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Intramesoporous silica structure differentiating protein loading density

Wen Qi^{1,2}, Xiaolin Li¹, Baowei Chen¹, Pei Yao^{2,*}, Chenghong Lei^{1,*}, and Jun Liu^{1,*}

¹Pacific Northwest National Laboratory, P. O. Box 999, Richland, WA 99352, USA

²School of Material Science and Engineering, Tianjin University, Tianjin 300072, P. R. China

Abstract

We report that hydrothermal aging temperature had a critical effect on intramesoporous structure of mesoporous silica and thus the intramesoporous structure affected protein loading in the mesoporous silica significantly. For a neutral protein Immunoglobulin G with a Y-like molecular shape, the larger desorption pore size allowed the larger protein loading. For a charged protein glucose oxidase with an elliptical molecular shape, the larger surface area resulted in the larger protein loading. Fluorescence emission spectra from tyrosinyl and tryptophanyl residues of the proteins in mesoporous silicas indicated that the charged protein was electrostatically attached inside the mesopores in a way of monolayer, while the neutral protein IgG could continue to aggregate after the monolayer occupancy.

Keywords

Mesoporous materials; Proteins; Drug delivery; Intramesoporous structure; Hydrothermal aging temperatures

1. Introduction

Mesoporous silica, with unique features of large surface areas, controllable porous structures, versatile functionalization accessibilities, and scalable productions, are ideal carriers for delivery of proteins [1–8], DNAs [9–13], and other drug molecules [14–17]. The drug molecules can be spontaneously entrapped in the mesoporous silicas via non-covalent interaction and can be also spontaneously released from the mesopores when the mesoporous silica-drug composites are dispersed in a fresh buffer solution in which a new thermodynamic balance can be reached [1–8]. Since the drug molecules are entrapped inside the mesopores, the intramesoporous structures, i.e. pore size (diameter), surface area and pore volume, would have great influence on the drug loading. In this work, when preparing as-made mesoporous silica (AMS), we carefully controlled the hydrothermal aging temperature (HAT) for the aging step of silica gel prior to its calcination. We found that HAT had a critical effect on the intramesoporous structure of AMS and the subsequently functionalized mesoporous silica (FMS) and thus on protein loading. For a neutral protein,

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*To whom correspondence should be addressed. Dr. Chenghong Lei Pacific Northwest National Laboratory Mail Stop J4-02 P.O. Box 999 Richland, WA 99352 Tel: 1-509-3716962 Fax: 1-509-3717304 Chenghong.Lei@pnl.gov. jun.liu@pnnl.gov. pyao@tju.edu.cn.

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the Y-like immunoglobulin G (IgG), the larger desorption pore size allowed the larger protein loading. For a charged protein, the elliptical glucose oxidase (GOX), the larger surface area resulted in the larger protein loading. The charged protein GOX was electrostatically attached inside the mesopores in a way of monolayer, while the neutral protein IgG could continue to aggregate in the mesopores after the monolayer occupancy.

2. Materials and Methods

As-made mesoporous silicas (AMS, SBA-15 type) were prepared according to procedures modified from our earlier work [18–20]. For a typical synthesis, 4.0 g of Pluronic P-123 (MW = 5,800) was dissolved in 2 M HCl solution (120 mL) at 35–40°C. Then 6.0 g of mesitylene and 8.5 g of tetraethylorthosilicate (TEOS) were added to the milky solution and stirred for 6 h at the same temperature. The mixture was transferred into a Teflon-lined autoclave container and heated up to the different HATs in the range from 80°C to 130°C for 24 h. The white precipitate was then collected by filtration, dried in air, and finally calcined at 550°C for 6 hours.

In this work, to prepare NH₂-FMS or HOOC-FMS, AMS were silanized with trimethoxysilane with the functional group NH₂ or HOOC [2–8]. In a typical synthesis for NH₂-FMS or HOOC-FMS, 0.5 g of AMS was first suspended in toluene (25 mL) and pretreated with water (0.16 mL) in a three-necked 125 mL round-bottom flask, which was fitted with a stopper and reflux condenser. This suspension was stirred vigorously for 2 h to distribute the water throughout the mesoporous matrix, during which time it became thick and homogeneous slurry. At this point, a corresponding amount of 3-aminopropyltriethoxysilane (APTES, MW = 221.37) or 2-cyanoethyl trimethoxysilane (CTS, MW = 175.26) to silanize 20% of the total available silanol groups (assuming 5.0 silanol groups per nm² [19–21]) of AMS was added and the mixture was refluxed at 120°C for 6 h. The mixture was allowed to cool to room temperature and the product was collected by vacuum filtration. The resulted NH₂-FMSs or NC-FMSs were washed with ethyl alcohol repeatedly and dried under vacuum. To hydrolyze cyano groups (CN- would be hydrolyzed into HOOC- as the functional group), 10 mL of 50% of H₂SO₄ solution was added to the mixture and stirred in an ice-bath for 3 h. The resulted HOOC-FMSs were filtered, washed with water extensively, and dried in vacuum. In this work, AMS prepared at HATs 80°C, 100°C, 110°C, 120°C and 130°C are termed as AMS-80, AMS-100, AMS-110, AMS-120 and AMS-130 and correspondingly FMSs as FMS-80, FMS-100, FMS-110, FMS-120 and FMS-130, respectively.

Typically, an aliquot of 1.0–2.0 mg of AMS or FMS was added in a 1.5-mL tube for incubation with 200–400 µL of the protein stock solution in the working buffer. pH 7.4, 10 mM sodium phosphate buffer (NaH₂PO₄-Na₂HPO₄) for GOX, and pH 7.4, 10 mM sodium phosphate buffer containing 0.14 M NaCl (PBS) was used as the working buffer for IgG. 0.4 mg protein was used for incubation per mg of the silica material. The incubation was carried out at 21 °C shaking at 1400 min⁻¹ on an Eppendorf Thermomixer 5436 for 24 h. The protein stock in the absence of mesoporous silica was also shaken under the same conditions for comparison. The mesoporous silica-protein composite was separated by centrifugation and the first supernatant (the elution number: 0) was removed. The amounts of proteins were measured by Bradford method using bovine gamma globulin as standards for rat IgG and BSA for GOX.

To test the in vitro gradual release of the loaded protein from FMS, 200 µL of a simulated body fluid that has ion concentrations nearly equal to those of human blood plasma (buffered at pH 7.4 with 50 mM Tris-HCl) as the elution buffer (the elution number: 1) was added and shaken with the mesoporous silica-protein composite at 1400 min⁻¹ on an

Eppendorf Thermomixer 5436 for 5 minutes and then the supernatant was separated by centrifugation. For each subsequent elution with the same elution buffer, the mesoporous silica-protein composite was repeatedly separated by centrifugation and the amount of the released protein in the supernatant was measured by UV at 280 nm using the diluted protein stock solutions as standards. Protein loading densities decreased along the series of elutions.

High resolution TEM was carried out on a Jeol JEM 2010 Microscope with a specified point-to-point resolution of 0.194 nm. The operating voltage on the microscope was 200 kV. Fluorescence emission spectra were measured with a Fluoro Max-2 fluorometer (SPEX, Edison, NJ). Nitrogen adsorption-desorption isotherms for surface-area and pore analysis were measured with a Quantachrome Autosorb Automated Gas Sorption Systems.

3. Results and Discussion

Figures 1a and 1b show transmission electron microscopy (TEM) images of AMS-100 and AMS-120. Although both AMS-100 and AMS-120 display the similar pore sizes of ~30 nm, AMS-100 has an ordered mesopore structure while AMS-120 has a large degree of disordered pores. Nevertheless, AMS-120 still reveals a more or less uniform cage-like porous structure. It is reported that AMSs prepared in this work have a foam-like intramesoporous structure with big cages connected by narrow pore entrances [18]. The adsorption pore size corresponds to the wide cage diameter while the desorption pore size is related to the narrow pore entrance size [22–25]. The results from N₂ sorption measurements show that AMS-80 has a Barrett-Joyner-Halenda (BJH) adsorption pore size of ~17 nm and a BJH desorption size of ~3.9 nm, while all other AMSs have very close adsorption pore sizes of ~30 nm but the desorption size increased from ~10 to 18 nm with the increased HATs from 100°C to 120°C and then decreased at 130°C (Figure 1c). However, the Brunauer-Emmett-Teller (BET) surface areas and pore volumes of AMSs show the different trends with varied HATs (Figure 1d). The surface areas were increased from ~300 to 800 m²/g with the increased HATs from 80°C to 100°C and then decreased from 100°C to 130°C (Figure 1d), while the pore volumes were increased from ~1.25 to 2.84 cm³/g with the increased HATs from 80°C to 110°C and then decreased from 110°C to 130°C (Figure 1d). Comparing to AMSs, FMSs showed the similar TEM images and the similar changing trend in pore sizes, surface areas and pore volumes (Supplementary Materials).

To study the intramesoporous structure effects on protein loading, AMSs and FMSs were tested with the neutral protein rat IgG (M.W.: 150 kDa; Dimensions: ~10 × 7 × 2 nm) with a Y-like molecular shape, one charged protein GOX (M.W.: 160 kDa; Dimensions: ~8 × 5 × 6 nm) with an elliptical molecular shape. In a standard procedure, ~1 mg of AMS or FMS was incubated with ~0.4 mg of the protein in pH 7.4, PBS for IgG and in pH 7.4, 10 mM sodium phosphate buffer for GOX, where the two proteins would be spontaneously entrapped in the mesopores (*Materials and Methods*). We defined the protein loading density (P_{LD}) as the protein amount (μg or mg) entrapped with 1 mg of FMS.

Figure 2a displays IgG loading density in AMS, NH₂-FMS and HOOC-FMS. As it shows, P_{LD} of IgG in AMS and FMS changed with and in good agreement with the trend of the varied desorption pore size corresponding to HAT at which AMS was prepared (Figures 1c and 2a). Relatively, AMS-120 and FMS-120 had the highest P_{LD} while AMS-80 and FMS-80 had the lowest loading density. Since AMS and FMS samples prepared at HATs 100–130°C have the similar adsorption size (~30 nm), it was the desorption pore size (corresponding to the narrow pore entrance size) that played a dominant role governing P_{LD} of IgG in AMS and FMS (Figure 2a), that is, the smaller desorption pore size limited the protein to be entrapped inside the pores presumably due to the steric hindrance of IgG's Y-like shape (PDB ID: 1IGT) formed from 4 peptide chains. It is worthwhile to note that AMS, NH₂-FMS and

HOOC-FMS shows the similar trends of loading density, indicating IgG as a neutral protein has no electrostatic (charge) selectivity since IgG is a neutral protein at pH 7.4, although HOOC-FMS displayed the highest affinity for loading IgG (Figure 2a). In addition, the surface area or pore volume of AMS and FMS was not a prominent limiting role affecting the IgG loading density because AMS and FMS prepared at HATs 100 °C and 110 °C had the largest surface area and pore volume respectively (Figure 1d) but AMS-120 and FMS-120 displayed the largest loading density (Figure 2a).

Figure 2b shows GOX loading density in AMS and FMS. Because GOX has an isoelectric point of 4.2 [26], therefore, at pH 7.4, GOX is a negatively charged protein. The negatively charged protein would be largely loaded in the positively-charged mesoporous silica but be expelled from loading inside the negatively-charged mesoporous silica. As expected, P_{LD} of GOX was very low in AMS and HOOC-FMS but much higher in NH₂-FMS (Figure 2b). Comparing NH₂-FMSs, NH₂-FMS-100 had the highest P_{LD} of GOX. P_{LD} of GOX decreased from NH₂-FMS-100 to NH₂-FMS-130, indicating that P_{LD} of GOX in NH₂-FMS changed with the varied surface area corresponding to HAT at which AMS was prepared (Figures 1d and 2b). Comparing NH₂-FMSs, we also found that NH₂-FMS-80 had the lowest P_{LD} due to its small pore sizes and pore volumes (Figures 1c and 1d), although its surface area was not the smallest. Therefore, as long as the pore size and the pore volume are large enough, from NH₂-FMS-100 to NH₂-FMS-130, it was the surface area that played the prominent role governing P_{LD} of GOX in NH₂-FMS, that is, the larger positively-charged surface area allowed the larger amount of the negatively-charged protein to be accommodated. For the elliptical GOX (PDB ID: 1GPE), which has no steric hindrance when loading in NH₂-FMS, the larger surface area is needed to ensure higher protein loading density.

Our previous results have showed that in vitro released IgG from FMS still maintained its activity and displayed similar fluorescence spectra to that of the free IgG prior to the entrapment, indicating that the interaction of FMS with IgG did not induce dramatic change on the IgG protein structure [6]. To study the possible entrapping states of IgG in FMS and the interaction of IgG with FMS, we studied the fluorescence emission of IgG in FMS at different loading densities comparing to the free IgG at the same protein concentrations with the excitation wavelength of 278 nm, allowing excitation of both tyrosinyl and tryptophanyl residues. Comparing the free IgG to FMS-IgG (Figure 3a), there was no dramatic emission peak shift but increased emission intensity at ~340 nm because the interaction of IgG with FMS resulted in less exposure of tyrosinyl and tryptophanyl residues of IgG to the aqueous environment along IgG attachment to the mesopore surface. Interestingly, when P_{LD} of IgG in FMS was increased to 0.17 mg/mg of FMS (Figure 3a), the fluorescence intensity of IgG in FMS was already decreased and closer to the emission spectra of the free IgG comparing to that of the lower P_{LD} of IgG in FMS (Figure 3a). This result demonstrated that, after all available silica surface was fully occupied with a monolayer of IgG molecules due to the interaction of IgG with FMS, more neutral IgG molecules may keep aggregating in FMS with no direct attachment to FMS (Figure 3b) as long as the pore volume is large enough because the states of the non-attached IgG molecules in FMS was closer to that of the free IgG in the aqueous environment. How IgG molecules were exactly attached and packed inside still need to be elucidated.

Fluorescence emission of GOX in FMS at different loading densities were compared to the free GOX at the excitation wavelength of 278 nm. Similarly to FMS-IgG (Figure 3a), we also found that there was no dramatic emission peak shift but increased emission intensity at ~340 nm because of the interaction of GOX with FMS (Figure 3c). However, even when P_{LD} of GOX in FMS was increased close to the largest P_{LD} (~0.09 mg/mg of FMS) (Figure 2b), its fluorescence intensity was still similar to that of GOX in FMS with the lower P_{LD} (~0.05 mg/mg of FMS), which was still far from the emission spectra of the free GOX (Figure 3c).

Therefore, after all available silica surface was fully occupied with a monolayer of GOX molecules (Figure 3d), we believe that the charged GOX molecules would not be able to accumulate in FMS due to the electrostatic expulsion. GOX consists of two identical monomers [27]. Each monomer of the dimeric GOX molecule is asymmetrically interfaced each other but each monomer having one same surface region exposed with much more concentrated negative charges than other regions [28]. For the whole GOX molecule, we believe that this negatively region (magenta color-circled area in the protein structure of GOX in Figure 3d) of one monomer would be attached to the mesoporous wall while the same region of the other monomer was pointed toward the inside axis of the mesopores. This way would provide the electrostatically expulsive microenvironment that can prevent GOX from further aggregation inside the mesopores after the monolayer occupancy. As expected, the spontaneously entrapped IgG and GOX were gradually released from FMS along the series of elutions with simulated body fluid because of their non-covalent interactions (Figure 4), demonstrating the potential applications of FMS for protein drug delivery under physiological conditions.

In summary, the protein loadings in AMS and FMS are correlated with the varied intramesoporous structure of mesoporous silicas with critical HATs as well as the biochemical structural characteristics of the protein (charge and shape). For the neutral Y-like IgG, the larger desorption pore size allowed the larger protein loading, while for the charged elliptical GOX, the larger surface area resulted in the larger protein loading when the pore size and the pore volume are large enough. Our results also showed that the neutral protein IgG could continue to aggregate in the mesopores after the monolayer occupancy, while the charged protein GOX was only attached inside the mesopores in a way of monolayers due to the electrostatic expulsion and the limit of pore volumes. A clear understanding how the intramesoporous structure affects the loading and release of proteins and other molecules would help the development of new mesostructured materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research Highlights

- For a neutral protein, the larger mesopore size allows the larger protein loading;
- For a charged protein, the larger surface area allows the larger protein loading;
- The charged protein may reside inside the mesopores in a way of monolayer;
- The neutral protein could continue to aggregate after the monolayer occupancy.

Figure 1. TEM images of AMS-100 (a) and AMS-120 (b); BJH adsorption and desorption sizes (c), BET surface areas and pore volumes (d) of AMS obtained at HATs from 80 to 130°C.

Figure 2.

Protein loading densities of rat IgG (a) and GOX (b) in AMS, NH₂-FMS, and HOOC-FMS at different HATs at which AMSs were prepared. 0.4 mg protein was used for incubation per mg of the silica material.

Figure 3.

(a) Fluorescence emission (excitation = 278 nm) of IgG in HOOC-FMS-120 at different P_{LD} (mg/mg of FMS); (b) Protein structure of the neutral IgG (PDB ID: 1IGT) with surface charge distribution (cationic-blue, anionic-red, uncharged-white) and a presumptive drawing for IgG loaded in AMS or FMS. The blue and red left-right arrows inside AMS represent the adsorption and desorption pore sizes respectively; (c) Fluorescence emission (excitation = 278 nm) of GOX in NH_2 -FMS-120 at different P_{LD} (mg/mg of FMS); (d) Protein structure of the negatively charged GOX (PDB ID: 1GPE) with surface charge distribution (cationic-blue, anionic-red, uncharged-white) and a presumptive drawing for GOX loaded in NH_2 -FMS, where the local region of the GOX structure most probably attached to the NH_2 -FMS is magenta color-circled [28]. pH 7.4, PBS was used as the working buffer for IgG, pH 7.4, 10 mM sodium phosphate buffer for GOX. [Protein] = 10 μ g/mL.

Figure 4. Gradually decreasing of P_{LD} of rat IgG (a) in HOOC-FMS and GOX (b) in NH_2 -FMS along continual elutions with simulated body fluid (*Materials and Methods*), where FMSs were derivatized from AMS prepared at different HATs.