Nanostructured Enrichment Materials

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Surfactant-Free Synthesis of SnO_2@PMMA and TiO_2@PMMA Core–Shell Nanobeads Designed for Peptide/Protein Enrichment and MALDI-TOF MS Analysis**

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Nanomaterials have achieved great success in biomedical research during the past twenty years,[1] which is represented by several landmark discoveries such as photoluminescent quantum dots for cell labeling,[2] polymer beads for drug delivery,[3] and carbon nanotubes as biological probes.[4] Recently, the use of nanomaterials for peptide/protein enrichment and analysis has also received much attention.[5] It is well known that peptide mapping by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) along with database searching is a major tool in modern protein analysis.[6] Although MALDI-TOF MS is highly sensitive to trace amounts of peptides/proteins, it seems insufficient when low-abundance peptides/proteins derived from practical biological tissues are involved, because these samples have extremely low concentrations of the target peptides/proteins (even below 1 nM), and the many contaminants such as buffer salts and surfactants (detergents) seriously interfere with the subsequent MS analysis. Therefore, pre-concentration and isolation of the target molecules from these complex mixtures are necessary before MS measurement. The several types of nanomaterials that have been developed to enrich and separate peptides/proteins from mixtures[5,7] include zeolite nanocrystals[8] and PMMA-modified $CaCO_3$ nanoparticles [PMMA = poly(methyl methacrylate)],[9] However, these materials have some drawbacks in practical applications. For example, incubation of peptides/proteins with zeolite nanocrystals is a time-consuming process. Solid zeolite particles can hinder the formation of uniform cocrystals between the matrix and peptides/proteins on the MALDI plates. The PMMA-modified $CaCO_3$ species, after enrichment with peptides/proteins, must be decomposed by an acid treatment that can cause considerable loss of the target molecules. Therefore, searching for proper enrichment materials applied in MALDI-TOF MS analysis remains a great challenge.

An ideal material for this purpose must have the following features. First, it can be suspended stably in water and it has a large surface area so that it will capture the target molecules to the greatest possible extent. Second, it must exhibit highly selectivity adsorption of the target molecules and exclude contaminants. Third, after adsorption, it should be easily separated from the mixture prior to redispersion in a small volume of water for application to the MALDI plate. Finally, it should not interfere with the signals of the target molecules during MS measurements. Polymer beads meet most of these criteria. Because their diameters are in the range of 0.1–10 μm, they can be suspended in water for months and recovered by high-speed centrifugation. In addition, many hydrophobic polymers can capture peptides, and their selectivity for the target molecules can be designed by controllable synthesis. However, their molecular weights are usually in the range of 10^2–10^4 Da, so their MS signals can seriously disturb the analysis of peptides and proteins. Although polymer beads have been tried by other researchers previously,[10] detection limits are not lower than 100 nM for peptides, and corresponding experiments with proteins are scarcely seen in the literature. A promising solution to this problem is to employ polymer beads containing inorganic nanocores, because the internal nanoparticles can bind the polymer chains and prevent their ionization during MS measurement. Unfortunately, the popular routes for synthesizing core–shell polymer beads at present always employ a large amount of surfactants,[11] which produce protein–surfactant adducts and surfactant aggregates. In an ideal pretreatment of peptides/proteins before MALDI-TOF MS analysis, conventional surfactants should be eliminated from the solution completely.[12]

Here we show a new method to prepare SnO_2@PMMA and TiO_2@PMMA core–shell nanobeads without using surfactants. These nanobeads effectively enrich intact horse heart myoglobin (MYO, 400 nM) and trypsin-digested MYO peptides (1 nM). After this optimized enrichment procedure, the MS signal intensities and the corresponding signal-to-noise (S/N) ratios of the intact MYO are increased by one order of magnitude, while those of the digested MYO are enhanced by three orders of magnitude. Because both the

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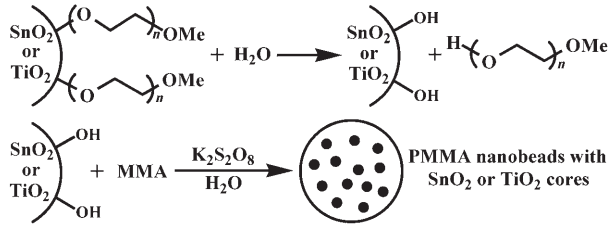
SnO_2 (2 nm) and TiO_2 (8 nm) cores of the nanobeads are much smaller than those of our previously reported PMMA–$CaCO_3$ nanoparticles (ca. 70 nm), they can form a homogeneous layer on the MALDI plate and do not hinder cocrystallization of the matrix and the peptides/proteins. Thus, unnecessary loss caused by acid treatment is avoided. Additionally, these SnO_2@PMMA and TiO_2@PMMA core–shell nanobeads show greater enrichment effects than other candidate nanoparticles.[8,9] Therefore, the significance of our method lies in utilizing the affinity between peptides/proteins and PMMA, the suspendability of the polymer beads, the strong interaction between the nanoparticles and the polymer shells, and excluding the drawbacks caused by surfactants.

To prepare core–shell nanobeads without any surfactants, inorganic nanoparticles should be synthesized with organic surface groups that help the nanoparticles disperse into monomers, and these groups must be removed after polymerization. Accordingly, we chose SnO_2 and TiO_2 nanoparticles modified with poly(ethylene glycol) methyl ether (PEGME) synthesized by solvothermal methods. These SnO_2 and TiO_2 nanoparticles are soluble in methyl methacrylate (MMA) monomer to form stable colloids. Our previous research[13] showed that the PEGME groups could be exchanged by hydroxy groups rapidly when these nanoparticles were dispersed in water. On this basis, we designed an experiment in which such hydroxy-exchange reactions took place on the PEGME–SnO_2 and PEGME–TiO_2 surfaces, and MMA monomers were simultaneously polymerized in boiling water to form PMMA beads. As a result, PMMA nanobeads containing SnO_2 and TiO_2 cores were produced (Scheme 1). Because PEGME molecules are miscible with



Scheme 1. OH-exchange reactions (top) and polymerization around nanoparticles (bottom).

water, they can be removed thoroughly by repeated washing/centrifugation of the nanobeads.

The average diameters of these pure PMMA,[14] SnO_2@PMMA, and TiO_2@PMMA core-shell nanobeads are all about 200 nm, and they are monodisperse in water (Figure 1). The large surface areas and the MMA component ensure their high adsorption capacity for peptides and proteins. The TEM images show that many nanodots are encapsulated in each core–shell bead, while no nanoparticles are found outside or nearby the beads. Since these nanocores have an abundance of OH groups on their surface[13] and both SnO_2 and TiO_2 nanocrystals are Lewis acids,[15] the interactions between the PMMA shells and the nanocores probably occur through hydrogen bonds or Lewis acid–base interactions.

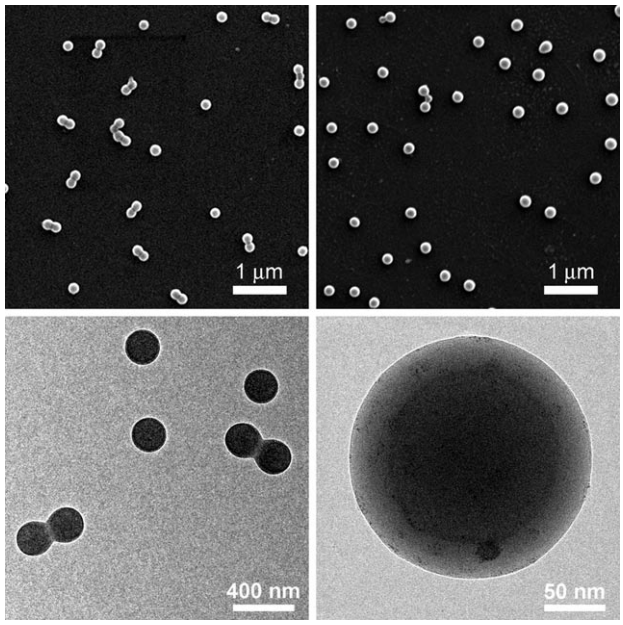


Figure 1. SEM images (top) of SnO_2@PMMA (left) and TiO_2@PMMA (right) beads, and TEM images (bottom) of the SnO_2@PMMA beads with different resolutions.

These interactions improved the decomposition temperature of the products significantly. Thermogravimetric (TG) measurements show that the pure PMMA samples decompose in the range of 200–300 °C, while the core–shell beads with 20 wt % nanocore decompose at about 300 °C. The TG analysis also provides the proportion of the nanoparticles for each core–shell material, which varies from about 2 to about 20 wt %.

In Figure 2, the IR spectra of the PMMA, PEGME-SnO_2, PEGME-TiO_2, and the core–shell nanobeads are compared. The asterisked peak at about 1100 cm^{-1} is due to the characteristic C-O-C vibration of PEGME.[13] Clearly, after centrifugation/washing, both SnO_2@PMMA and TiO_2@PMMA exhibit no C-O-C absorbance, that is, the PEGME groups have been removed thoroughly from the PEGME–SnO_2 and PEGME–TiO_2 nanoparticles. The Sn–O bands at

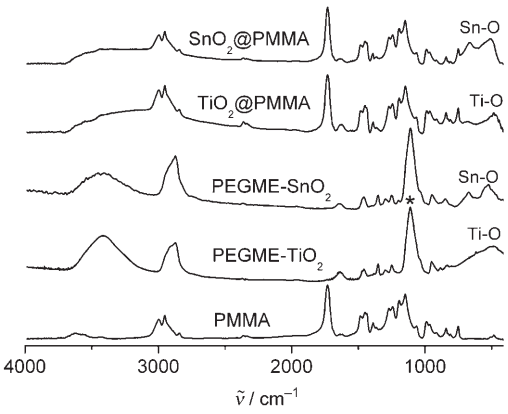


Figure 2. IR spectra of the pure PMMA beads, PEGME–SnO_2 and PEGME–TiO_2 nanoparticles, and the final core–shell nanobeads. The asterisked peak is the C-O-C band of PEGME.

670 and 520 cm^{-1} and the Ti–O band at 490 cm^{-1} remain for the core–shell nanobeads,[13] which proves the presence of the nanoparticles in the PMMA beads. Therefore, the final products SnO_2@PMMA and TiO_2@PMMA have only two simple components: PMMA shells and SnO_2 or TiO_2 nanocores. The merit of our preparation route is the exclusion of any surfactants, emulsifiers, cross-linkers, or phase-transfer agents that would interfere with subsequent MALDI-TOF MS analysis. Moreover, these nanobeads are stable in water for months, and their enrichment function remains unchanged.

Figure 3 shows the MALDI-TOF mass spectra for the 1 nM digested MYO and 400 nM intact MYO samples before and after enrichment. Pure PMMA, SnO_2@PMMA, and TiO_2@PMMA beads were used as enrichment materials. For comparison, the feature peaks of the target molecules are marked by asterisks. Among them, the highest MS peaks are labeled with intensity (S/N ratio). Before enrichment, the MS intensity and S/N ratio are very weak for both digested and intact MYO. After enrichment, all of the nanoparticles exhibit significant improvement in MS intensity, although a range of S/N ratios is observed. The samples enriched by core–shell nanobeads show S/N ratios that increase by 1–3 orders of magnitude, while those enriched by pure PMMA have rather low S/N ratio enhancements, especially the MYO digest. The mass spectrum for digested MYO enriched by pure PMMA shows many interfering signals in the range of 700–1400 Da, that is, PMMA itself may be ionized under the measurement conditions. Pure PMMA beads were also tested by MALDI-TOF MS, and the result verified this speculation. Therefore, incorporation of the nanocores and polymer shells produces ideal enrichment materials.

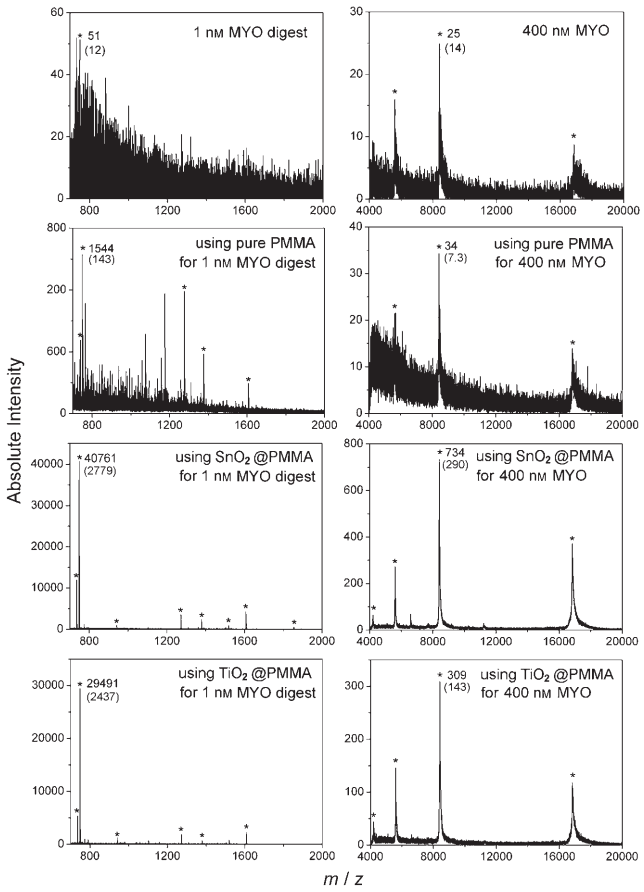


Figure 3. MALDI-TOF MS of digested MYO and intact MYO samples without enrichment and those enriched by pure PMMA, SnO_2@PMMA with 22.3 wt% SnO_2, and TiO_2@PMMA with 20.6 wt% TiO_2. The MS intensity and the corresponding S/N ratio (in parentheses) are labeled for the highest peak.

To elucidate the relationship between the nanocores and PMMA shells, a range of core–shell beads with different core contents were synthesized and tested. The results are listed in Table 1. The core–shell nanobeads show a clear correlation between inorganic content and improved S/N ratios, that is, the nanocores hinder PMMA ionization during measurement. Table 1 also illustrates the superior qualities of SnO_2@PMMA compared to TiO_2@PMMA when they both have a similar weight percentage of cores. This phenomenon may be due to the difference in size between SnO_2 (2 nm) and TiO_2 (8 nm), because the smaller particles have a relatively larger surface area and hence a higher capacity to bind the PMMA molecules.

Although an excess of core–shell nanobeads was added to each test solution, and the supernatants have no MS signals at all after centrifugation, the MS intensity of the target molecules is not the same. Indeed, in both Figure 3 and Table 1, the peak intensity increases significantly with increasing nanocore content. This may have two explanations. One is competitive ionization between PMMA and the target molecules and competition for absorption of the laser energy between PMMA and the matrix.[5,7,9] When the PMMA signals are suppressed by the nanocores, the MS signals from the target molecules are improved. The other explanation arises from the core–shell nanostructure. Like other typical polymer microspheres,[16] the PMMA beads are porous and they adsorb the target molecules. But if the target molecules are trapped deep inside the beads, it will be very difficult to detect them by MALDI-TOF MS, so the intensity and S/N for samples enriched by pure PMMA are rather low. However, in the case of the core–shell beads, the SnO_2 and TiO_2 nanoparticles will block the target molecules and constrain them to the shallow outer layers of the bead (see Figure 1). Hence, the

Table 1: MS intensity and S/N ratio of the labeled peaks for 1 nM digested MYO and 400 nM intact MYO after enrichment with different materials. These labeled peaks are at m/z 748 (peptide sequence ALELFR) for digested MYO and m/z 8471 (MYO+2 H^+) for intact MYO.

Core content	Digested MYO		Intact MYO	
in PMMA beads	Intensity	S/N ratio	Intensity	S/N ratio
0 (unenriched)	51	12	25	14
0 (pure PMMA)	1544	143	34	7.3
1.8 wt% SnO_2	1554	190	40	11
4.6 wt% SnO_2	5109	484	143	56
11.2 wt% SnO_2	18560	1808	276	105
22.3 wt% SnO_2	40761	2779	734	290
2.0 wt% TiO_2	1775	148	26	9.5
5.1 wt% TiO_2	5819	444	77	44
9.5 wt% TiO_2	10031	1344	176	87
20.6 wt% TiO_2	29491	2437	309	143

samples treated with the core–shell beads exhibit higher MS intensity, and the MS intensity increases with increasing core content.

Our enrichment process has been proven to be effective in a real proteomic analysis using proteins extracted from the brain of Sprague–Dawley rat. Through a glutathione-S-transferase pulldown experiment, protein bands were analyzed by MALDI-TOF MS after separation by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Ten protein bands, which could not be identified by traditional lyophilization, were successfully identified by means of the present nanobead enrichment process (the results are deposited in the Supporting Information), and this indicates the excellent potential of our method for real proteomic applications.

In summary, we have developed a surfactant-free route to prepare SnO_2@PMMA and TiO_2@PMMA core–shell nanobeads with controlled size and composition. These nanobeads can effectively enrich extremely low concentrations of peptides/proteins from complex aqueous solutions, and significantly improve their MS intensity, S/N ratio, and peptide sequence coverage. Such positive effects are ascribed to interactions between the nanocores and the polymer shells, which depend on the concentration and diameter of the nanocores in the polymer beads. Therefore, to produce better core–shell enrichment materials, the interactions between the two components should be strengthened, and polymer shells with higher selectivity should be synthesized. The present explorative research points to a new direction for designing highly efficient enrichment nanomaterials for use in the MALDI-TOF MS analysis of peptides and proteins.

Experimental Section

SnO_2 and TiO_2 nanoparticles modified with PEGME ($M = 350$, Fluka) were prepared according to our previous research.[13] They can be dispersed in solution of MMA monomer to form stable colloids. In a typical preparation of polymer beads, 5 g MMA and 80 g distilled water were mixed together and heated with stirring under nitrogen atmosphere. When the temperature rose to 75 °C, 0.5 g of $K_2S_2O_8$ initiator was added. After 3 min, another 5 g of MMA and an appropriate amount of nanoparticles were injected into the reaction system immediately. The mixture was heated to reflux for 1 h to obtain a milky suspension. After cooling to room temperature, the product was centrifuged at 6000 rpm for 6 min to remove any precipitates, and then the supernatant milky suspension was centrifuged at 12000 rpm for 30 min to obtain the gel-like polymer beads. These gels could be redispersed in water by sonication, and in this way they were purified by washing/centrifugation. To investigate the morphology and composition of the polymer beads, a Philips XL30 scanning electronic microscope was used to obtain SEM images, a JEM-2010 transmission electron microscope operating at 200 kV was employed to obtain TEM images, and a Perkin–Elmer TGA 7 thermal analyzer was used to record the thermogravimetric data. The infrared measurements were carried on a Nicolet Impact 360 FTIR spectrometer.

To enrich the digested MYO (1 nM) or the intact MYO (400 nM) from 1 mL of aqueous solution, 0.5 μL of nanobead suspension (250 $\mu g\,\mu L^{-1}$ for the digested MYO and 1 $mg\,\mu L^{-1}$ for the intact MYO) was added. This mixture was then incubated with shaking for 10 min at 37 °C, followed by centrifugation at 16000 rpm for 20 min. After the supernatant was decanted, 0.5 μL of matrix (10 $\mu g\,\mu L^{-1}$ solution of α-cyano-4-hydroxycinnamic acid in acetonitrile/water (1/1 v/v) containing 0.1 % trifluoroacetic acid, Sigma) was added to the residue and mixed under sonication. Then the mixture was directly dropped onto the stainless steel MALDI plate for MS analysis. All mass spectra were acquired by a 4700 Proteomics Analyzer MALDI-TOF/TOF-MS (Applied Biosystems, Framingham, USA) under the same conditions. The instrument was operated at an accelerating voltage of 20 kV with a 200-Hz pulsed Nd:YAG laser (355 nm).

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