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Improved controlled release of protein from expanded-pore mesoporous silica nanoparticles modified with co-functionalized poly(*n*-isopropylacrylamide) and poly(ethylene glycol) (PNIPAM-PEG)



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ABSTRACT

Novel pore-expanded mesoporous silica nanoparticles (MSNs) with pore sizes of approximately 11 nm were synthesized and modified with thermoresponsive, poly(*n*-isopropylacrylamide) (PNIPAM) gating groups on the nanoparticle exterior surface and in addition with poly(ethylene-glycol) (PEG) within the porous interior to minimize protein adsorption. PEG traditionally has been grafted to the nanoparticle exterior to minimize non-specific binding and interactions with the biological environment, but due to the templating mechanism of MSN synthesis, both the pore interior and nanoparticle surface can be separately modified. Here, an improved control release behavior of bovine hemoglobin (Bhb) was observed after PEGylating the interior porous framework, compared to the release Bhb from unmodified MSNs. This can be attributed to the reduced protein denaturation on PEGylated silica that was observed using circular dichroism spectroscopy.

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1. Introduction

MSNs as a drug delivery agent has been explored in the past two decades but it has only been recently further modified as a potential agent for the delivery of proteins [1–4] and genetic material [5–7]. The pore size of typical MCM41, or SBA type MSNs, is the limiting factor in the effective entrapment and delivery of larger peptide and protein species. To work around this, hollow MSNs have been used to deliver larger compounds [8], as with other pore-expanded MSNs but with sizes upward of 200–300 nm [6]. In an earlier work [9] pore-expanded, core-shell MSNs were synthesized that combined both a superparamagnetic core with a mesoporous silica shell. It was observed however that the presence of the iron oxide core reduced the effectiveness of the pore-expansion process with TMB. In other instances however [6], depending on when and how the MSN sol-gel is modified with trimethylbenzene (TMB), pore sizes upwards of 20 nm can be achieved, although with compromises in the porous structure of the MSN. Nonetheless, the potential in pro-

ducing large pore MSNs capable of delivering large proteins on the order of 5–10 nm is desirable for many therapeutic drug delivery applications.

One consideration involving peptide and protein delivery is the structural stability of the species, which determines the activity and effectiveness of the peptide or protein after its release into the local environment. Protein adsorption onto surfaces has been explored in planar [10] and spherical studies [11], but its adsorption into porous silica environments has not been as thoroughly discussed. In particular, it was observed that proteins do in fact deform and change their secondary structures when adsorbed onto glass surfaces [12]. Several methods to reduce the negative effects of this include modifying the surface with polymers such as PEG, which has long been noted to reduce non-specific binding of proteins onto surfaces [13,14]. However, designing a robust MSN platform for protein delivery that also exclusively modifies the internal porous structure with PEG is ideal for reasons stated above.

In the scope of designing nanoparticles for protein delivery, a gating mechanism is necessary to eliminate any premature release of the species. PNIPAM has been previously demonstrated [15] to be an effective gating mechanism and chosen here. The lower critical solution temperature (LCST) of PNIPAM is typically 31–32 °C, which is situated between room and physiological temperature, making it ideal for a thermo-responsive gating mechanism for drug delivery

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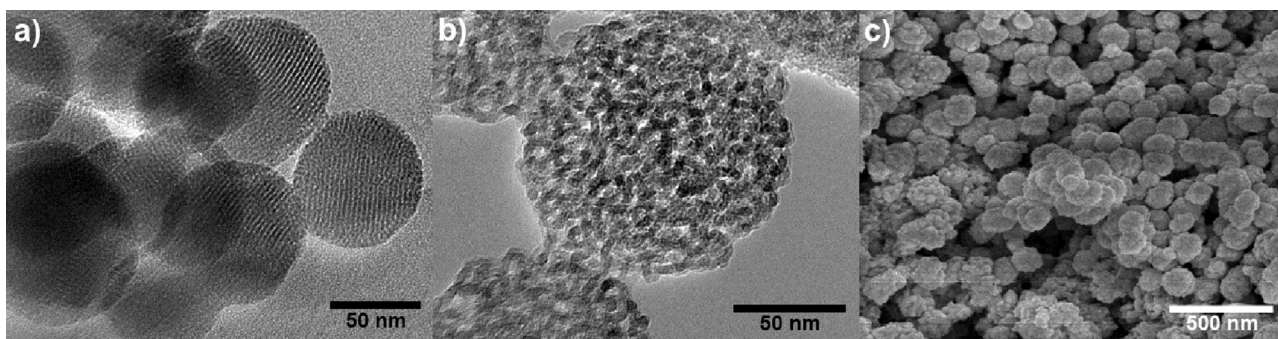


Fig. 1. (a) TEM of the MSNs, (b) expanded-pore, PNIPAM-PEG-MSNs, and (c) SEM images of the PNIPAM-PEG-MSNs.

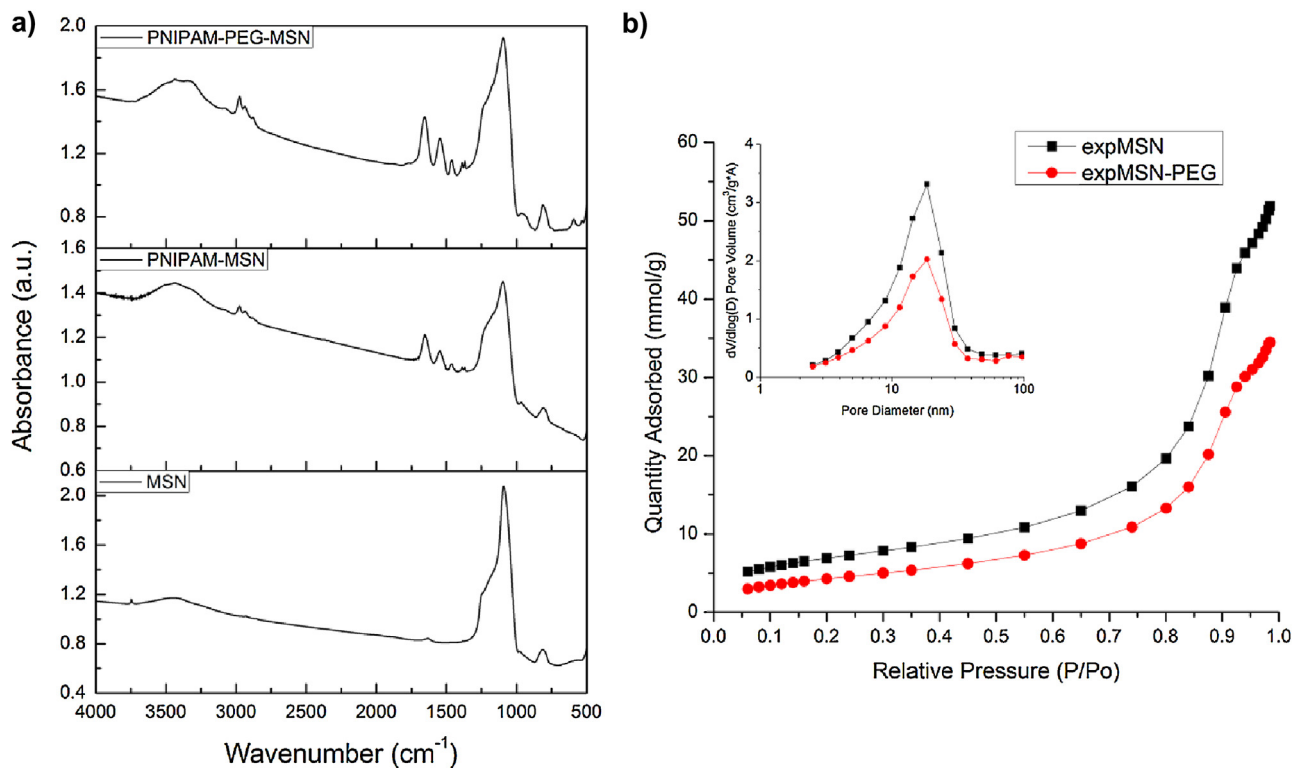


Fig. 2. (a) IR spectra of the MSN, PNIPAM-MSN, and PNIPAM-PEG-MSNs. (b) Nitrogen gas adsorption curves and pore size distribution of the expanded pore MSNs (expMSN) and PEGylated, expanded pore MSNs (expMSN-PEG). The adsorption isotherm and pore size distribution data are from single measurements.

media. Due to a straightforward and well-controlled functionalized methodology, PNIPAM is robust and can be combined with many other functionalization methods [16]. As such, a co-functionalized PEG/PNIPAM MSN is illustrated here for the delivery of protein, with bovine hemoglobin (BHb) as the model protein.

2. Materials and methods

Expanded-pore MSNs were produced by the sol-gel process, followed by a hydrothermal expansion process using TMB, similar to a previous work [17]. Briefly, first 1.75 mL of 2 M NaOH was added to 240 mL of DI water and increased to 80 °C. Afterwards, 500 mg of CTAB was slowly dissolved in the solution. 2.5 mL of TEOS was then added dropwise and the reaction ran for 2 h. The reaction was then cooled and aged overnight. The centrifuged MSNs were dispersed into 10 mL each of ethanol, water, and TMB, and placed into a Teflon autoclave. The hydrothermal pore expansion was carried out at 140 °C for 3 days and afterwards, the MSNs were collected rinsed in ethanol. To functionalize the exterior surface

of the MSNs, a 3-aminopropyltriethoxysilane/ α -bromoisobutyryl bromide (BIBAPTES) precursor was synthesized as done by Vasani et al. [18]. Atom transfer radical polymerization (ATRP) of PNIPAM was done using a previous methodology [9] with half the *n*-isopropylacrylamide (NIPAM) content. Prior to PEGylating the pore interior, the remaining surfactant template was removed through refluxing in 1 M ethanolic-HCl for 24 h. Afterwards, approximately 25 mg of PNIPAM-MSNs were added to 10 mL of EtOH and 146 μ L of PEG-silane (Gelest SIM6492.7). The reaction was run for 7 h at 37 °C. For protein release studies, BHb loading was done with a 1:1:0.1 ratio of BHb (mg) to PNIPAM-PEG-MSNs (mg) to PBS 0.25 \times (mL) for 24 h.

3. Results and discussion

The pore-expansion process of MCM41-type MSNs has yielded a variety of porous structures in literature, depending on the pore-expansion agent and synthesis conditions. In the work done here, Fig. 1a and b show the microstructure of MCM41 MSNs before

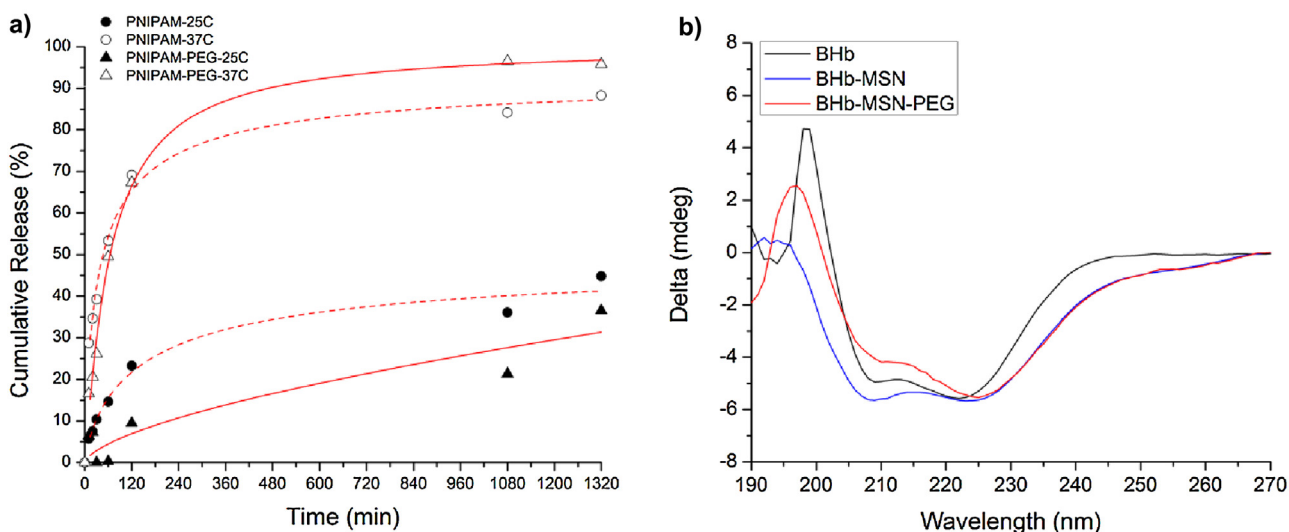


Fig. 3. (a) Release of BHB from PNIPAM-MSNs and PNIPAM-PEG-MSNs at 25 and 37 °C. (b) CD spectroscopy of free BHB, BHB in PNIPAM-MSN, and BHB in the PNIPAM-PEG-MSN. A reversible Langmuir adsorption isothermal model (OriginPro 8.5 LangmuirEXT1) was used to fit the release curves of the BHB in the silica environments [19]. The BHB release data are the average from three data points ($n=3$, maximum SD $\pm 19.2\%$, minimum $\pm 5.9\%$) while the CD spectroscopy curves are from single measurements.

and after the pore expansion process. The inclusion of TMB during the hydrothermal treatment drastically alters the porous structure, often resulting in either radial pores or random, worm-like structures as seen here [17]. For loaded proteins, such as BHB which has a diameter of 5 nm, the average pore size of 15 nm is sufficient to entrap BHB.

Due to the presence of the surfactant template after MSN synthesis, the internal porous environment of the nanoparticles is blocked. This allows for interesting subsequent or co-functionalized nanoparticles. Peptides, proteins, and genetic material are sensitive to conformational changes due to their secondary and higher hierarchical structure. In order to preserve the structure of these species, PEG can be used to functionalize the interior surface of these MSNs. Fig. 2 confirms the strong presence of PNIPAM (carboxyl at 1750 cm^{-1}), but because the Si-O peaks completely dominate any indicator of C-O bonds present in PEG, it is not possible to confirm this alone with IR data. Instead, from BET/BJH porosity measurements, Fig. 2 indicates that through PEGylating the internal porous structure, a reduction in the pore volume is observed. The pore size is not affected substantially, as the average pore diameter decreases from 12.0 to 11.3 nm. Further PEGylating the porous structure results in a reduced pore volume and surface area as expected. Fig. 2 illustrates the relatively small 0.7 nm reduction in pore diameter, but noticeably reduced the pore volume from $1.82\text{ cm}^3/\text{g}$ to $1.21\text{ cm}^3/\text{g}$ and the surface area from $564.4\text{ m}^2/\text{g}$ to $361.5\text{ m}^2/\text{g}$. This change in the pore volume is noticeable in the maximum amount of BHB released between the PEGylated and non-PEGylated case.

Despite the lower amount of BHB released, PEGylation of the MSN reduces BHB adsorption to the silica surface and as a result, improves the protein release behavior, especially at shorter times. Fig. 3 reveals that the inclusion of PEG surface modifiers within the MSN allow for a greater relative BHB release at 37 °C, but a decreased effect at 25 °C. It is likely steric hindrance is minimizing release at 25 °C, but at the greater temperatures where the PNIPAM gating mechanism is on, it suggests that PEG is decreasing the non-specific BHB adsorption onto the silica and as a result, improving the release behavior. It is worth noting however, as demonstrated by the BET/BJH data, that the maximum loading capacity of the PNIPAM-PEG MSNs is lower than the PNIPAM-MSN alone.

For all cases, the BHB follows a well behaved release characteristic but in the case of PNIPAM-PEG-MSN at 25 °C, the curve seems

to indicate a greater maximal value at longer timeframes. This is indicative that the PNIPAM gating may not be completely effective. However, in the timeframe of 24 h, a five-fold release of the PNIPAM-PEG at 37 °C reaches an asymptotic value.

The BHB structure was investigated with circular dichroism (CD) spectroscopy to determine if the adsorption conformation of the protein was indicative of the difference in the release behavior. It was expected that the improved release (percent released BHB of the total loaded amount) would be due to the reduced non-specific adsorption of the BHB onto the silica surfaces. Fig. 3b shows the CD spectroscopy data confirming that BHB contained within silica environments does alter the stable conformation. Free BHB in PBS0.25X shows a clear structure confirmed in literature [20], with both a strong presence of alpha-helix and beta-sheet secondary structures. In the other two instances, BHB was loaded into both expanded-pore MSNs with PNIPAM, and PNIPAM-PEG. The CD spectroscopy was run at 25 °C, indicating that the BHB should have minimal release, but as the UV-vis spectroscopy data indicates, some free BHB in both cases is likely present in the solution. However, BHB loaded into PEGylated MSNs improves the conformation, although there is still deviation from the free BHB structure. The alpha-helix near 200 nm is present in PEGylated MSN, whereas in solely MSN, this peak is nearly absent. Minor peak shifts in the beta-sheet structure are also observed. Despite the improved conformation, particularly at low wavelength alpha-helices, there is still substantial deviation which may be due to BHB entrapped in the PNIPAM surface layer. Further viability assays would help support and confirm noticeable differences due to the PEGylation.

4. Conclusion

A PEG and PNIPAM co-functionalized, expanded-pore MSN was synthesized and loaded with BHB in order to demonstrate a potential protein delivery medium. The enlarged pore structure of sub-100 nm MSNs allows for the storage of proteins while the inclusion of PNIPAM provides a thermo-responsive gating mechanism that triggers protein release at physiological temperatures. Through PEGylation of the interior, porous surface, improved BHB release is observed due to reduced protein adsorption and deformation.

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