDI-MS (Fig. 7a). Graphene as such does not bind phosphopeptides however abundant non-phosphopeptides at m/z 1267 (YLGYLEQLLR 106-115 α-S1), 1758 HQGLPQEVLNENLLR, α-S1) and 2187 (DMPIQAFLLYQEPVLGPVR, 184-202, β) are enriched (Fig. 7b). Upon enrichment with G-NiO nanocomposite, 22 phosphopeptides are enriched which belong to all three casein variants (Fig. 7c). The phosphopeptides enriched are given in Table 2. The enrichment by the nanocomposite is attributed to the negative graphene surface finely holding positively charged NiO without causing agglomeration of oxide particles.

Bottom-up Identification of Serum Phosphoproteins

Serum being a source of information is employed to identify the phosphoproteins. Serum digestion is carried out to study the phosphorylation sites. Digested serum is used as a sample to enrich phosphopeptides via G-NiO (Fig. S4). Two phosphoproteins related to tumours are identified through bottom-up approach using mascot search engine. The one is apoptosis-associated Speck-like protein containing a CARD, known for its apoptosis initiating ability in tumour cells. It is involved in inhibition of tumorigenesis in primary melanoma whereas in

metastatic melanoma, this effect is diminished[25]. Phosphopeptides with phosphorylation at serine and threonine are identified. Second protein is tumour protein D52 (TPD52) which is overexpressed in prostate and breast cancer. It is interesting to study the phosphorylation of tumor protein D52 as the nanocomposite has enriched phosphopeptides related to this protein[26]. Phosphopeptides enriched from serum digest are listed in Table S1.

Conclusion

Graphene nanofoam is derivatized with NiO and used in phosphoproteomics. During the fabrication of nanocomposite, the graphene strongly interacts with NiO without the agglomeration of oxide particles. This contributes to high enrichment efficiency, selectivity and sensitivity. G-NiO NPs bind mono- and multi-phosphopeptides as compared to ZrO_2 and TiO_2. The performance of NiO NPs is enhanced in the form of G-NiO nanocomposite because of the better surface area. Egg yolk, non-fat milk and serum digests are used as sample for the phosphopeptides enrichment. From serum, two phosphoproteins related to tumours are identified using bottom-up approach. It can be concluded that the performance of NiO is enhanced by using graphene support in the form of G-NiO nanocomposite.

Experimental

Reagents and Materials

α-casein, β-casein, ammonia solution, nickel oxide (<50 nm particle size (TEM), 99.8% trace metals basis), zirconium (IV) oxide (<100 nm particle size (TEM), titanium (IV) oxide (21 nm particle size (TEM), iodoacetamide, dithiothretol, ethanol, bovine serum albumin (BSA), tri-fluoro acetic acid (TFA), 2,5-Dihydroxybenzoic acid, acetonitrile, ammonium bicarbonate (NH_4HCO_3), ammonium hydroxide solution, tetraethyl ortho-silicate, hydrochloric acid, surfactant pluronic F108, dimethyl disulfide and nickel nitrate were purchased from Sigma Aldrich. Trypsin was obtained from Promega whereas Milli Q water was used to prepare solutions.

Synthesis of Graphene-Nickel Oxide

Silica spheres were synthesized by Stober's method[27]. A mixture of 2.3 mL of tetraethyl ortho silicate, NH_4OH solution (29%, 2.59 mL in 410 mL distilled water), 1 mL of water and 60 mL of ethanol were stirred for 6 hours at 50 ºC. The mixture (37.5 mL) was dialyzed for 48 hours (diluted to 75 mL with water). The modification was made by HCl (15 mL, 12 M), F108 (surfactant pluronic, 0.6 g) and dimethoxydimethylsilane (DMDMS, 0.875) for 48 hours and neutralized by 29% ammonium hydroxide solution. Graphene oxide (GO) sheets were fabricated as previously reported[28] and GO suspension (1 mg/mL) was prepared by stirring 0.3 g of GO in 300 mL of water. After sonication (2 hours) silica spheres (55 mL) were added to the suspension, stirred for 12 hours and dried at 50 ºC. It was followed by the calcination at 900 ºC for 5 hours in the presence of argon at 2 ºC/min. The material was washed with hydrofluoric acid (HF, 5%, 20 mL) and 0.0730 g NGF (nano-porous graphene foam) was mixed with 0.1460 g $Ni(NO_3)_2.6H_2O$ by impregnation method in 30 mL ethanol and stirred at room temperature till solvent evaporated. It was calcinated in N_2 at 600 ºC for 6 hours at 2 ºC/min to get graphene-NiO nanocomposite.

Sample Preparation

Digestion of Standard Proteins

α- and β-casein were dissolved in Milli-Q water (1 mg/mL). The standard solution was aliquoted to five fractions, 200 µL each. 50 µL of 45 mM DTT and 160 µL of 1 M NH_4HCO_3 solution (to disrupt disulphide bonds) were added to each of the fraction. The incubation of aliquots was done for 15 minutes by using an Eppendorf thermomixer at 50 °C. After incubation, 50 µL of 100 mM iodoacetamide was added and solutions were cooled down to room temperature. In the next step, sample solutions were incubated again for 15 minutes at room temperature in dark. After cooling, the solution was diluted using 1400 µL of Milli-Q water and 20 µL of trypsin (0.1 µg/µL), was added to solutions and all the aliquots were kept overnight at 37 °C for digestion. Finally, the enzymatic digestion was quenched by diluting the solution with 0.15% TFA for sample of 1 pmol/µL and pH was adjusted to more than 3. Protein digest was stored at -20 °C after placing it in thermomixer for 5 minutes.

Digestion of Non-Fat Milk/Egg Yolk

Non-fat milk (0.2 mL, containing 3 g proteins/100 mL, α- and β-caseins (~66% of the total proteins as per values given by the company) purchased from local market was lyophilized to dryness. Egg yolk (1 mL) was lyophilized to powder. The lyophilized powder was dissolved in one molar ammonium bicarbonate solution to a final concentration of 50 mM. For the reduction of disulfide bonding, the solution was mixed with 200 µL of 45 mM DTT and the mixture was incubated for 20 minutes at room temperature. Solution was again incubated for half an hour after adding iodoacetamide (50 µL of 100 mM) at room temperature in dark. For enzymatic digestion milk protein solution was mixed with 80 µL trypsin solution (0.1 mg/mL) and incubated overnight at 37 °C. Finally, 100 µL of 0.15% TFA solution in ACN/water was added to the protein solution to stop digestion process.

Digestion of Serum

The serum was diluted in 50 mM NH_4HCO_3 (pH~8) with final concentration of 1 pmol. For reduction, 50 µL of 100 mM dithiothretol (DTT) was added and heated at 56 °C for 15 minutes. The solution was cooled to room temperature by adding of 50 µL of 100 mM iodoacetamide (IAA) for alkylation. The whole mixture was incubated in dark for 15 minutes at room temperature. Afterwards, the obtained solution was diluted with 1400 µL of water. The digestion was preceded with a mixture of trypsin and Lys-C in a ratio of 1:50 for 14 hours at 37 °C. 10 µL of 0.1% TFA was added to stop the digestion.

Selectivity/Sensitivity Assessment

The protocol for preparation of dephosphorylated HeLa cell (de-HeLa cell) is given in supporting information. Spiked samples for the selectivity study were prepared. Five samples were prepared in the ratio of 1:100, 1:300, 1:500, 1:700 and 1:1000 using β-casein digest in de-HeLa cell. Sensitivity was measured with concentrations of 5, 3 and 1 fmol.

Phosphopeptides Enrichment by Graphene-NiO

Enrichment was performed by the batch extraction. The nanocomposite particles (20 µg) were activated by activation buffer by adding 100 µL 80% ACN in 0.1% TFA. The mixture was vortexed for 1 minute, centrifuged at 12000 rpm for 3 minutes and supernatant was removed. Following similar steps the particles were conditioned with 100 µL of 0.1% TFA. Peptide samples, 1 mg/mL (tryptic digests of standard proteins and non-fat milk) acidified with 0.1% TFA were added to the nanocomposite material. The mixture was vortexed and incubated for half an hour at 37 °C with gentle shaking. Removal of non-phosphopeptides was carried out by 10 µL of 70% ACN in 0.1% TFA. Finally, phosphopeptides elution was done by 20 µL of 2% ammonia solution (pH 11). The eluted phosphopeptides were subjected to MALDI-MS analysis. Egg yolk digest and non-fat milk were also applied to graphene

nanofoam prior to the derivatization. Zirconia, titania and nickel oxide nanoparticles were applied to β-casein digest using their reported protocols.

MALDI-MS Analysis with Database Searching for Phosphopeptides

For the peptide samples, MS analyses were carried out on Ultraflex III MALDI TOF/TOF MS (Bruker Daltonics) operated in reflector and positive ion mode. The matrix solution was prepared by dissolving DHB in 0.1% TFA: 50% acetonitrile (1:1) spiked with 1% phosphoric acid. The fractions which were collected i.e. eluted phosphopeptides, prior to enrichment and non-phosphopeptides were mixed with matrix solution in equal volumes and spotted on MALDI target plate. Calibration standard I (Bruker) was used for calibration. The mass spectra were exported using Flex analysis software (Version 3.3) based on the BLAST algorithm. The parameters for the BLAST search were as follows: enzyme, trypsin; cystein modification, iodoacetamide; missed cleavages, one; fixed modification, C-carbamidomethyl; variable modifications, oxidation(M), Phosphorylation (ST), Phosphorylation (Y) and peptide tolerance, 3.0 Da. Mono-isotopic mass list was used in Mascot (www.matrixscience.com) to query the Swiss-Prot database which identifies the phosphorylated peptides for α (αS1 and αS2) and ß-casein (Swiss-Prot accession number P02662, P02663 and P02666 respectively). Generated data was converted to MS spectra file and exported to BioTools for identification using peak picking method suppressed up to 700 Da. With access to Swiss-Prot (http://web.expasy.org) database, the program was set up to provide the information about phosphopeptides as m/z, amino acid sequence, hit score, protein accession number, missed cleavage, error in Da and error in ppm.

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Additional information regarding the preparation of HeLa cell extract, Fig. S1 for phosphopeptides enrichment of β-Casein, Fig. S2 for comparison to metal oxides NPs, Fig. S3 for bottom-up identification of serum phosphoproteins and Table S1 for enrichment from serum is given in Supporting Information.

Table 1 Peptide fragments identified from raw egg yolk digest, enriched entities by graphene and graphene-nickel oxide nanocomposite.

Derived protein from egg yolk	m/z for the peptide fragments	Peptide sequence	MS spectra for fractions		
			Raw	GNF	NiO-GNF
Phosvitin	1084.600	***SSSSSS***VLSKI	-	-	*
	1170.111	AKTSSSSSSASSTAT***SSSSSS***ASSPN	-	-	*
	1256.711	D***SSSSSSSS***VL***S***K	-	-	*
	1337.926	KPMDEEENDQ	-	-	*
	1419.677	GTEPDAKTSSSSSSASSTAT***SSSSSS***ASSPN	-	-	*
	1678.892	SGHLEDD***SSSSSS***VL	-	-	*
	1726.810	LEDD***SSSSSS***VLSKI	-	-	*
	1856.920	EDD***SSSSSS***VLSKIWG	-	-	*
Lipovitellin	1575.567	EVNPE***S***EEE	-	-	*
	2073.567	EVNPE***S***EEEDE***SS***PYEDI	*	-	*

Table 2 Phosphopeptides of milk casein variants enriched by nano-porous graphene-NiO nanocomposite.

Milk Casein Variants	Amino-acid Position	Amino acid sequences	No of phosphate groups	$[M+H]^+$
α-S2	153-162	TVDMMES*TEVF	1	1254.94
	141-151	EQLS*TSEENS*K	2	1329.52
	153-164	TVDMES*TEVFTKK	2	1594.28
	17-36	NTMEHVS*S*S*EES*IISQETYK	1	2616.23
	61-85	NANEEEYSIGS*S*S*EES*AEVATEEVK	4	3006.05
α-S1	121-134	VPQLEIVPNS*AEER	1	1660.57
	57-73	KDIGES*ES*TEDQAMEDIK	1	1847.62
	43–58	DIGS*ES*TEDQAMEDIK	2	1927.21
	118–137	KYKVPQLEIVPNS*AEERLHS	1	2202.02
	104–138	KYKVPQLEIVPNS*AEERLHSM	1	2289.11
	51–76	KVNELSKDIGS*ES*TEDQAMEDIKQME	2	2857.43
β Casein	47-48	KFQS*EEQQQ	1	975.83
	47-49	KFQS*EEQQQT	1	1104.10
	33-48	FQSEEQQQTEDELQDK	1	2061.02
	22-40	NVPGEIVESLS*S*S*EES*ITR	4	2352.61
	47-67	FQS*EEQQQTEDELQDKIHPF)	1	2556.23
	44-67	KIEKFQS*EEQQQTEDELQDKIHPF	1	2779.95
	16-40	RELEELNVPGEIVES*LS*S*S*EESITR	4	2965.63
	29-52	KIEK FQS*EEQQQTEDELQDKIHPF	1	3054.42
	26-41	RELEELNVPGEIVES*LS*S*S*EESITRI	3	3122.63
	26-39	RELEELNVPGEIVES*LS*S*S*EESIT	4	3180.54

Figure and Table captions

Fig.1 TEM (a) Graphene nanofoam; (b) Nickel oxide embedded in graphene nanofoam.

Fig. 2 (a) X-ray diffraction (XRD) pattern; (b) Thermogravimetric (TGA) analysis; (c) BET surface area and (d) Pore volume calculated for graphene-NiO nanocomposite.

Fig. 3 MALDI-MS Spectra of tryptic β-casein digest: (a) Direct analysis (b) Analysis of eluted fraction after enrichment by graphene-NiO nanocomposite.

Fig. 4 MALDI-MS spectra of tryptic β-casein digest spiked in dephosphorylated HeLa cell extract using Graphene-Nickel oxide nanocomposite in different ratios as: (a) 1:100; (b) 1:300; (c) 1:500; (d) 1:700; and (e) 1:1000. The symbol **β** represents phosphopeptides derived from β-casein.

Fig. 5 Sensitivity study at femto-molar concentration of β-casein: (a) 5 femtomole, (b) 3 femtomole and (c) 1 femtomole.

Fig. 6 MALDI-MS spectra of tryptic egg yolk digest: (a) Without enrichment (b) After enrichment with graphene (c) After enrichment with graphene-NiO nanocomposite. PV represents enriched phosphopeptides from phosvitin and LP from lipovitellin.

Fig. 7 MALDI-MS spectra of tryptic digest of non-fat milk: (a) Without enrichment (b) After enrichment with graphene (c) After enrichment with graphene-NiO nanocomposite.

Table 1 Peptide fragments identified from raw egg yolk digest, enriched entities by graphene and graphene-nickel oxide nanocomposite.

Table 2 Phosphopeptides of milk casein variants enriched by graphene-NiO nanocomposite.

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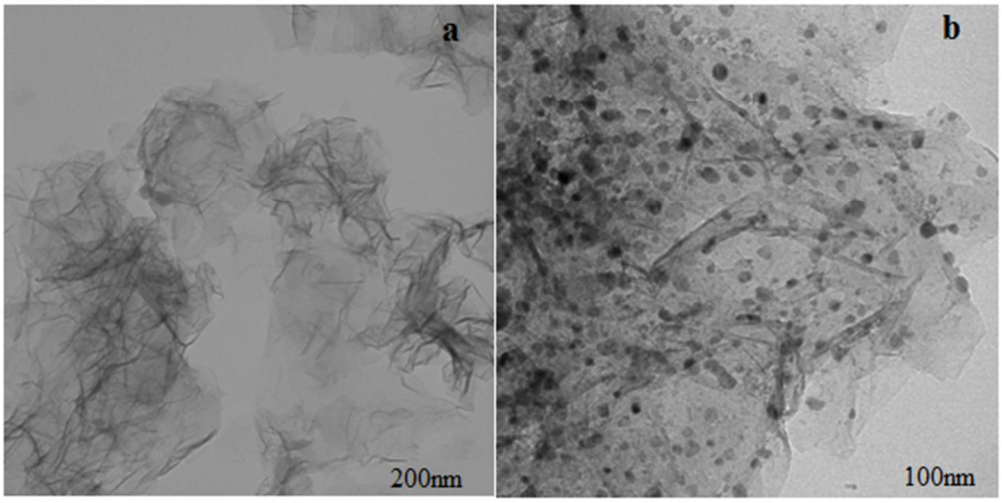

Fig.1 TEM (a) Graphene nanofoam; (b) Nickel oxide embedded in graphene nanofoam.
155x78mm (96 x 96 DPI)

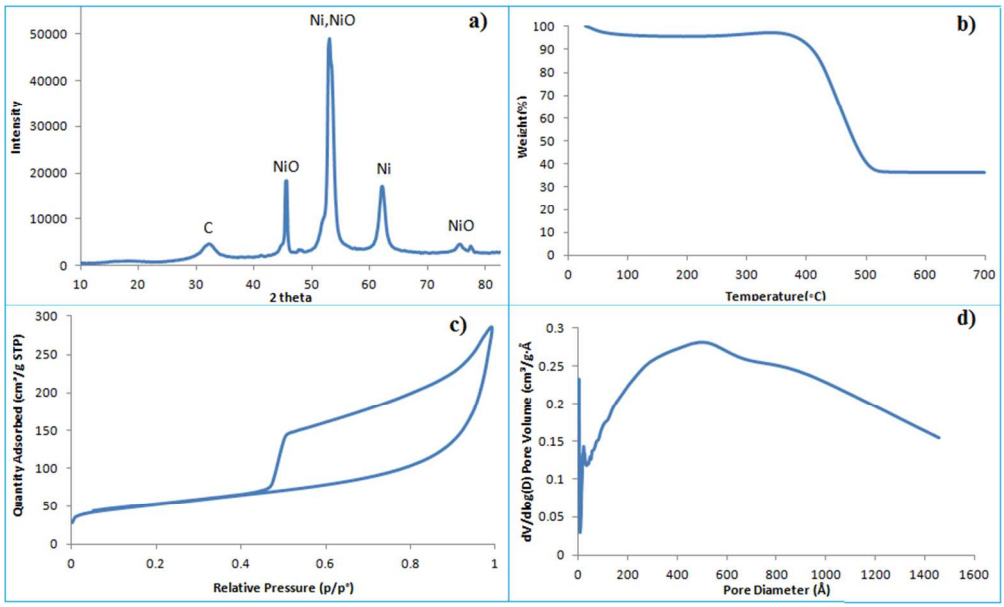

Fig. 2 (a) X-ray diffraction (XRD) pattern; (b) Thermogravimetric (TGA) analysis; (c) BET surface area and (d) Pore volume calculated for graphene-NiO nanocomposite.
244x146mm (96 x 96 DPI)

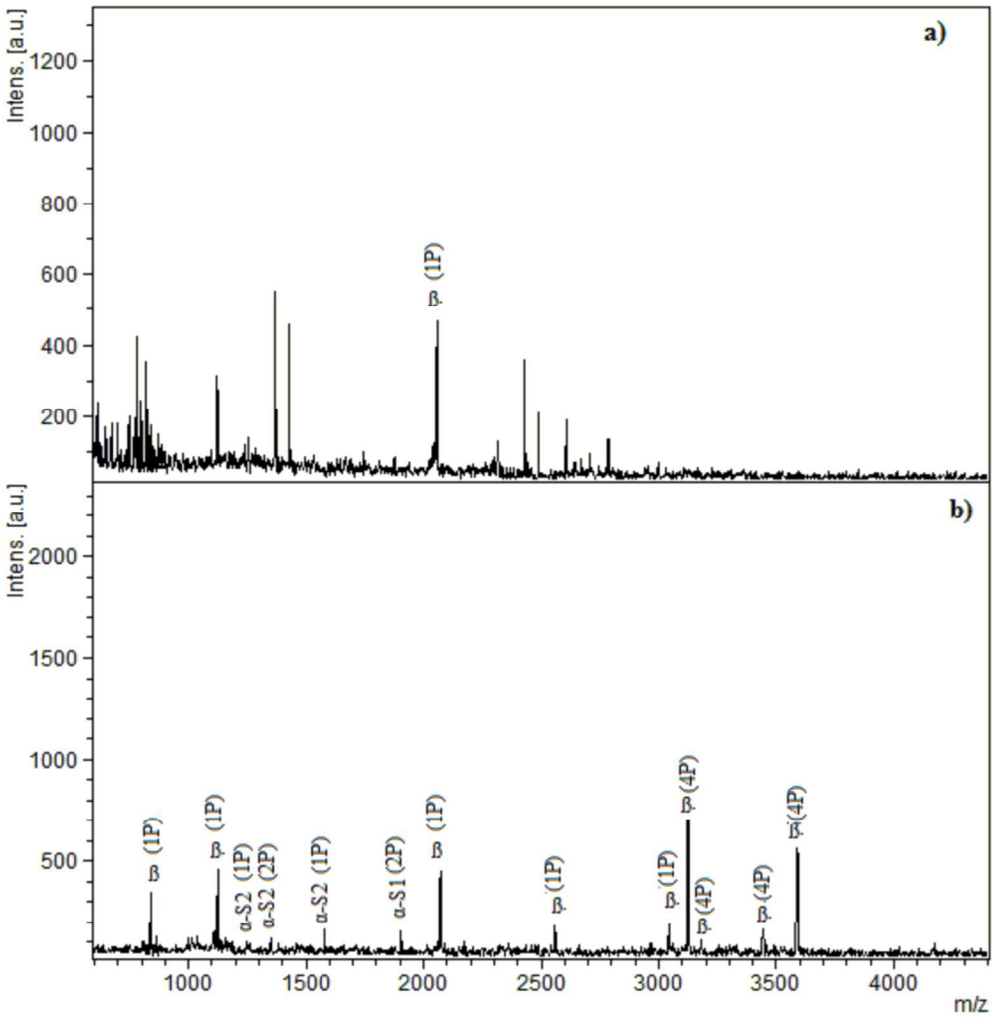

Fig. 3 MALDI-MS Spectra of tryptic β-casein digest: (a) Direct analysis (b) Analysis of eluted fraction after enrichment by graphene-NiO nanocomposite.
153x157mm (300 x 300 DPI)

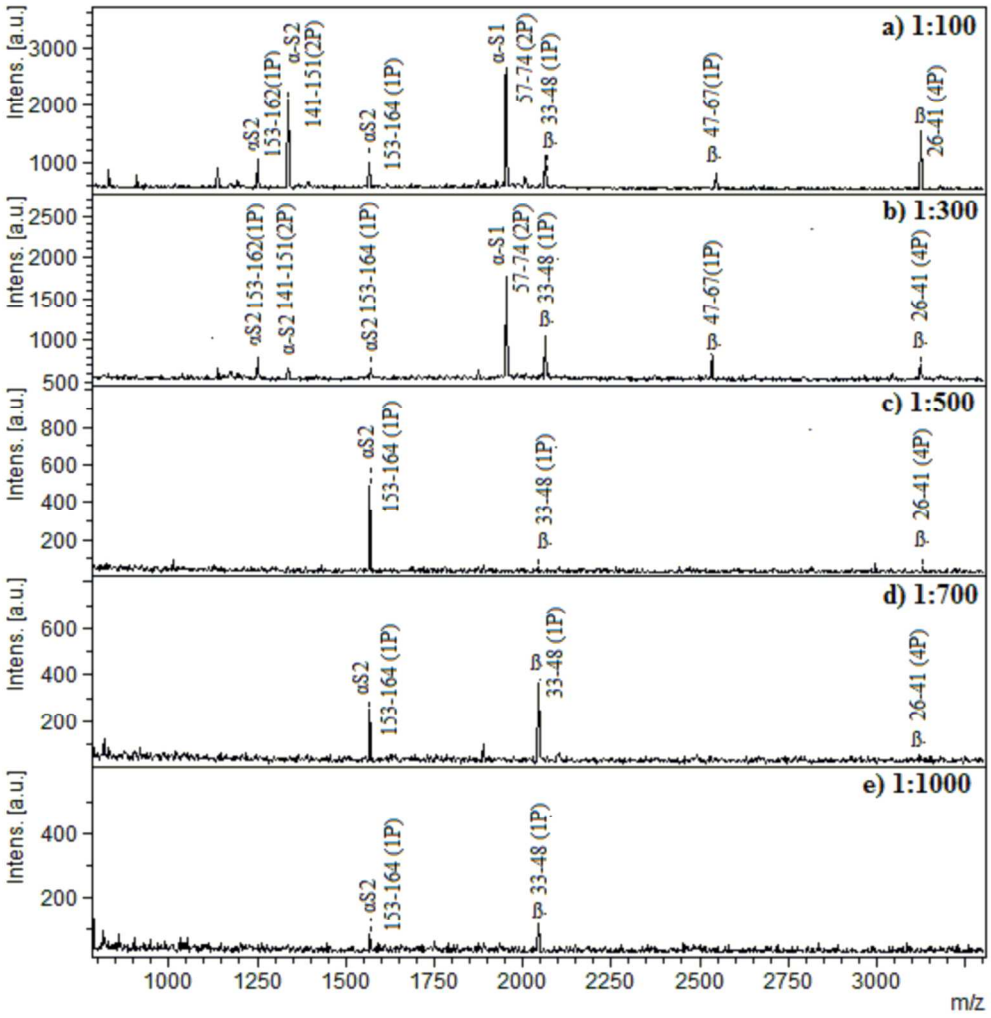

Fig. 4 MALDI-MS spectra of tryptic β-casein digest spiked in dephosphorylated HeLa cell extract using Graphene-Nickel oxide nanocomposite in different ratios as: (a) 1:100; (b) 1:300; (c) 1:500; (d) 1:700; and (e) 1:1000. The symbol β represents phosphopeptides derived from β-casein.
153x156mm (300 x 300 DPI)

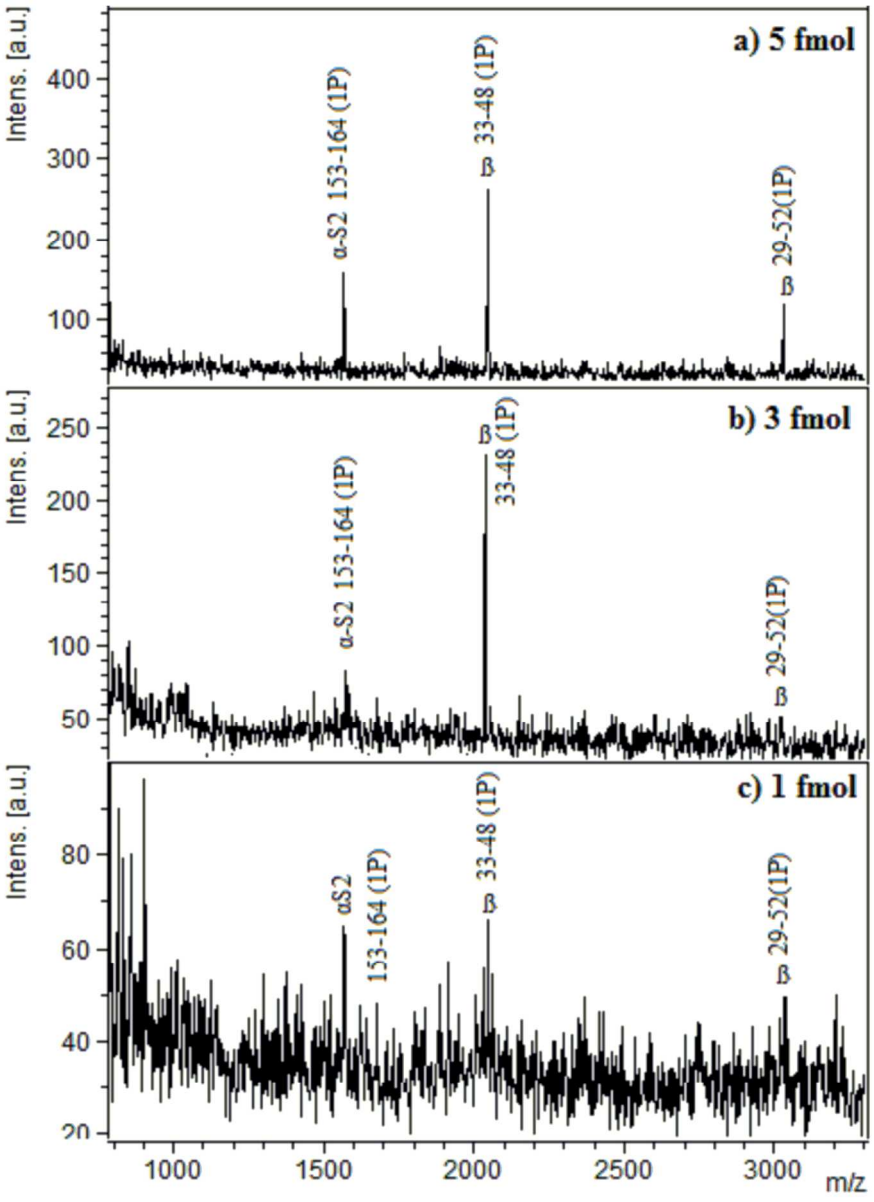

Fig. 5 Sensitivity study at femto-molar concentration of β-casein: (a) 5 femtomole, (b) 3 femtomole and (c) 1 femtomole.
151x205mm (300 x 300 DPI)

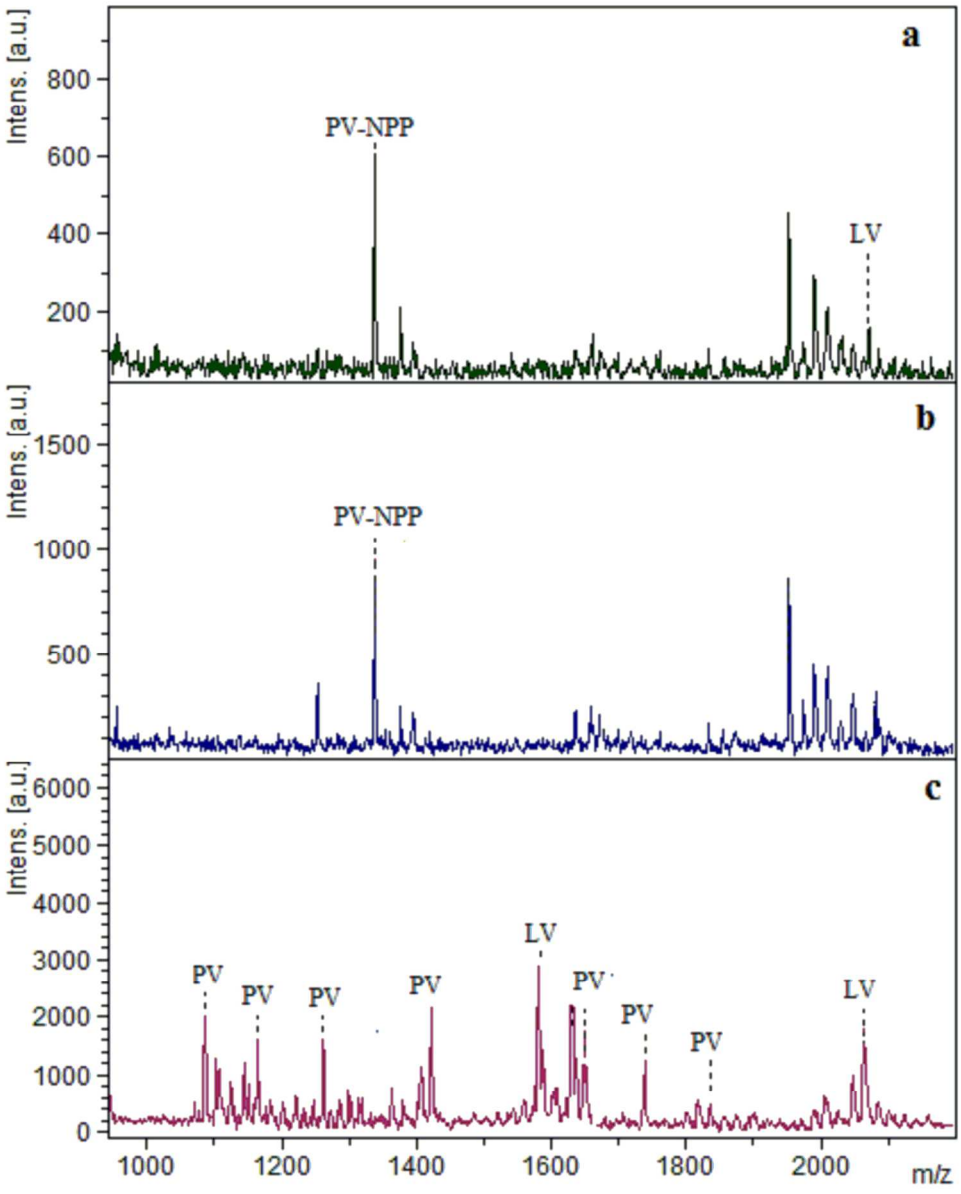

Fig. 6 MALDI-MS spectra of tryptic egg yolk digest: (a) Without enrichment (b) After enrichment with graphene (c) After enrichment with graphene-NiO nanocomposite. PV represents enriched phosphopeptides from phosvitin and LP from lipovitellin.
151x186mm (300 x 300 DPI)

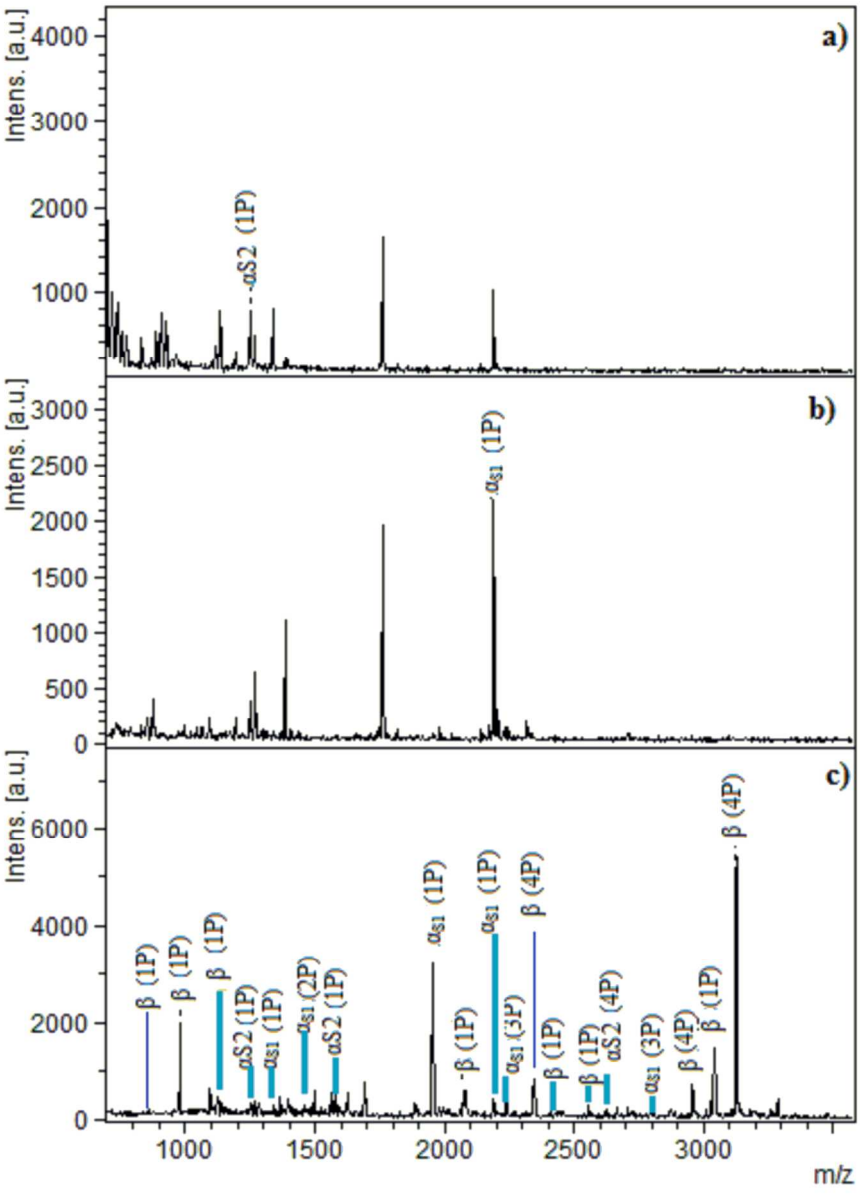

Fig. 7 MALDI-MS spectra of tryptic digest of non-fat milk: (a) Without enrichment (b) After enrichment with graphene (c) After enrichment with graphene-NiO nanocomposite.
153x211mm (300 x 300 DPI)