pH responsive *in vitro* cargo release of PEG coated MSNs is governed by buffer composition

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Abstract

The effect of different buffers on *in vitro* release of PEG coated mesoporous silica nanoparticles is discussed in this work. We prepared Rhodamine loaded and PEG coated mesoporous silica nanoparticles (PEG coated RhB@MSNs) of ~ 650 nm size and studied their release behaviour for 5 hours in various buffer solutions (both Na+ and K+ versions) prepared by standard procedure. We observed that Rhodamine release was highest in the citrate buffer. A comparison of different buffer effects on the *in vitro* release reveals that it is crucial to choose the right buffer for determining the loaded cargo release in each pH zone (acidic, neutral and basic). Clearly, choosing the wrong combination of buffers for different pH zones would result in completely different interpretation of the release behaviour.

Introduction

To overcome the limitations of low permeability and poor water solubility, nanoparticle-based systems have emerged as a preferred alternatives to traditional tools in drug delivery,[1,2] imaging techniques,[3–5] toxicology research,[6,7] etc. Characteristics such as small size, high loading capacity, low density and on-demand drug release at a specific target site make nanoparticle based systems highly desirable.[2,8–12] Among the various organic, inorganic and hybrid (organic-inorganic) nanoparticle-based systems discovered, inorganic silica and silica-based nanoparticles are the most popular due to their biocompatibility and biostability.[2,6,7,12,13] In general, silica nanoparticles can be easily synthesized, have high porosity, high surface area, small size, tunable pore size and above all, the ability to be functionalized ie., small ligands or charged molecules can be easily grafted on the silica surface to achieve desired functionality.[14–16] Properties such as high cargo loading capacity,[11,17] stimulus (pH, temperature, solvent) responsiveness,[18,19] change in cargo release kinetics,[20] increased stability[2,21] etc., could be achieved by functionalizing silica nanoparticles. Among the various silica nanoparticles synthesized to date, MCM-41[22,23] and

SBA-15[24–26] are the two most popular nanoparticles that have been used either in native form or functionalized to achieve required function.

Once synthesized, the cargo loaded nanoparticles have to be evaluated for *in vitro* release before proceeding to the more complex *in vivo* release behaviour. For pH sensitive systems, in order to determine whether the cargo-loaded nanoparticles are pH sensitive, their release behaviour is tested in typically three different buffer solutions; each representing either the acidic, neutral or basic zone. Most often, standard buffers are chosen;[11,27–30] for example, acetate for the acidic zone, PBS for the neutral zone and tris for the basic zone. However, not much attention is paid to the choice of Na/K salts for the buffer preparation or monitoring release behaviour in different buffers for the same pH zone. The effects of different ions in solution on silica nanoparticles is well documented;[26,31–34] also, it is already known that MSNs degrade in PBS.[35,36] Despite these facts, not much attention is paid to the choice of buffer solutions for *in vitro* release experiments. In this work we report the effects of 23 different buffers on PEG coated RhB@MSNs and perform a comparative study of the release behaviour of the nanoparticles.

Materials and Methods

Chemicals

Synthesis: Tetraethyl ortho-silicate (TEOS) [cat. No, 8.00658], Hydrochloric acid [cat. No.1.93401.0521], Hexadecyl trimethyl ammonium bromide (CTAB) [cat. No. 8.14119] , PEG-400 [cat. No. 8.07485] were purchased from Merck, India, Ammonia solution [cat. No. 78719] was bought from SRL chemicals, India, Rhodamine B [cat. No. GRM980] was purchased from Hi-media, India and Ethanol [cat.No. 32221] was purchased from Honeywell, India.

Buffer preparation: Sodium phosphate di-basic anhydrous (Na_2HPO_4-$7H_2O$) [cat. No.1949146], sodium dihydrogen orthophosphate anhydrous ($NaH_2PO_4H_2O$) [cat. No. 61707], potassium phosphate di-basic anhydrous (K_2HPO_4-$7H_2O$) [cat. No.1649318], potassium dihydrogen orthophosphate anhydrous ($KH_2PO_4H_2O$) [cat. No. 1649201], sodium citrate dihydrate [cat. No 94968], potassium citrate [cat. No. 74176], NaCl [cat. No. 1940103], KCl [cat. No. 1644133] was purchased from SRL chemicals, India, hydrochloric acid [cat. No.1.93401.0521] was purchased from Merck, India],Tris-base [cat. No.103133] was bought from MP Biomedicals, India, sodium acetate, Potassium acetate [cat. No. GRM1091] was bought from Hi-media, India, glacial acetic acid [product code 00004], NaOH

pellets [product code 05898], KOH pellets [product code 05378] from Loba Chemie Pvt. Ltd.,India] and Citric acid [cat. No. C0759] was purchased From Sigma, India.

Synthesis of mesoporous silica nanoparticles (MSNs): Synthesis of MSNs was performed by using the standard sol-gel method. 1.6 g of CTAB was dissolved in 35 % w/v ethanol-water mixture containing ammonia (25 wt %,10 ml) and 10 ml of TEOS was added rapidly under vigorous stirring. The mixture was stirred at 35 ℃ for 24 hrs. After 24 hrs, the white product was collected by vacuum filtration and was washed thrice with 35 % ethanol-water mixture. The product was dried under vacuum at 60 ℃, overnight. The obtained white product was then dispersed in acidified ethanol (200 ml of absolute ethanol containing 400 μl of concentrated HCL) and was stirred for 2 hrs at 30 ℃. This process was repeated thrice for removal of the template. The final white product was collected by vacuum filtration and washed thrice with water and dried under vacuum at 60 ℃, overnight.

RhB loading (RhB@MSNs): The Rhodamine solution was prepared by dissolving 5 mg of RhB in 100 ml of water. 1 g of as-synthesized MSNs were dispersed in this lRhB solution by sonication for 5-10 mins and then the mixture was rotated for 24 hrs. After 24 hrs, the pink product was collected and washed thrice with Mili-Q® by centrifugation at 4000 rpm at 25 ℃ for 10 minutes. The final product was dried under vacuum at 40 ℃, overnight. This uncoated solid sample was used as control for all *in vitro* experiments.

PEG coating (PEG coated RhB@MSNs): PEG coating was performed by dispersing 1 g of RhB@MSNs in 100 ml of water by sonication for 5-10 mins. Then 10 % of PEG-400 was added to this solution and the whole mixture was kept on a rotating mixture for six hrs. After six hours the mixture was washed five times with water and centrifuged at 4000 rpm at 25 ℃ for 10 mins. The final product was collected and dried under vacuum, overnight.

Preparation of different buffers:

Citrate buffer

Using sodium salts

pH 3.0: 0.1 M stock solution was prepared by dissolving 2.714 g of sodium citrate dihydrate (0.0092 M) and 17.437 g of citric acid (0.0908 M) 800 ml Mili-Q® water. The pH was checked and found to be 3.0. The volume was then made upto 1 L using Mili-Q® water. The stock solution was diluted to 0.01 M for *in vitro* experiments.

pH 4.5: 0.1 M stock solution was prepared by dissolving 16.968 g of sodium citrate dihydrate (0.0577 M) and 8.127 g of citric acid (0.0423 M) 800 ml Mili-Q® water. The pH was checked and found to be 4.5. The volume was then made upto 1 L using Mili-Q® water. The stock solution was diluted to 0.01 M for *in vitro* experiments.

Using potassium salts:

pH 3.4: 0.1 M stock solution was prepared by dissolving 2.984 g of potassium citrate (0.0092 M) and 17.442 g of citric acid (0.0908 M) in 800 ml Mili-Q® water. The pH was checked and found to be 3.4. The volume was then made upto 1 L using Mili-Q® water. The stock solution was diluted to 0.01 M for *in vitro* experiments.

pH 5.4: 0.1 M stock solution was prepared by dissolving 18.718 g of potassium citrate (0.0577 M) and 8.125 g of citric acid (0.0423 M) in 800 ml Mili-Q® water. The pH was checked and found to be 5.4. The volume was then made upto 1 L using Mili-Q® water. The stock solution was diluted to 0.01 M for *in vitro* experiments.

Acetate buffer:

Using sodium salts

pH 3.4: 0.1 M stock solution was prepared by dissolving 0.396 g of sodium acetate (0.0048 M) and 5.715 g of glacial acetic acid (0.0952 M) in 800 ml Mili-Q® water. The pH was checked and found to be 3.4. The volume was then made upto 1 L using Mili-Q® water. The stock solution was diluted to 0.01 M for *in vitro* experiments.

pH 4.7: 0.1 M stock solution was prepared by dissolving 5.524 g of sodium acetate (0.0673 M) and 1.961 g of glacial acetic acid (0.0327 M) in 800 ml Mili-Q® water. The pH was checked and found to be 4.7. The volume was then made upto 1 L using Mili-Q® water. The stock solution was diluted to 0.01 M for *in vitro* experiments.

Using potassium salts

pH 3.4: 0.1 M stock solution was prepared by dissolving 0.471 g of potassium acetate (0.0048 M) and 5.716 g of glacial acetic acid (0.0952 M) in 800 ml Mili-Q® water. The pH was checked and found to be 3.4. The volume was then made upto 1 L using Mili-Q® water. The stock solution was diluted to 0.01 M for *in vitro* experiments.

pH 5.0: 0.1 M stock solution was prepared by dissolving 6.605 g of potassium acetate (0.0673 M) and 1.963 g of glacial acetic acid (0.0327 M) in 800 ml Mili-Q® water. The pH was checked and found to be 5.0. The volume was then made upto 1 L using Mili-Q® water. The stock solution was diluted to 0.01 M for *in vitro* experiments.

Using no salts

1 mM HCL solution: Firstly a 1 M solution of HCL was prepared by using 8.25 ml of concentration HCL in 100 ml Mili-Q®. Then, the solution was diluted 100 times to obtain a 1 mM HCL for experiments. *The pH of 1 mM HCl was 3.6 and was used as it is for in vitro experiments at pH 3.6.*

pH adjustments using NaOH: A stock solution of 1 M of NaOH was prepared and diluted 100 times to obtain a 1 mM solution. This NaOH was then slowly added to 1 mM HCL solution with constant stirring and the solutions of different pH **(5.0, 7.4 & 9.0)** were prepared. *Note: The pH of 1 mM HCl was 3.6 and was used as it is for in vitro experiments at pH 3.6.*

pH adjustments using KOH: A stock solution of 1 M of KOH was prepared and diluted 100 times to obtain a 1 mM solution. This KOH was then slowly added to 1 mM HCL solution with constant stirring and the solutions of different pH **(5.0, 7.4 & 9.0)** were prepared. *Note: The pH of 1 mM was 3.6 and was used as it is for in vitro experiments at pH 3.6.*

Phosphate buffer (pH 7.4 and 9.0):

Using sodium salts:

pH 7.6: 1 L of 0.1 M stock solution was prepared by dissolving 20.214 g of sodium phosphate dibasic anhydrous (Na_2HPO_4-$7H_2O$ - 0.0754 M) and 3.394 g of sodium dihydrogen orthophosphate anhydrous ($NaH_2PO_4H_2O$ - 0.0246 M) in 800 ml Mili-Q® water. The solution was made upto 1000 ml after checking the pH. The stock solution was diluted to 0.01 M for *in vitro* experiments.

pH 9.0: Exact procedure as above was followed to prepare a stock solution and before making up the volume to 1000 ml, 1 M NaOH was added with continuous stirring until the pH reached 9.0. The final stock solution was diluted to 0.01 M for *in vitro* experiments.

Using potassium salts:

pH 7.6: 1 L of 0.1 M stock solution was prepared by dissolving 13.133 g of potassium phosphate dibasic anhydrous(K_2HPO_4.$7H_2O$ - 0.0754 M) and 3.347 g of potassium dihydrogen orthophosphate anhydrous ($KH_2PO_4H_2O$ - 0.0246 M) in 800 ml Mili-Q® water. The solution was made upto 1000 ml after checking the pH. The stock solution was diluted to 0.01 M for *in vitro* experiments.

pH 9.0: Exact procedure as above was followed to prepare a stock solution and before making up the volume to 1000 ml, 1 M KOH was added with continuous stirring until the pH reached 9.0. The final stock solution was diluted to 0.01 M for *in vitro* experiments.

Tris (pH 9.0 & 11.0):

pH 9.0: 0.1 M stock solution was prepared by dissolving 12.11 g of tris base in 800 ml of Mili-Q®. The pH was checked and then adjusted to 9.0 by adding concentrated HCL with continuous stirring. Once the desired pH was achieved, the volume was made upto 1 L using Mili-Q®. The stock solution was diluted to 0.01 M for *in vitro* experiments.

pH 11.0: 0.1 M stock solution was prepared by dissolving 12.11 g of tris base in 800 ml of Mili-Q®. The pH was checked and was found to be 11.0. The solution was then made upto 1000 ml with Mili-Q® water. The stock solution was diluted to 0.01 M for *in vitro* experiments.

Tris buffered saline (TBS - pH 9.0 and 11.0):

Using sodium salts:

pH 11.0: 0.1 M stock solution was prepared by dissolving 8.2 g of NaCl and 12.1 g of tris base in 800 ml Mili-Q® water. The pH was checked and the volume was made upto 1 L using Mili-Q®. The stock solution was diluted to 0.01 M for *in vitro* experiments.

pH 9.0: 0.1 M stock solution was prepared by dissolving 8.2 g of NaCl and 12.1 g of tris base in 800 ml Mili-Q® water. The pH was checked and then adjusted to 9.0 using concentrated HCL with continuous stirring. Once the desired pH was achieved, the volume was made upto 1 L using Mili-Q®. The stock solution was diluted to 0.01 M for *in vitro* experiments.

Using potassium salt:

pH 11.0: 0.1 M stock solution was prepared by dissolving 8.2 g of KCl and 12.1 g of tris base in 800 ml Mili-Q® water. The pH was checked and the volume was made upto 1 L using Mili-Q®. The stock solution was diluted to 0.01 M for *in vitro* experiments.

pH 9.0: 0.1 M stock solution was prepared by dissolving 8.2 g of KCl and 12.1 g of tris base in 800 ml Mili-Q® water. The pH was checked and then adjusted to 9.0 using concentrated HCL with continuous stirring. Once the desired pH was achieved, the volume was made upto 1 L using Mili-Q®. The stock solution was diluted to 0.01 M for *in vitro* experiments.

Experimental method

***In vitro* release experiments:** The *in vitro* cargo release experiments were performed in the prepared buffers at different pH. 5 mg of PEG coated RhB@MSNs were weighed separately in different vials and 2 ml of buffer solution was added to these vials and rotated for different time periods (0 to 5 hrs). After 5 hrs, all the vials were centrifuged at 4000 rpm at 25 ℃ for 10 mins and the absorbance of the supernatants was measured at 553 nm. Blank measurements were also performed for each set of samples and the absorbance was deducted from the measured value.
Control experiments were performed using uncoated RhB@MSNs.

Instruments used

The spectral measurements for *in vitro* release studies were performed on SpectraMax 250 Microplate reader. pH measurements of different buffers were done using Hanna (HI-5521-02) pH meter.

Results and Discussion

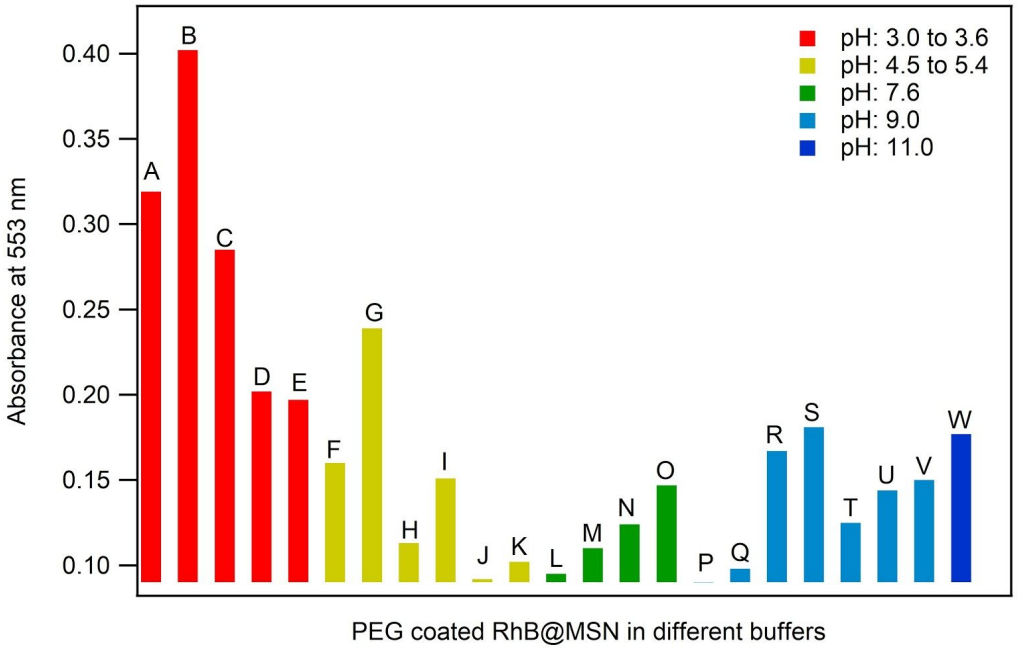
While attempting to understand the pH responsive behaviour of PEG and PDA coated RhB@MSNs in our previous work,[37] we realized that changing the buffer or the salt used in preparation of the buffer (Na + or K+ salts) results in either increase or decrease of the amount of cargo released. So, in order to clearly understand the release mechanism, we performed a series of measurements for PEG coated RhB@MSN with uncoated RhB@MSN as a control in different pH solutions/buffers. Considering the large number of buffers and sample handling, we limited our study to the PEG coated sample and not the PDA coated RhB@MSN as well.
Figure 1A and B show the *in vitro* release of PEG coated RhB@MSN and uncoated RhB@MSN in different buffers after one hour. Table 1 provides a quick reference to the different buffers used. Our *in vitro* release experiments brought about an interesting observation. We found that the results obtained could be interpreted differently depending upon what pH solution or buffer used in the experiments for acid/neutral/base zones were compared. For example, the PEG coated samples showed acid sensitivity in the presence of K+ or Na+ while using the pH solutions prepared by adjusting the pH of 1 mM HCl solution (pH 3.0) with KOH or NaOH to higher pHs. Now, instead of using this recipe, if phosphate buffer solutions (PBS - pH 7.4 and 9.0) were used to perform release experiments in the basic pH zone, high release of RhB was observed at pH 9.0. This means that, if one uses a simple HCl solution (pH adjusted by addition of a strong base KOH/NaOH) for the acidic

zone and PBS for the basic zone, then, it would seem like the nanoparticle is base sensitive; and if only the strong base is used to adjust pH for all zones, then the results would indicate that the nanoparticle is acid sensitive. Similarly, for release measurements performed in an acetate buffer, if one chooses to use acetate buffer (Buffer H) for pH 5, phosphate buffer solution (Buffer N/O) for pH 7.4 (neutral pH) and Tris (Buffer W) for pH 9.0 (basic pH), then the results would indicate that the sample is sensitive at neutral pH. One other example is; if one chooses to perform *in vitro* release experiments at pH 5.0 (Buffer H - for acid pH), pH 7.4 (Buffer N - for neutral pH) and pH 9.0 (Buffer R - for basic pH), then the results would clearly indicate that the sample is base sensitive. As can be clearly seen, different comparisons of the results obtained could be interpreted differently depending on the choice of buffer one uses to perform the release experiments. The control measurements using uncoated RhB@MSNs always showed higher release compared to that of the PEG coated sample as expected. Individual *in vitro* release results of PEG coated RhB@MSN in comparison to the control uncoated RhB@MSN sample in different buffers can be found in the supplementary information.

Table 1: List of different buffers prepared for the in vitro RhB release experiments.

Sl. No	**pH**	**Buffer**	**Notes**
A	3.0	Citrate buffer	Only sodium salts used
B	3.4	Citrate buffer	Only potassium salts used
C	3.6	HCl	1 mM HCl; no pH adjustment
D	3.4	Acetate buffer	Only sodium salts used
E	3.4	Acetate buffer	Only potassium salts used
F	5.0	Citrate buffer	Only sodium salts used
G	5.0	Citrate buffer	Only potassium salts used
H	5.0	Acetate buffer	Only sodium salts used
I	5.0	Acetate buffer	Only potassium salts used
J	5.0	1 mM HCl	pH adjusted by adding 1 mM NaOH
K	5.0	1 mM HCl	pH adjusted by adding 1 mM KOH
L	7.4	1 mM HCl	pH adjusted by adding 1 mM NaOH
M	7.4	1 mM HCl	pH adjusted by adding 1 mM KOH
N	7.6	Phosphate buffer solution	Only sodium salts used
O	7.6	Phosphate buffer solution	Only potassium salts used
P	9.0	1 mM HCl	pH adjusted by adding 1 mM NaOH
Q	9.0	1 mM HCl	pH adjusted by adding 1 mM KOH
R	9.0	Phosphate buffer solution	pH adjusted by adding 1 M NaOH

S	9.0	Phosphate buffer solution	pH adjusted by adding 1 M KOH
T	9.0	Tris	pH adjusted by adding conc. HCl
U	9.0	Tris buffered saline	NaCl used
V	9.0	Tris buffered saline	KCl used
W	11.0	Tris	No pH adjustment



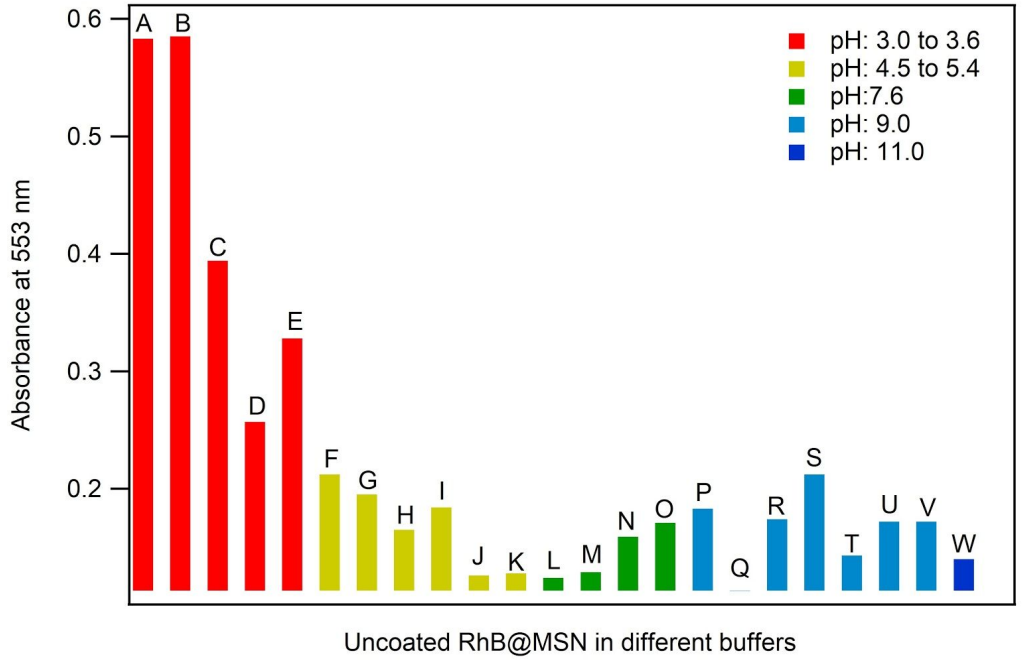


Figure 1: *In vitro* RhB release of A) PEG coated RhB@MSN and B) control sample - uncoated RhB@MSN in different buffers after 1 hour.

Conclusions

We performed a series of *in vitro* cargo release experiments for PEG coated RhB@MSN and uncoated RhB@MSN samples in different buffers for acid, neutral and basic pH zones. Our results clearly show that cargo release from the polymer coated samples can be different in different buffers in either acid, neutral or base pH and it would be wise to perform the *in vitro* cargo release experiments for pH sensitive samples in at least two or three different buffers per pH zone before concluding on the pH sensitivity of the sample.

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Conflicts of interest

There are no conflicts to declare

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