Electronic Supplementary Information

A protein corona complex for the controlled pharmacokinetic release of probucol from mesoporous silica particles

Kalpeshkumar Giri, Michael Lau, Inga Kuschnerus, Irene Moroni and Alfonso E. Garcia-Bennett

Experimental Details **Page 1**

Material Characterisation **Page 2**

Figure S1: Structural properties of mesoporous particles of AMS-6. **Page 5**

Figure S2: Drug loading and formation of a hard lysozyme corona. **Page 6**

Figure S3: Surface enhanced Raman spectra and FT-Infrared spectra **Page 7**

Figure S4: δ-potential and particle size distribution (dynamic light scattering) **Page 8**

Figure S5: Full Kinetic dissolution profiles in SGF and SIF. **Page 8**

Table S1: Fitted Dissolution kinetic parameters**.** **Page 9**

References **Page 8**

EXPERIMENTAL DETAILS

All chemicals were purchased from Sigma-Aldrich (Sydney, Australia) and used as received. Male Sprague Dawley rats were purchased from the Animal Resources Centre (ARC), Perth, Australia.

Mesoporous Particle Synthesis

A method for the synthesis of AMS-6 has been described previously.[1] In the synthesis, the surfactant, *N*-Lauroyl-L-Alanine (1.25 g), was first added to 250 ml deionized water in a PVC bottle and kept in this bottle at 80 °C (200 rpm) for 12 hours. The surfactant solution was stirred for 10 min at 1000 rpm before adding a co-structure directing agent 3-aminopropyl triethoxysilane (1.25g APES) and TEOS (6.25 g) as the silica source. After addition, above the solution was stirred at 1000 rpm for 1 hour. The speed was reduced to 500 rpm after 12 hours and stopped stirring and kept the bottle at RT for 12 hours. The as-synthesized MCM-41 material was filtered and dried overnight at RT; the surfactant was removed by calcination at 550 °C (3 hours inflowing air) to give the final mesoporous particle.

MCM-41 mesoporous silica particles were synthesized according to a previously reported method with a slight modification.[2] In a typical synthesis, 2.5g cetyltrimethylammonium bromide (CTAB) was dissolved in 50 g of deionized water in a PVC bottle. Aqueous ammonia (13.5 g) and absolute ethanol (60 g) was added to the CTAB solution and stirred for 20 min (250 rpm) at room temperature (RT), tetraethyl orthosilicate (4.7 g TEOS) was added in one resulting in a gel. After stirring for 2 hours, the white precipitate produced was kept in the oven at 100 °C for 24 hours. The products were recovered by filtration and calcined as described above for AMS-6.

MATERIAL CHARACTERISATION

Powder X-ray diffraction (XRD)

Powder X-ray diffraction (XRD) studies were performed on loaded samples and free drug to evaluate the crystallinity by a powder diffractometer (Bruker D8 Discover diffractometer) using Cu-Kα radiation as X-Ray source (λ = 1.5406Å). The diffraction patterns were recorded between 1 and 70 2θ degrees.

Nitrogen adsorption/desorption isotherms

Textural properties were characterised using N_2-adsorption desorption isotherms measurement on calcined and drug-loaded silica samples at liquid nitrogen temperature (-196°C) using a Micromeritics TriStar II volumetric adsorption analyser (Micromeritics Instrument Corporation, GA, USA). Before the measurements, all samples were dried and degassed for 12 hours at 100°C. (40°C for drug-loaded samples to avoid drug degradation). Specific surface areas of material were calculated by applying the Brunauer–Emmett–Teller (BET) method in the relative pressure range between 0.05 and 0.2. The total pore volume was considered from the amount of gas adsorbed at P/Po = 0.95.

Thermogravimetric analysis (TGA)
TGA was used to determine the loading amount of the drug in mesoporous silica carrier by using the instrument model TGA-2050 (TA instruments, Delaware, USA). The analysis was done by using a temperature ramp between 20–700°C at a heating rate of 20°Cmin^{-1} from 20 to 800°C. The sample weights varied from 5 mg to 10 mg. The derivative weight loss calculation was performed using TA instruments software (TA instruments, Universal analysis 2000, version 3.0 G).

Scanning electron microscopy (SEM)
SEM was used to study morphology and topography of the particles and their surfaces as well as their size by using a JSM-7401F scanning electron microscope (JEOL Ltd., Tokyo, Japan) operating at 1–2 kV with no gold coating.

Transmission electron microscopy (TEM)
TEM was used to determine the pore structure of mesoporous silica particles complementing low angle XRD, since low angle XRD provides limited structural information that is not sufficient to refine the structure of a mesoporous material. All images of samples were taken with a JEOL-3000F microscope (JEOL Ltd, Tokyo, Japan), operating at 300 kV (spherical aberration: 0.6 mm; resolution 1.7 Å). Images were recorded using a charge-coupled device camera model Keen View, SIS Analysis Specialized Imaging (Olympus Soft Imaging Solutions, Olympus Corporation, Münster, Germany; size: 1024 × 1024; pixel size: 23.5 × 23.5µm) at x30,000–100,000 magnification using low dose conditions on calcined samples.

Surface Enhanced Rama Spectroscopy (SERS)
For SERS, gold substrate microscope slides (Dynasil, USA) were used and prepared with approximately 1mg of lyophilised sample. Measurements were immediately performed with a Sierra Raman instrument (Snowy Range Instruments, USA) with a laser power of 30mW, a wavelength of 785nm and an integration time of 60 seconds. For each spectrum, 10 measurements were recorded and averaged. Background measurements were obtained under the same condition as for the sample measurements and subtracted.

Fourier Transform Infrared Spectroscopy
Fourier transform IR (FTIR) spectra of lyophilized samples were obtained using a Thermo Scientific Nicolet iS5 FT-IR Spectrometer with iD5 ATR accessory, in transmittance, from 4000cm^{-1} to 400cm^{-1} was averaged over 32 scans for each curve. All samples were measured without dilution.

Dynamic light scattering (DLS) and δ-potential

These were performed on a Zetasizer ZS (Malver Instrument, UK) at 25 °C with a He-Ne laser (633 nm, 4 mW output power) as a light source, in filtered distilled water (10µL, 1 mg/mL).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)
To evaluate the LYS corona adsorbed onto PB loaded mesoporous silica surface, 1 mg of each AMS-6PB34.8LYST$_{10}$ and AMS-6PB34.8LYST$_{120}$ samples were mixed with 20µl of 2xSDS loading buffer. In

case of control samples, 1mg of LYS was suspended in 1 ml PBS first and then from this solution 20 µl was pipetted out in a new Eppendorf tube and mix with 2xSDS loading buffer. All the samples were boiled for 5 min at 95°C and centrifuged in a bench-top centrifuge for 5 min to settle down the particles. 10µl of supernatant from each sample was carefully loaded in NUPAGE 4-12% BT GEL of 12 wells (Life Technologies, Thermo Fisher Scientific, Australia), and the gel was run for 55-60 min at 200mV in 20 times diluted MOPS SDS Running Buffer (10×, Thermo Scientific). Staining was performed with Coomassie brilliant blue R-250 staining solutions Kit (Bio-Rad) for 2 h, followed by washing in Milli-Q water for overnight.

KINETIC RELEASE MEASUREMENTS

Media for dissolution experiments

Simulated intestinal fluid (SIF) was prepared by dissolving NaOH (0.896g, 0.0224mmol) and KH2PO4 (6.805g, 0.05mmol) in purified water (1 L) giving a pH of 6.8. Simulated gastric fluid (SGF, pH1.2) was prepared by dissolving 2.0 g of sodium chloride in 7.0 ml of hydrochloric acid and sufficient water to make 1 L solution. All chemicals were purchased from Sigma-Aldrich. Purified water was obtained using a Millipore filtration system (Sydney, Australia) to a resistivity of 18.2 MΩ × cm.

Drug loading

A wetness impregnation procedure was applied to load PB (TCI, USA) into the mesoporous silica. In brief, a sample of mesoporous silica was added into ethanol and stirred for 20 min with sonication. Then different drug solution was added to get different amount of loading and stirred for 1 hour in a round bottom flask, and the solvent was subsequently removed by rotary evaporation (150 rpm) at 40 °C under reduced pressure (66Pa). Samples dried at atmospheric pressure for 1 hour after which they were stored in airtight containers. A low loading capacity was specifically targeted in order to minimise any effects of drugs crystallizing on the outside of the particle and having a large effect on the dissolution curves. Samples were labelled according to the drug loading percentage. For instance, sample contains 34.8% of PB in AMS-6 was labelled as AMS-6PB34.8% and in case of 21.8% drug in MCM-41 was labelled as MCM-41PB21.8%.

Protein corona preparation

1 mg of AMS-6 mesoporous silica particles with and without drug were incubated with, 1ml of lysozyme 50% w/w for different time points (10min and 120min) at 37°C. The hard corona was isolated via centrifugation for 15min, 13200rpm (4°C). The supernatant was discarded, and the remaining mesoporous silica particles pellets re-dispersed in water and this process (washing) was repeated 3 times to remove soft corona. To prepare the incubated sample for characterisation by TGA, and nitrogen adsorption/desorption isotherm it was lyophilised. For lyophilisation, the sample was frozen after the last washing process at -80°C and then placed in a for lyophilisation suitable glass container by Martin Christ Freeze Dryers (1200ml). The sample was then lyophilised overnight at -50°C at 0.1mbar in a Martin Christ Freeze Dryer for 24 hours.

Drug release and drug dissolution experiments

Dissolution studies of drug-loaded and pure crystalline drug were performed in SIF (pH 6.8) and SGF (pH 1.2) containing 0.25% (w/v) CTAB as a wetting agent. In the case of drug release from AMS-6PBLYS samples containing a lysozyme corona: 1ml of lysozyme (conc. 15mg/ml) was added to each microtube containing 30mg of AMS-6PB34.8 (2:1 ratio AMS-6PB to LYS). The colloidal suspension was incubated for 10 min (T_{10}) and 120 min (T_{120}) at 37°C. The in vitro release was conducted using a USP II paddle method (50rpm, 37°C, and 900ml dissolution medium) equipped with a UV/Vis Cary 60 Spectrophotometer (Agilent) connected to an Agilent G7926A Model 708-DS Dissolution Station. The

Higuchi kinetic parameter (K_H) were obtained excluding the first two points for the SIF dissolution curves and up to 2 hours of release, which showed an initial burst effect followed by a slow release from of the drug from inside the porous matrix.[3] All the experiment were conducted in triplicates.

***In vivo* pharmacokinetic study**

Male Sprague–Dawley rats with a mean bodyweight of 200 ± 20g were fasted overnight prior to experiments, with free access to water. The experimental protocol was approved by the Animal Ethics Committee (AEC) of the Macquarie University, Sydney, Australia (AEC Reference No.: 2018/023-3). All animals were allowed to acclimatize for at least 7 days prior to the experiments. The rats were divided randomly into four groups (n=8 for each studied group). Group 1 (control) was treated with only water. PB solution and AMS-6PB34.8 was administered orally at a dose of 10mg kg^{-1} of PB in group 2 and 3 respectively. In the case of group 4, AMS-6PB34.8 was incubated with 1 ml of lysozyme (9 mg/ml) to maintain the ratio of 2:1 (AMS-6PB34.8: LYS) for 10 min (AMS-6PB34.6T_{10}) to form a corona complex. All formulations were administered by oral gavage as suspension form with water.

An approximately 0.15ml aliquot of the blood sample was collected via the tail vein injection at specified time intervals (0.25, 0.5, 1, 2, 3, 4, 8, 12 and 24 hours) in microcentrifuge tubes containing disodium ethylenediaminetetraacetate dihydrate (Na_2EDTA). The blood samples were immediately centrifuged for 10 min at 6000rpm to collect the plasma, and the plasma samples were immediately frozen in an upright position and stored at −80°C±10 until LCMS/MS analysis of PB.

Sample Analysis

Plasma samples were thawed, mixed at room temperature and processed together with quality control samples, reagent blank, and calibrators. Eight point calibration curve (CC) was prepared by serial dilution of PB stock solution (1.1mg/ml) in the range of 0.51-660 ng/ml. Calibration standards and Quality control (QC) were prepared by spiking 45µL of blank plasma with 5 µL of the appropriate working solution resulting in concentrations of 0.51, 1.01, 13.2, 44.0, 176.0, 264.0, 440.0 and 660 ng/ml of plasma for the calibration curve. And 1.62, 271.0 and 441.0ng/ml plasma respectively for quality control (QC) samples of low-quality control (LQC), medium quality control (MQC), high-quality control. A volume of 50 µL study sample was transferred to the pre-labelled Eppendorf tubes from the corresponding samples and 250 µL IS (internal standard, Irbesartan) solution working (167.5 ng/ml) was added to all the samples (CC's, QC's and samples except blank). Whereas for blank samples added 250µL of precipitation solution (100% acetonitrile) and vortexed for 5 minutes, centrifuged for 15 minutes at 10000 rpm at 4°C. Transferred the supernatant to pre-labelled HPLC vials and injected into the LC-MS/MS system for estimation of the analyte.

Mass spectrometry

Electrospray ionization LC-MS/MS analysis was carried out using an AB Sciex 4000 triple quadrupole mass spectrometry instrument with a Shimadzu LC-20 AD VP HPLC system. Chromatographic separation was achieved at 40 °C using a reversed-phase column Agilent Poroshell EC-C18, 2.7µ, 4.6×50mm. The analyte was eluted from the column using an isocratic flow of Mobile Phase A (0.1% formic acid in 80% methanol): Mobile phase B (0.1% formic acid in 5mM ammonium format (98:2) for 5 min. The flow rate and injection volumes were 0.600 mL/min and 10 µL, respectively. The total probucol was detected by Multiple Reaction Monitoring (MRM) in the negative ion mode, quantified by the ion transition m/z 515.228 → 235.900 for PB and 427.22 → 193.000 for Internal standard. The ion spray voltage and source temperatures were 5550 V and 500°C, respectively. Decluttering, entrance and exit potentials were -80.0 V, 10.0 V and -13.0 V, for PB and for internal standard -90.0 V, 10.0 V and -13.0 respectively. Curtain gas, ion source gas 1, ion source gas 2, and collision gas were set to 30 psi, 30 psi, 45 psi, and 6 psi, respectively. The collision energies for quantitation ions for the probucol internal standard were -30eV and -36eV respectively.

Figure S1: Properties of mesoporous particles of AMS-6. (a) Powder X-ray diffraction (XRD) pattern of calcined AMS-6 and PB-AMS-6 loaded at 34.8wt% showing mesoscale diffraction peaks associated with the cubic mesostructure (a_0=120.7Å for the calcined AMS-6). (b) High angle XRD region of PB-AMS-6-34.8% showing the absence of scattering peaks from crystalline drug and compared to the pure PB compound. (c) and (d) show representative TEM and SEM images of calcined AMS-6 particles.

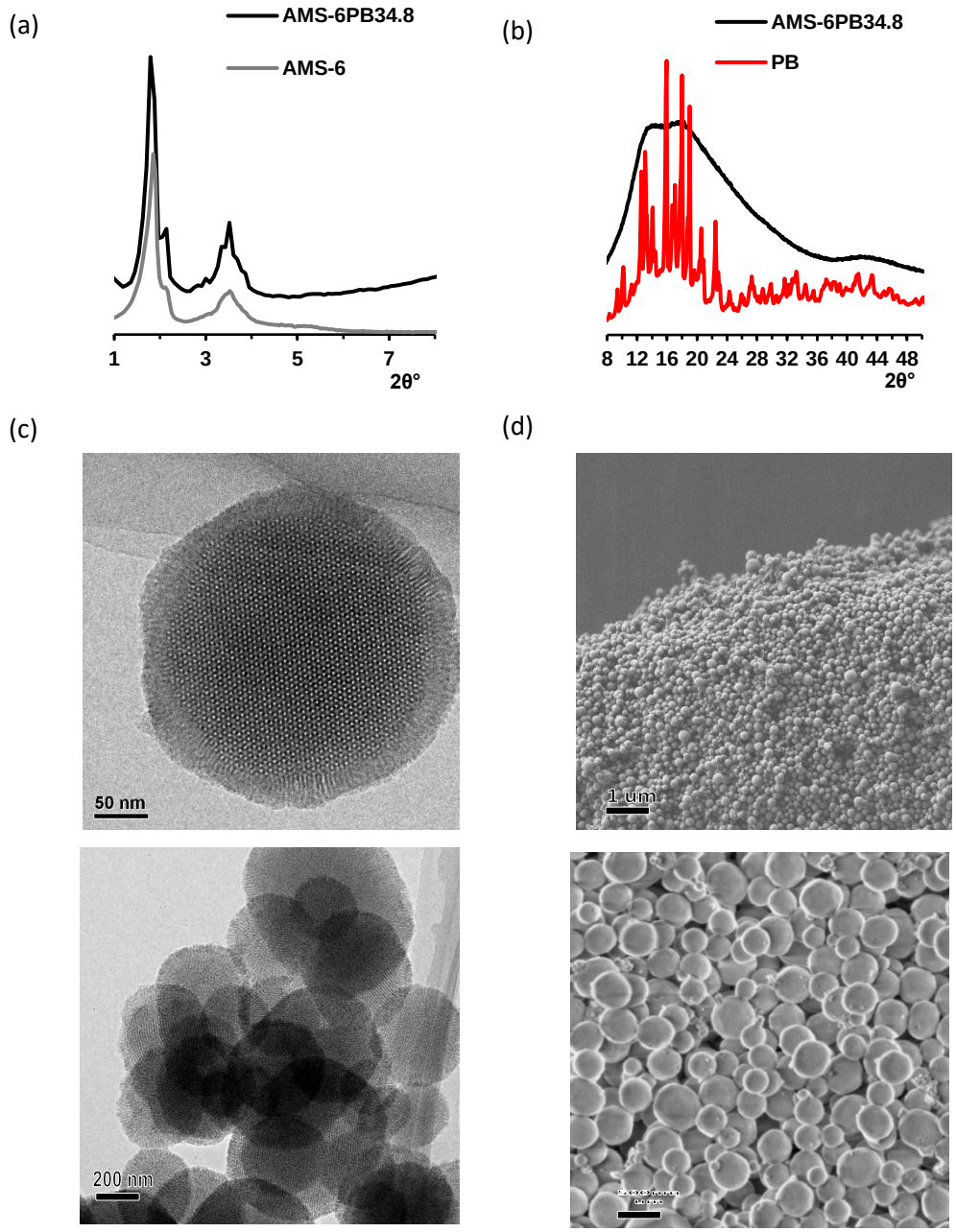
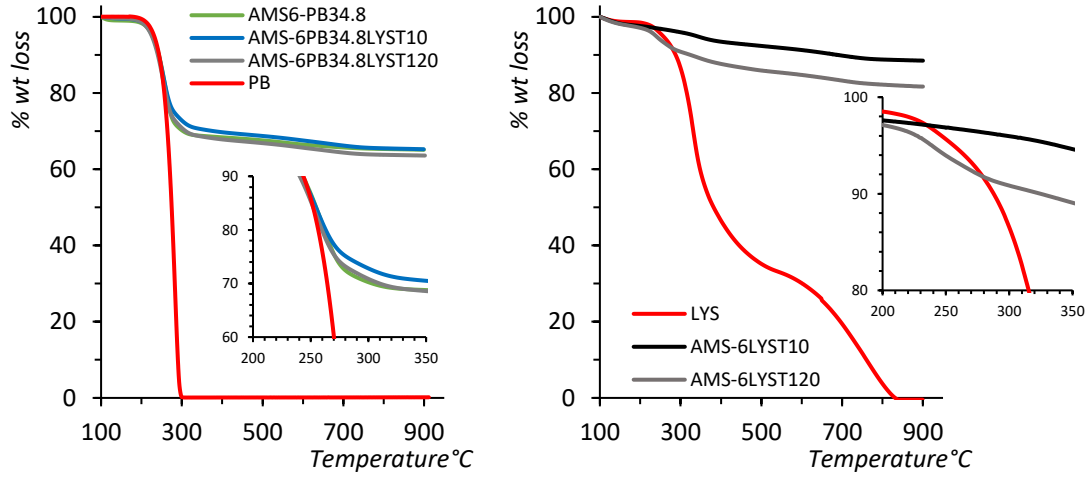


Figure S2: Drug loading and *hard* Lysozyme corona. (a)Thermogravimetric analysis curves of AMS-6PB34.8 and AMS-6PB34.8LYS samples as a function of incubation time compared to PB and AMS-6LYS samples in the absence of PB compared to LYS. Insets show enlarged areas at the onset of decomposition of LYS and PB. (b) Nitrogen adsorption isotherms curves of AMS-6PB34.8, AMS-6PBLYS and AMS-6PB34.8LYS samples prepared at different LYS incubation times.

(a)



(b)

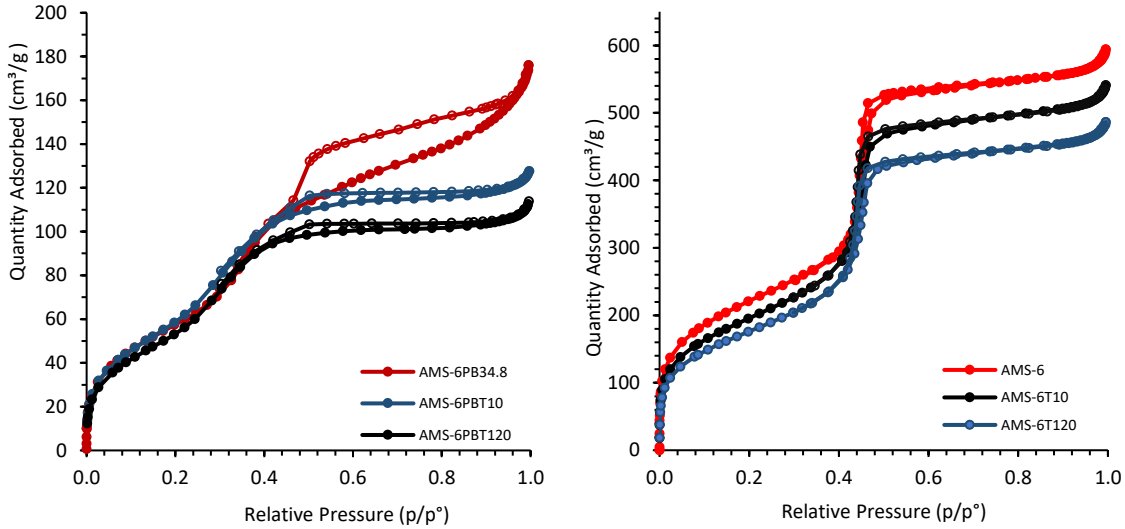


Figure S3: (a) Surface enhanced Raman and (b) Fourier Transform Infrared (FT-IR) spectra of AMS-6PB34.8, AMS-6PB34.8LYST10 and AMS-6PB34.8LYST120 samples as a function of incubation time. For comparison pure PB and LYS spectra are also shown.

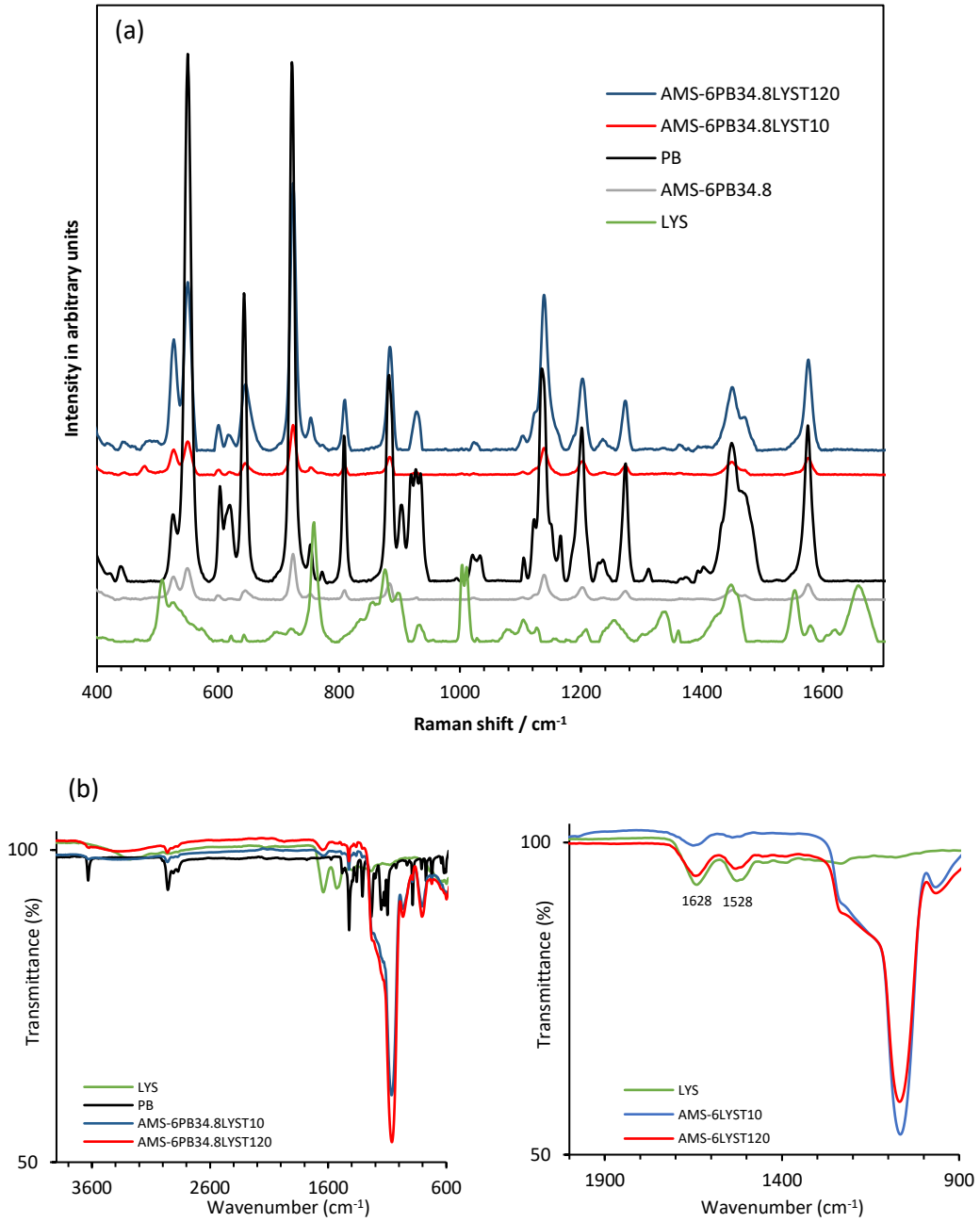


Figure S4 Effect of Lysozyme PC on net surface charge (δ-potential) and particle size distribution (dynamic light scattering) of AMS-6 particles in water after incubation at different times in lysozyme.

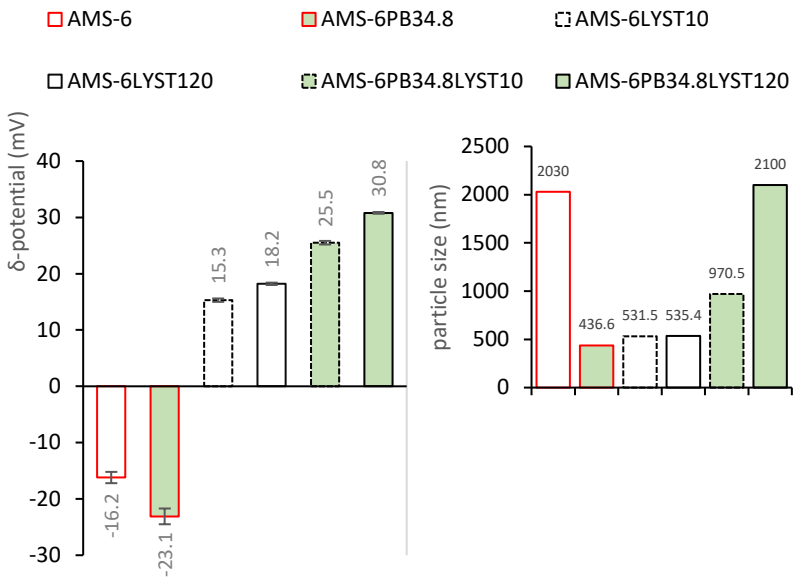


Figure S5: Kinetic dissolution profiles of crystalline PB, AMS-6PB34.8, AMS-6PB34.8LYST$_{120}$ and AMS-6PB34.8LYST$_{10}$ in SGF (pH 1.2) and SIF (pH 6.8).

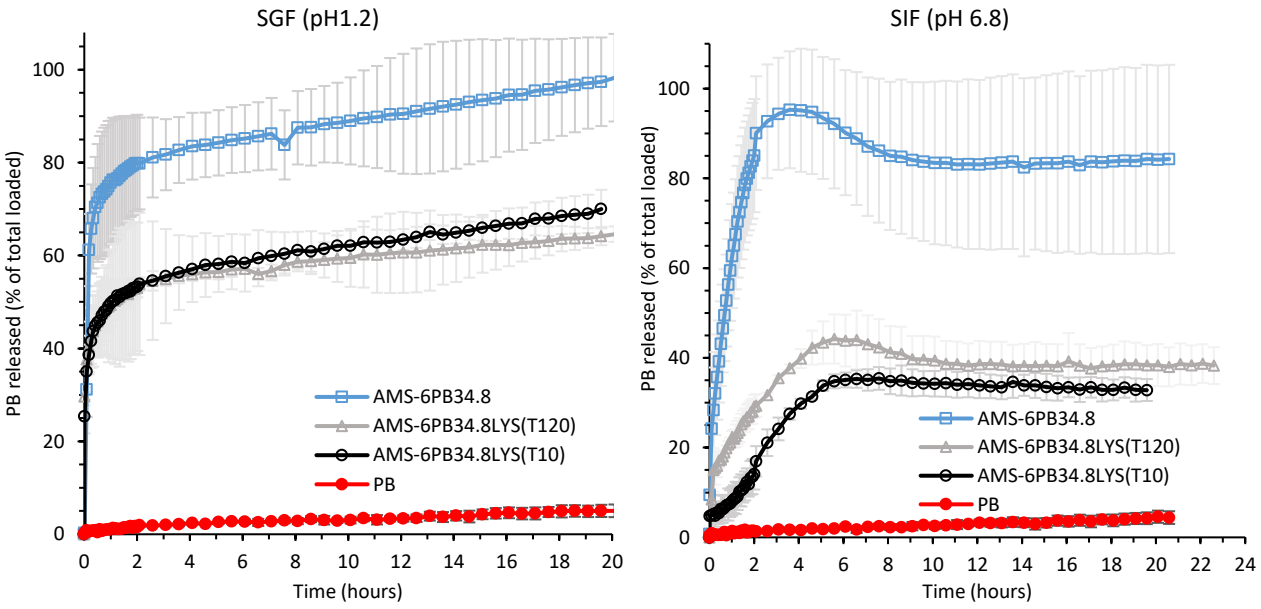


Table S1 Fitted drug release kinetic parameters, using Higuchi (H) model, obtained from dissolution experiments for PB loaded mesoporous silica samples. Values for AMS-6PB34.8LYS samples in SIF are calculated excluding the first two points (<10 minutes release) which demonstrate a burst release over this period.

Material	Incubation Medium	Release Media	Incubation time T_{min}	R^2	K_H	$T_{1/2}$ min
Probucol (PB)	-	SIF	-	0.98	0.9^{ϕ}	>1300
	-	SGF	-	0.97	0.3^{ϕ}	>1300
AMS-6PB34.8	-	SIF	-	0.99	59.2	45.4
		SGF	-	0.97	79.6	20.5
	Lysozyme	SIF	T_{10}	0.97	8.65	>1300
			T_{120}	0.96	21.7	>1300
		SGF	T_{10}	0.98	47.8	72
			T_{120}	0.90	48.3	78

$^{\phi}$ values for zero order release rate

References

1. R. Atluri, N. Hedin and A. E. Garcia-Bennett, *Chemistry of Materials*, 2008, **20**, 3857-3866.
2. M. Manzano, V. Aina, C. O. Areán, F. Balas, V. Cauda, M. Colilla, M. R. Delgado and M. Vallet-Regí, *Chemical Engineering Journal*, 2008, **137**, 30-37.
3. G. Frenning, *Int J Pharm*, 2011, **418**, 88-99.