Bio-inspired silica offers a novel, green and biocompatible alternative to traditional drug delivery systems

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ABSTRACT

The development of a drug delivery system (DDS) is essential in many cases to remedy the limitations of free drug molecules. Silica has been of great interest as a DDS due to being more robust and versatile than other types of DDS (e.g. liposomes). Using ibuprofen as a model drug, we investigated bio-inspired silica (BIS) as a new DDS and compared it to mesoporous silica (MS); the latter have received much attention for drug delivery applications. The BIS is synthesised under benign conditions and without the use of hazardous chemicals, which enables controllable in situ loading of drugs by carefully designing the DDS formulation conditions. Here we systematically studied these conditions (e.g. chemistry, concentrations and pH) in order to understand BIS as a DDS and further achieve high loading and release of ibuprofen. Drug loading into BIS could be enhanced (up to 70%) by increasing the concentration of the bioinspired additive. Increasing the silicate concentration increased the release to 50%. Finally acidic synthesis conditions could raise loading efficiency to 62% while also increasing the total mass of drug released. By identifying ideal formulation conditions for BIS, we were able to produce DDS that were able to release fivefold more drug per weight of silica when compared with MCM-41. Biocompatibility of BIS was also investigated which found that, although ~20% of BIS was able to pass through the gut wall into the blood stream, it was non-haemolytic (~2% haemolysis at 500 µg ml^{-1}) when compared to MS (10% haemolysis at the same concentration). Overall for DDS, it was clear that BIS has several advantages over MS (ease of synthesis, controllability and lack of hazardous chemicals) as well as being less toxic, making BIS a real potentially viable green alternative to DDS.

Keywords:

Nanomaterials, nanomedicines, pharmaceuticals, cytotoxicity, biomedical devices.

1. INTRODUCTION

Drug molecules currently on the market, while effective, can have a whole range of limitations which reduce the efficacy of the drug. Some limitations include poor solubility, in vivo degradation and short systemic circulation times [1]. Due to these factors, in order to achieve efficacy, drugs may require higher doses, which can result in higher toxicity [1]. One method of improving drug efficacy is by developing drug delivery systems (DDS) [2]. Aside from the obvious potential medicinal benefits of DDS, there are also large economic benefits to be gained as new DDS take significantly less time and investment to develop than a new drug molecule (3-4 years and approximately $20-50 million for DDS [1] vs $500 million and 10-12 years for a new drug[3]).

Many materials have been investigated for the use of DDS, e.g. liposomes, polymeric nanoparticles (e.g. dendrimers) and "hard" nanoparticles, mainly consisting of metals (e.g. gold), metal oxides (e.g. iron oxide, titanium oxide and silica) or carbon [4-6]. However, relatively few DDS are currently on the market [7]. The main limitations for any DDS becoming a clinical product are the long regulatory journey coupled with issues with biocompatibility, efficacy and manufacturing processes. Briefly, a DDS must first be proven to work and be safe in vitro and then in vivo, manufacture should be straightforward and it should provide significant benefits over risks before it can gain support from patents and financial backing. Next human clinical trials are carried out and if these are passed then the product will go on to become commercialised [7]. This long, multi-step process can create obstacles for new DDS and results in the failure of many of them. Due to the high failure rate of DDS there is huge potential for new developments in this field.

Here we focus on silica as a DDS because there has been increasing interest in the use of silica nanoparticles for the purpose of drug delivery since 2001, when Vallet-Regi, et al.,

described the effective loading and release of ibuprofen from a type of mesoporous silica nanoparticle (MCM-41) [8]. The successful use of a silica DDS over other systems (e.g. liposomes) has been attributed to its thermal and chemical stability as well as versatility compared to conventional drug delivery systems [9,10] [11,12]. Further, silica offers a versatile platform for functionalisation with biomolecules in order to tailor drug release as well as targeting the delivery. One of the most common methods of controlling drug release is through functionalising silica to create stimuli-responsive DDS. This opens up a wide range of external stimuli which can be used to manipulate these materials, ranging from magnetism, ultrasound and light, to the more conventional, temperature and pH [13-16]. The functionalisation has also shown promise in targeted drug delivery. For example, an interesting avenue is using silica functionalised with cell penetrating peptides for targeted delivery directly into cytoplasm.[17]

Silica can be functionalised with various chemical groups, making it compatible with a range of drugs. Examples of various drugs investigated with silica range from anti-inflammatories like ibuprofen or aspirin to antibiotics such as gentamicin and erythromycin, anti-malarials and anticancer drugs such as doxorubicin and campothecin [8-10,18-27]. While a gold coated silica product (Auroshell[28]) is in the first stage of development to be available as an anti-cancer agent, there are currently no silica based drug delivery systems on the market, despite the fact that MS showed some promise as effective DDS nearly 15 years ago. This delay is due to several limitations including long and laborious synthesis (synthesis of MCM-41 can take between 10 and 146 hours[29-32]), the use of harsh chemicals, toxic surfactants, hazardous precursors and harsh conditions (extremes of temperatures and pH [31]). These imply that drug loading can only occur post-synthesis, which adds another step (and extra time) to the synthesis of this type of DDS. Therefore, a greener, economical, scalable and safer method of synthesising silica with potential for in situ drug loading would be highly favourable.

Biomineralisation of silica is observed in several species of aquatic unicellular organisms, such as diatoms (a class of algae) [33], as well as in more complex organisms, such as some sponge species and even in some plants [34-36]. It was found that specific proteins and biomolecules were involved in the condensation of biosilica, such as silicatein and silaffin [34-36]. By understanding the chemistry and the role of these biomolecules, we have developed analogues of these biomolecules ("additives", typically amines) which have been shown to rapidly condense silica under benign conditions [37,38]. As such, this has enabled the discovery of bio-inspired silica (BIS) which can be controllably synthesised at room temperature, at neutral pH, in water and within 5 min [39]. This also opens the possibility of in situ drug loading, thus allowing a one step, green, DDS formulation [40]. Further, amine-ibuprofen interactions have been reported to be favourable for drug delivery [41-43], which provides another potential benefit of BIS over MS: a possible additional function of the amine additives.

As yet only five papers have been published on the use of BIS synthesis for drug delivery applications (including one from our group [40]), suggesting a vast potential for future research. Li, et al, utilised a so-called "biomimetic" synthesis route, however, this method retained all the issues of synthesising MCM-41 (i.e. long synthesis time, high temperatures and requirement for calcination) [44] . Begum et al. made use of surfactants to create porosity, thus their system still requires an energy intensive calcination step as well as post-synthesis drug loading [45]. Sano et al. designed a drug molecule which had the dual function of pharmacological activity and silica condensation ability (not all drug molecules will have this dual ability) meaning that the system was limited to only a small set of drug molecules [46]. Lechner et al. linked their cargo molecule to a silica condensing peptide; however, they were not able to fully control drug release. Conjugating peptide with drug has many other issues, such as loss of drug activity, use of hazardous chemicals and also an extra synthesis step[47]. Preliminary work from our group reported the green synthesis of silica with in situ drug loading

of calcein (a hydrophilic drug-like molecule) [40]. This synthesis required no calcination and the amine additive was separate from drug molecule. BIS did not show any significant toxic effects to either fibroblasts or human monocytes in the resting state, even at high silica concentrations. However, mesoporous silica particles showed substantially reduced cell viability, even at low concentrations. For example, the silica concentration required to reduce cell viability to 50% (IC_{50}) was 5-10 times more for BIS than MCM-41. Further, BIS did not induce secretion of inflammatory cytokines at the concentrations proposed for use in DDS.[40] From these results, it is evident that despite the use of amine additives, the BIS are safe and do not cause concerning cytotoxicity.

In the present study we aimed to further extend BIS to a pharmaceutically active drug molecule (ibuprofen) and create a DDS formulation which, through carefully investigating and understanding the formulation chemistry, would have the ability to control the loading and release of pharmaceutically active drugs. Ibuprofen was chosen since it is a commonly used model drug for DDS development due to its small molecular size (1.0 x 0.6 nm^2) [8], stability [48], ease of detection (UV absorbance at ~220 nm), and available literature on ibuprofen-silica systems for comparison. The main aim of this research is to primarily understand in situ drug loading into the BIS system. Specifically, we plan to determine predictive rules, investigate the effects of amine additive, drug interactions and silica chemistry on DDS performance (drug loading and release profiles). Further, in order to make BIS a viable DDS, it should exhibit similar or improved loading and release profiles for ibuprofen when compared to the competitor MCM-41 based DDS.

2. MATERIAL AND METHODS

2.1. Chemical Reagents

All reagents were purchased from Sigma unless otherwise stated.

Acetronitrile (HPLC Plus, ≥99.9%), ammonia (NH_3, anhydrous, ≥99.98%), anhydrous sodium sulphite (97%), ammonium molybdate $\cdot 4H_2O$, calcium chloride hexahydrate (USP testing specifications), concentrated hydrochloric acid, diethylenetriamine (DETA) (99%), dinitrophenol (≥98.0%) (DNP), Dulbecco's PBS, formic Acid (≥95%), glucose (≥99.5%), heparin, hexadecyltrimethylammonium bromide (CTAB), hydrochloric acid solution 1M (HCl, Fisher), ibuprofen (≥98%), Immu-mount, magnesium sulphate heptahydrate, oxalic acid $\cdot 2H_2O$ (≥99.5%), pentaethylenehexamine (PEHA) (technical grade), potassium chloride (≥99.0%), phosphate buffered saline (PBS) (tablets pH 7.4), poly(allylamine hydrochloride) average M_w ~17,500 (PAH), poly(fluorescein isothiocyanate allyamine hydrochloride) (Poly(allylamine hydrochloride) : Fluorescein isothiocyanate 50:1), potassium phosphate monobasic, sodium chloride (≥99.5%), sodium metasilicate pentahydrate (technical) (Fisher), sulphuric acid (98%), tetraehylenepentamine (TEPA) (Acros organics), tetraethoxysilane (TEOS) (99.999% trace metals basis), Triton X-100 (laboratory grade).

2.2. In situ drug loading into BIS and drug release

To a solution of sodium metasilicate in deionised water a solution of amine additive (in water) was added followed by an ibuprofen solution (in 70% ethanol). Then a known volume of 1M HCl (the volume of HCl required varied depending on the amine additive used) was added to reduce the pH of the solution to the desired pH (pH 7, unless otherwise stated). The concentrations of the reactants in the final solution were 30mM of sodium metasilicate, 1mg ml^{-1} PAH and 1mg ml^{-1} ibuprofen, this ratio was termed 1:1:1. For a 50ml batch of 1:1:1, 0.3182g sodium silicate, 0.05ml of PAH and 0.05g of ibuprofen were used. When synthesising BIS with other amines (DETA, TEPA and PEHA), a molar ratio of [Si]:[N] of 1:1 was used. This equates to 0.05155g of DETA, 0.05678g of TEPA and 0.05809g of PEHA for a 50ml

batch. Once acid was added, silica precipitated within seconds and the solution was left for 5 minutes before being centrifuged at 8000 rpm for 15 minutes in order to stop the reaction. The supernatant was stored at 4^{o}C in order to determine the drug loading efficiency (% of drug which was loaded into the silica) and drug content (% weight of drug in the silica-drug complex) via the method described in section 2.4. The silica pellet was resuspended, washed in deionized water and centrifuged, twice more (no detectable drug was observed in these supernatants) and finally dried at 45 ^{o}C for at least 5 hours.

Once dried, 10 mg of the silica was suspended in 1.4ml of PBS (pH 7.4) and incubated at 37 ^{o}C to measure the drug release. At each time point (1, 3, 5, 7 and 24 hour time points), samples were centrifuged at 8000 rpm for 15 minutes and 1 ml of the supernatant was used for HPLC analysis and replaced with fresh PBS in order to satisfy the perfect sink conditions for the determination of the diffusion parameters. Release is expressed as the % of the loaded drug which has been released from 10mg of silica. Each sample was prepared in triplicate and release profiles were measured from each sample in triplicate.

2.3. Synthesis of MCM-41 and post synthesis drug loading

MCM-41 was synthesised by first dissolving CTAB in 300 ml of 25 % ammonia at 35 ^{o}C. While stirring, 20 ml of TEOS was slowly added. This solution was then stirred for 3 hours and then aged for 24 hours at room temperature in a closed container to allow silica to form. The product was then vacuum filtered and washed with 1 litre of distilled water and finally dried overnight at 85 ^{o}C. To remove the surfactant (CTAB), MCM-41 was calcinated at 500^{o}C for 5 hours. This was based on previously published methods [12].

To load drug, 10mg of MCM-41 was immersed in a 1 mg ml^{-1} solution of ibuprofen (in 70% ethanol) at 37 ^{o}C overnight. Samples were centrifuged at 8000 rpm for 15 minutes and the supernatant was removed (and supernatant drug concentration was measured to determine loading efficiency) and replaced with fresh PBS for a release experiment. At each time point

samples were centrifuged at 8000 rpm for 15 minutes and 1ml of the supernatant was taken for HPLC analysis and replaced with fresh PBS.

2.4. Drug detection via high performance liquid chromatography (HPLC)

Drug loading and release were determined via an HPLC analysis method. A DIONEX system was used with an auto-sampler (GINA50), a pump (P580) and variable wavelength detector (UVD170S), along with an ACE 5 C-18 column (150X4.6 nm with 5 µm particle size) at room temperature. An isocratic reverse phase HPLC method was used with 30 µl injection volume and a mobile phase of acetonitrile: 0.1% formic acid (70:30) at a flow rate of 1ml min^{-1}. Ibuprofen retention time was approximately 4.7 minutes and was detected at a wavelength of 220 nm (λ_{max} wavelength of ibuprofen). Data were collected using Chromeleon V6.80 software and peaks were integrated to determine drug concentration. Data were fitted with a single exponential equation (Eq. 1) where Y_0 is the final % release, A is a constant and R_0, is the slope. By multiplying A and R_0 the maximum rate of release (% release per hour) was deduced.

$$y = Y_0 + A\, e^{R_0 X} \qquad \text{[Eq. 1]}$$

2.5. Materials characterisation

Silica samples were characterised using nitrogen adsorption in a micromeritics ASAP 2420 Accelerated Surface Area and Porosimetry system. Samples were first weighed and degased in optimum pressure and temperature conditions (120 ^{o}C). They were then held at the boiling point of nitrogen and evacuated allowing for nitrogen gas to enter the sample tubes while the pressure was monitored. Analyses of the data included BET (Brunauer Emmett Teller [49]) theory, used to characterise the surface areas of the silica particles, and the BJH (Barrett Joyner Halenda [50]) theory which allowed for the characterisation of the silica pore size distributions.

Silica samples were imaged by scanning electron microscopy (SEM) using a Hitachi SU6600 field electron-SEM. Samples were mounted on sample holders using sticky carbon tape and then gold splutter coated under vacuum to prevent the charging of the sample. Micrographs were taken using a 20kV potential difference and a working distance of 8.7mm.

2.6. Measuring movement of silica across the gut wall

Rats (200-250g, male, Sprague Dawley) were anesthetised via intraperitoneal injection with pentobarbitone (60mg/kg) and sacrificed for the experiment. The small intestine was removed and washed through with 37ºC Krebs solution (made from distilled H_2O, 16.09% (w/v) NaCl, 1.1% (w/v) KCl, 0.22M KH_2PO_4, 2.74% (w/v) $MgSO_4.7H_2O$, 0.12M $CaCl_2.6H_2O$). Intestines were then inverted and bathed in Krebs solution, ensuring 37ºC temperature was kept constant. Small sections of gut (~5-6 cm) were cut and tied closed at one end with thread, filled with 1ml of fresh Krebs solution, and then the open end was also tied closed.

In order to verify the health of the sections of gut, a control experiment was set up which measured the passage of glucose across the gut wall. Sections of gut were either immersed in 6ml of 1mM glucose solution or in 1mM DNP (dinitrophenol) solution (to inhibit the active transport of glucose [51]) for 15 minutes at 37ºC before a glucose solution (to make a final concentration of 1mM) was added. Sections of gut were then incubated at 37ºC for an hour, before being cut open and their contents removed. Glucose concentrations were measured by using a Glucose (gluc-pap) assay kit purchased from Randox.

To measure the passage of silica through the gut wall, fluorescent silica was prepared using the same method in section 2.2 except that PAH-FITC was used as the amine additive,

thus creating fluorescently labelled silica. Fluorescence was measured on a RF-530IPC fluorometer at excitation wavelength of 495nm, and emission wavelength of 515nm. Tubes of inverted rat gut sections were incubated in 1 mg/ml silica solution (in Krebs) or 1mg ml^{-1} silica solution and 1mM DNP for an hour at 37^{o}C. Gut sections were then cut open and contents removed and the fluorescence measured; the sections were then fixed in a formalin solution (neutral buffered 10%) for 30 minutes followed by two PBS (pH 7.4) washes. The inside and outside surfaces of the gut sections were then imaged using a Carl Zeiss Axio Imager Z1 with 10x/0.30 lens. Sections of gut were mounted either by stretching the gut and pinning the edges or compressing gut sections under Immu-mount and coverslips.

2.7. Haemolytic activity of silica

To measure the haemolytic activity of silica, rats (Sprague Dawley) were bled and the blood was stabilised with heparin (100 µl of 1000 units ml^{-1}). 4ml of heparin stabilised blood was diluted with 9ml of Dulbecco's PBS and centrifuged at 2250g for 5 minutes. The supernatant was carefully removed and the blood was washed five times with Dulbecco's PBS (D-PBS). After the last wash the red blood cells (RBC) were diluted with 40ml of D-PBS. 0.2ml of diluted RBC were then added to 0.8ml of silica suspension at the desired concentration to make a final silica suspension. Positive and negatives controls were set up by adding 0.2ml of RBC to either 0.8ml D-PBS or 0.8ml of 0.2% Triton X-100 respectively. All samples were prepared in triplicate and briefly vortexed before being left static at room temperature for 4 hours. Samples were then vortexed again and centrifuged at 10,000g for 2 minutes. 10µl of supernatant was used to the absorbance of haemoglobin using an anthos2020 plate reader at 577nm with a reference wavelength of 655nm. Haemolysis was calculated as % haemolysis = [(sample absorbance – negative control)/(positive control – negative control)] x 100 [52].

3. RESULTS AND DISCUSSION

In order for BIS to be developed as an effective DDS, one must understand the synthesis chemistry and the mechanisms that dictate the loading and release of drug molecules from the system. There has been little published on the loading mechanics of the BIS system and it has been speculated, but not proven, that embedded amine, originally employed to facilitate silica condensation, also helps to functionalise the silica [40]. If this is the case then the BIS DDS can be synthesised, functionalised and drug loaded all in one step which is a vast improvement on the long multi-step process involved in MS. All these possibilities were investigated herein.

3.1. The effect of the amine additive on the loading and release of ibuprofen

The effect of the choice of amine additive for the synthesis of BIS upon its ability to load and release calcein (a non-pharmaceutically active but "drug-like" molecule) has previously been reported[40]. As these effects are drug specific, we investigated them for an active drug molecule (ibuprofen) in the BIS system and compared earlier results for calcein, with those for ibuprofen.

In order to screen most suitable systems, four additives were investigated– three small amines and one polyamine. These were chosen based on their silica precipitation performance and previous investigations into BIS [37,38,40,53]. We measured the loading efficiency (amount of drug loaded on DDS when compared to the concentration used for loading), drug content in the DDS (amount of drug loaded per weight of DDS) and total amount of drug released (mg drug/10 mg DDS). Diethylenetriamine (DETA), a small amine, was immediately excluded for use as it had a loading efficiency of only <5% (Figure 1A). The other amines used were pentaethylenehexamine (PEHA), tetraethylenepentamine (TEPA)) and poly(allylamine hydrochloride) (PAH) and they exhibited loading efficiencies of 20-30%, while MCM-41 showed ~40% loading efficiency (Figure 1A). These differences between BIS and MCM-41

are likely due to the different methods by which the drug was loaded into these two types of silica. For BIS, ibuprofen was loaded in situ and so the drug would have been entrapped within the silica particles, followed by some surface physisorption. With MCM-41, only post-synthesis loading was possible and so drug loading was entirely reliant on physisorption (hence surface area and porosity is important in this system).

Focussing on drug release from these DDS, despite having loading efficiencies similar to BIS-PAH, BIS-TEPA or BIS-PEHA released <2% of loaded drug and as such these amines must also be discarded (Figure 1 B and C). Approximately 22% of loaded ibuprofen was released from BIS-PAH, compared to the 39% released from MCM-41 (Figure 1B and Table S1). The release data appeared to fit well using a single exponential equation with >0.9 R^2 values in all cases (Table S1). The fitting showed that BIS-DETA, BIS-TEPA and BIS-PEHA all had a very low release rate (Figure 1B and C). However, the rates of release (Table S1) from MCM-41 and BIS-PAH were similar (15 and 17% per hour respectively).

The loading efficiency of drug on MCM-41 was found to be 41%, while the loading efficiency for BIS-PAH was 23% (Figure 1A). Despite this, MCM-41 released around half the amount of drug when compared to BIS-PAH (0.12mg compared to 0.28mg for 10 mg DDS, respectively). This implies that for a dose of 1 mg of ibuprofen, a patient would have to take ~83 mg of MCM-41 compared to only ~54 mg of BIS-PAH. High doses of MCM-41 silica can result in serious toxicity issues unlike BIS[40,52], which highlights a key benefit of using BIS.

The differences in release profiles between BIS synthesised with the different amines are likely to be due to the porosity and morphology characteristics of the silica synthesised (Figure 1D and S1B). Adsorption of drugs is a function of pore size, pore volume and surface area; particle size does not have any impact on release but rather pore morphology.[54,55] In the case of MCM-41 (a mesoporous silica), it is generally accepted that porosity is a major factor in

controlling the release of drugs, and so further investigation was needed as to whether this was the case for BIS [43,56,57]. BIS-DETA, TEPA and PEHA all have a very small pore volume (~0.1 cm^3/g) and low surface areas (~20-40 m^2/g), see Figure 1D, The pore volume and surface area for BIS-PAH (0.74 cm^3/g and of 129 $m^{2/}g$ respectively) were higher than that of silica synthesised with the other three amines. This suggests that silica particles synthesised with any of the small amines were dense when compared to BIS-PAH, which explains the higher release from BIS-PAH within the BIS series. These observations explain why BIS-DETA, TEPA and PEHA samples exhibit poor drug loading/release when compared with BIS-PAH. Interestingly, MCM-41 has a much larger surface area (989 m^2/g) than any of the BIS, but it demonstrated loading efficiency comparable with BIS-PAH. Scanning electron microscopy (SEM) revealed that BIS-PAH particles were fairly uniform in shape and sizes exhibiting a range between 72±17 nm and 78±18 nm (Figure S2A & B), without and with the drug, respectively, thus suggesting that the presence of the drug did not affect the particle sizes significantly. On the other hand, MCM-41 samples used herein were not only very large in comparison (3340±1013 nm, Figure S1A & B) but also non-uniform with large variations in sizes and shapes. Further, it is interesting to note that despite the differences in particle sizes between MCM-41 and BIS-PAH, the amounts of drug released were often comparable. At this point in time, a direct comparison between these two DDS is not possible simply based on SEM results because of their distinctly different drug loading mechanisms and further analysis in future is necessary.

Along with porosity altering the release of ibuprofen, it has been reported that amine-ibuprofen interaction is important in loading [40,42,58]. Since BIS-TEPA and BIS-PEHA showed over 30% drug loading efficiency, it is possible that the amine additives facilitate ibuprofen loading through favourable amine-drug interactions as reported elsewhere[41-43,58] but they also form non-porous silica by fully encapsulating ibuprofen within the dense silica particles thus

resulting in very low release. PAH, however, allows ibuprofen loading through favourable interactions with amine groups and release occurs through the silica pores. These observations are consistent with the literature where it has been reported that these small amines lead to the formation of dense and non-porous silica, while PAH forms porous silica [37,38].

3.2. Altering reactant concentrations to understand the silica-drug system

The main aim here is to understand the DDS and investigate how controllable it is with ibuprofen so that this knowledge can be implemented for other drugs. As such, our next step was to study the effects of reaction chemistry on DDS performance. There had been some evidence that altering reactant concentrations can alter the loading and release profiles of calcein from BIS synthesised with PAH [40]; however, the reasons behind this effect were not fully investigated. Therefore, a systematic approach by varying synthesis conditions and evaluating their effects on drug loading and release has been taken while keeping the starting concentration of ibuprofen in the reaction mixture constant (1 mg ml^{-1}).

Figure 2A and Table S2 show that for MCM-41 (as reported in the section above), the loading efficiency was ~40% and the drug content was ~3 wt%. The loading efficiency and drug content for the 1:1 BIS-PAH sample (30mM solution of sodium metasilicate and a 1mg ml^{-1} solution of PAH) were ~22% and 13 wt%. When the concentrations of silicate and PAH were doubled (2:2) there was a doubling of ibuprofen loading efficiency (Figure 2A). This was attributed simply to more silica being formed (Table S2) since the drug content did not change (Figure 2A). When only the silicate concentration was increased, but the PAH concentration was kept at 1mg ml^{-1} (2:1), there was a slight increase in ibuprofen loading efficiency (Figure 2A) but drug content remained unchanged which was attributed simply due to an increased silica yield (Table S2). Producing more silica means that more ibuprofen was loaded (and so less was wasted by being left in the reaction mixture). Interestingly, when a synthesis ratio of 1:2 (increasing PAH concentration but maintaining silicate concentration) was investigated,

drug loading efficiency increased three fold to 75% (Figure 2A). This loading efficiency (which was significantly higher than that found for MCM-41 (~40%)) was produced from a significantly lower silica yield (Table S2). The drug content also increased substantially from ~10% for 1:1 to ~70% for 1:2. This is likely due to a drug-amine interaction, suggesting that the amine can have a dual function of facilitating silica condensation as well as acting as a functionalisation agent to facilitate drug loading (see section 3.3 for further discussion). These loading studies highlight that the synthetic conditions can readily modulate the loading efficiency of BIS and even reach loadings that are significantly higher than what is achievable with MCM-1.

Finally, the release of ibuprofen from these samples was investigated and it was found that the overall release of ibuprofen from different silica varied. BIS-PAH (1:1) released 22% of the loaded ibuprofen and 2:2 and 2:1 both achieved higher releases (45% and 50% respectively), which were greater than the 39% released from MCM-41 (Figure 2B & C). It is possible that release was higher from 2:2 and 2:1 than 1:1 due to faster silica condensation since the silica precursor concentration used was doubled [59]. This resulted in lower pore volumes and smaller pores (Figure 2D and S3B), leading to less drug being entrapped within the silica, remaining mainly as surface bound, making release easier. In contrast, a 1:2 ratio released only 6% of loaded ibuprofen (Figure 2B), despite a very high loading efficiency and a larger pore size (Figure 2D & S3B).

When the release profiles were considered (Figure S3A), all but the 2:1 samples exhibited burst release, where the majority of drug was released over the first five hours and very little release was observed after this point (Table S2). This suggests that the ibuprofen that is able to escape is mainly surface bound and any ibuprofen embedded within the silica particles is trapped and unable to be released. This idea is supported by Figure S3A where all the BIS release profiles were similar to the release profile of MCM-41, which only had surface bound

ibuprofen loaded. However, the 1:2 system had a much lower maximum release rate than the other systems (Table S2) as well as low total release (Figure 2B). Table S2 also shows that the mass of ibuprofen released from all the BIS systems were higher than from MCM-41, some BIS samples releasing 5x more drug per weight of silica than MCM-41. This is important since if more mg of drug is released then less silica will need to be administered to a patient.

3.3. Understanding additive-drug interactions to control DDS formulation.

Ibuprofen contains a carboxylic acid group, which is expected to interact with amines. Several studies have exploited these favourable amine-ibuprofen interactions by post-synthetically functionalising MS [41-43,58]. In addition, from the results presented above, there was an indication that the PAH-ibuprofen interactions are important for the drug loading and release. Therefore, we investigated whether drug loading and release could be controlled by tuning PAH-ibuprofen interactions by varying the synthesis pH (and in turn the protonation). In this study silica was usually formed at pH 7 since silica formation is the quickest at neutral pH for this synthesis method [40,59]. BIS will not readily form outside the pH ranges of pH 5-9, hence we have focused on exploring drug loading under this pH range and monitored the effect of formulation pH on the drug release (Figure 3).

When silica was condensed at pH ≥7, the loading efficiency was not altered (remaining at ~20%, Figure 3A). When synthesis pH was more acidic, on the other hand, ibuprofen loading efficiency could be enhanced up to three times, to 60%, at pH 5. A similar picture was observed for the drug content (wt %) shown in Figure 3A. The release for samples formulated at pH ≤7 was similar (Figure 3C & Table S3), whereas DDS formulated at pH ≥7 had greatly diminished release. It should be noted that all release experiments were carried out in PBS at pH 7.2. Interestingly, despite the higher drug loading at pH5, there was not a correspondingly higher release observed when compared with DDS formulated at pH7 (Figure 3 B). Despite this, the

total ibuprofen (mg) released per weight of silica was 10 times higher for the pH 5 sample than MCM-41 (Figure 3D)

When release was plotted as a fraction of total release over time, two different release profiles became apparent (Figure 3C). BIS-PAH synthesised at pH ≤7 exhibited similar burst release profile observed for BIS samples reported above (also evident from high release rates, Table S3), where the majority of ibuprofen was released from the silica in the first 5 hours and very little was released after this. This burst release profile was similar to that seen for MCM-41, suggesting that the main mechanism for release in these systems was release from the surface. However, silica synthesised at pH >7 appeared to have a slow and sustained release profile, which was also reflected in slow release rates (Table S3). Release did not plateau for 24 hours and ibuprofen maintained a slow release over the course of the experiment. This slow release suggested that the loaded ibuprofen was embedded within the silica rather than bound to the surface, making release more prolonged. While the total amount of ibuprofen released from these samples under the 24 hour observation window was low, this system does show some promise as a prolonged release system.

It is clear from the results presented that the DDS formulation pH controlled the loading and release of ibuprofen. This could be caused by differences in porosity, morphology and/or additive-drug interaction. SEM results suggested that pH did not have a significant effect on the morphology or the particle sizes of DDS (Figure S 2A & B). When surface area and pore volume were measured for BIS-PAH DDS formulated at different pH conditions, there were no significant differences observed (Figure 3E & S4). The differences in ibuprofen loading in these systems can then likely be attributed to the ionisation of the three components present (silica, amine additive and drug) in the reaction mixture as well as the silica formation pathways. A scheme showing how the proportions of ionised reactants vary as the reactant pH is altered can be seen in Figure 4 and Table S4. The results here suggest that the negative

charge on silica can have an inhibitory effect on loading efficiency. Both the silica surface and ibuprofen are negatively charged at pH ≥7 (Table S4) and so silica and drug will repel one another, thus explaining low loading efficiencies at pH ≥7 (only ~20% of ibuprofen was loaded under these conditions, Figure 3A and Table S3). With DDS formulations prepared under acidic conditions, and particularly at pH5, the silica and ibuprofen both are significantly less charged, thus allowing for ibuprofen to be more efficiently loaded (30-60% of ibuprofen was loaded under acidic conditions, Figure 3A and Table S3).

It is clear that pH has a drastic effect on the loading efficiency of ibuprofen into BIS, with more acidic conditions resulting in increased loading. There is also strong evidence of an amine-drug interaction playing a major role in the ability of BIS to load drug. This interaction, when too strong, can also inhibit drug release.

3.4. Biocompatibility of BIS

Due to ease and non-invasive nature of administration, oral delivery of drugs is the most preferred route for patients [60]. Silica is an ideal material for oral drug delivery due to its stability under the conditions found in the GI tract, especially the low pH found in the stomach (pH1-3) and so it is able to protect loaded drug molecules from the changes in pH as well as degradative enzymes and bile salts [61,62]. While amine functionalisation is beneficial for drug loading and controlling release, exposure of amine-functionalised MS to cells has been reported to result in a higher cytotoxicity[40], higher level of plasma membrane damage, and higher haemolytic activity[52]. BIS were reported to be either non-cytotoxic, toxic only at extremely high concentrations, or when internalised into activated macrophages[40]. In order to further improve our understanding of BIS, it is important to uncover the fate of orally administered silica.

A simple and effective experiment was set up using sections of rat gut and measuring the movement of fluorescently tagged BIS-PAH (FITC-BIS-PAH) across the gut wall over an hour. FITC-BIS-PAH was synthesised using FITC-tagged PAH, so that its movement through the gut wall could be measured. We observed that ~22% of silica moved across the gut wall during the hour long incubation (Figure 5). This movement was through passive diffusion since it was not affected by the addition of an inhibitor of active transport (DNP). To further observe the movement of silica particles through the gut wall, fluorescence microscopy images of the inner and outer surfaces of the rat gut were taken (Figure 5). It is clear that when no silica is present there are no defined points of fluorescence but in the gut sections exposed to silica and silica with DNP, defined points of silica are observed. Silica was clearly localised on both sides of the gut wall, confirming its movement. Due to the ability of BIS-PAH to pass through the gut wall, it became important to investigate its biocompatibility with other cell types, particularly red blood cells (RBC).

The effect of BIS on RBC was determined through haemolytic activity of BIS, when exposed to red blood cells (RBC). Figure 6 shows that BIS-PAH had very low haemolytic activity, only lysing 2% of RBC at the highest concentration used (500 µg/ml) and only 0.6% lysis at the concentration which passed through the gut wall (~250 µg/ml). MCM-41 exhibited a higher haemolytic activity, rising to 10% at 500 µg/ml. The reasons behind this difference are initially unclear but may be related to the size of the particles. It has been reported that silica particle size affects haemolysis [63]. BIS-PAH particles were spherical (78±18 nm in diameter, S2A & B) and significantly smaller than the irregular MCM-41 particles used (3340±1013 nm in diameter), which could partly explain the difference in haemolytic activity between BIS and MCM-41. SEM data also show that although BIS primary particles were <100 nm, they form micron sized agglomerates and rapidly precipitate (hence DLS was not possible/useful). It is thus expected that BIS particles are as toxic as MCM-41 simply based on

their sizes, but this was not observed. Although further work is required on BIS to fully understand their biocompatibility, our present and previous results show that BIS are more biocompatible when compared to MS.

4. CONCLUSIONS

Our primary aim was to develop an in situ drug loading and release system using bioinspired silica (BIS). The BIS system can be controlled using many factors such as the choice of amine additive, pH of synthesis, kinetics of synthesis and eventual location of drug within the silica (Figure 7). Our results identified that the ideal formulation would be BIS-PAH synthesised with a reactant ratio of 2:2. Formulation under acidic pH was found to be suitable for designing DDS for faster targeted release, while basic pH was preferred for sustained release (Figure 7). Although a small portion of BIS-PAH was able to pass through the gut wall into the blood stream, due to its low haemolytic activity that does not appear to be an issue, in contrast to MCM-41. Ultimately, BIS appears to have several advantages over MCM-41 (such as one step formulation, simple controllability and lack of hazardous chemicals) and it was found that BIS has similar or improved drug loading and release profiles to MCM-41, in addition to superior biocompatibility. These benefits give BIS real potential as a viable DDS to be further investigated. We believe that the understanding of the DDS formulation using BIS that has emerged from this work can enable the discovery and development of a wide variety of DDS.

5. ACKNOWLEDGEMENTS

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6. SUPPORTING INFORMATION

Supporting information contains data for loading and release profiles, mathematical fitting of release data, release presented as % of final concentration and pore size distributions for BIS synthesised with different amines, with different reactant concentrations and at different pH. Also contains SEM images and particle size measurements and % ionisation data.

7. ABBREVIATIONS

BIS, Bio-inspired silica; BIS-PAH, bio-inspired silica synthesised with poly(allylamine hydrochloride); CTAB, hexadecyltrimethylammonium bromide; DDS, drug delivery system; DETA, diethylenetriamine; DLS, dynamic light scattering; DNP, dinitrophenol; D-PBS, Dulbecco's phosphate buffered saline; HPLC, high pressure liquid chromatography; MCM-41, Mobil Composition of Matter No. 41; MS, mesoporous silica; PAH, poly(allylamine hydrochloride); PAH-FITC, poly(fluorescein isothiocyanate allyamine hydrochloride); PBS, phosphate buffered saline; PEHA, pentaethylenehexamine; RBC, red blood cell; SBA-15, Santa Barbara Amorphous type material 15; SEM, scanning electron microscope; TEOS, tetraethoxysilane; TEPA, tetraehylenepentamine; UV, ultraviolet.

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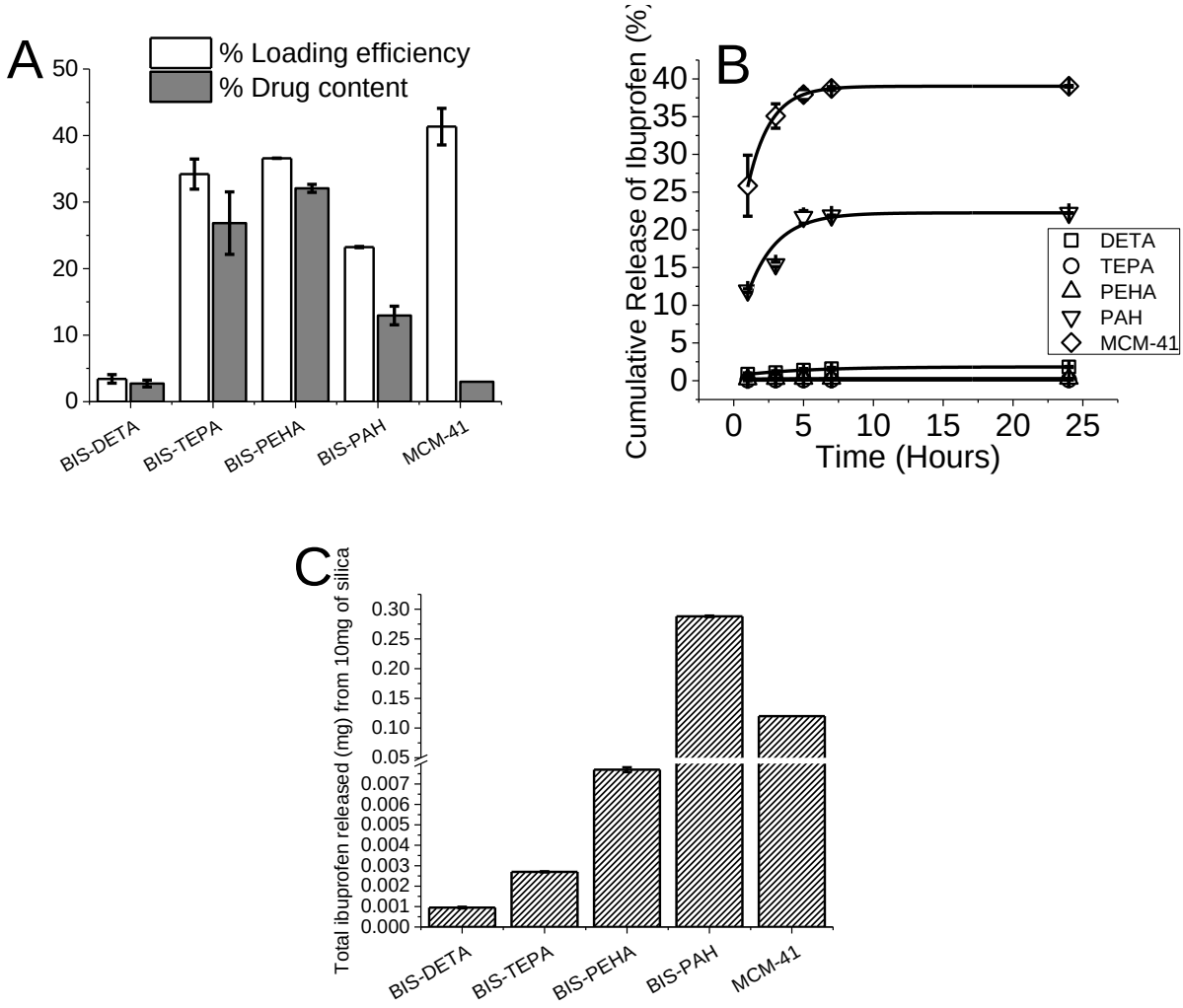
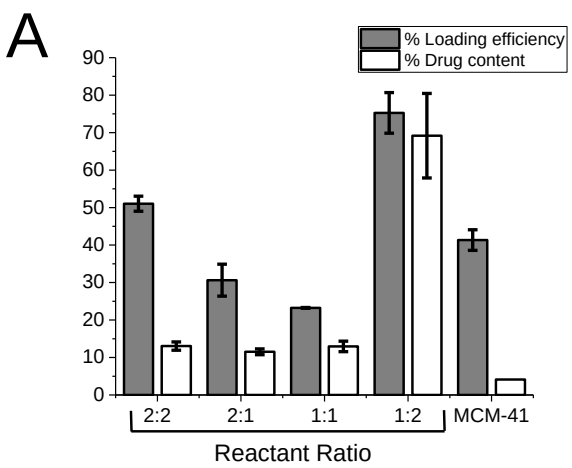
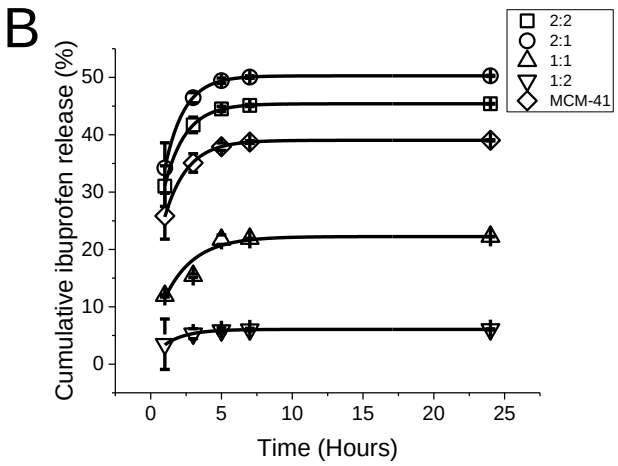
FIGURES AND TABLES

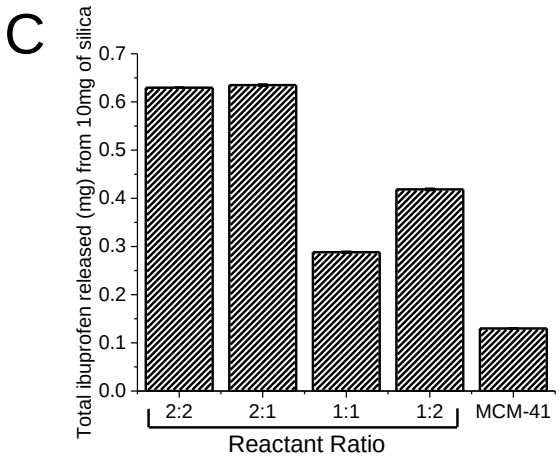


D	Surface area (m^2/g)	Pore volume (cm^3/g)	Pore size (nm)
BIS-DETA	16	0.095	*
BIS-TEPA	33	0.095	*
BIS-PEHA	36	0.131	*
BIS-PAH	129	0.91	23
MCM-41	983	0.74	2

Figure 1 :- BIS synthesised with different amines (A) % loading efficiency and % drug content (wt/wt) of ibuprofen loaded into four different BIS and MCM-41, (B) The % release of loaded ibuprofen from four different BIS and MCM-1, (C) Total mass of ibuprofen (mg) released from 10mg of silica sample, (D) Surface area, pore volume and pore size figures for four different BIS and MCM-41 (* due to broad pore size distributions, specific pore sizes are not applicable). For A, B and C, n=3, error bars represent one standard deviation. For D, n=1.

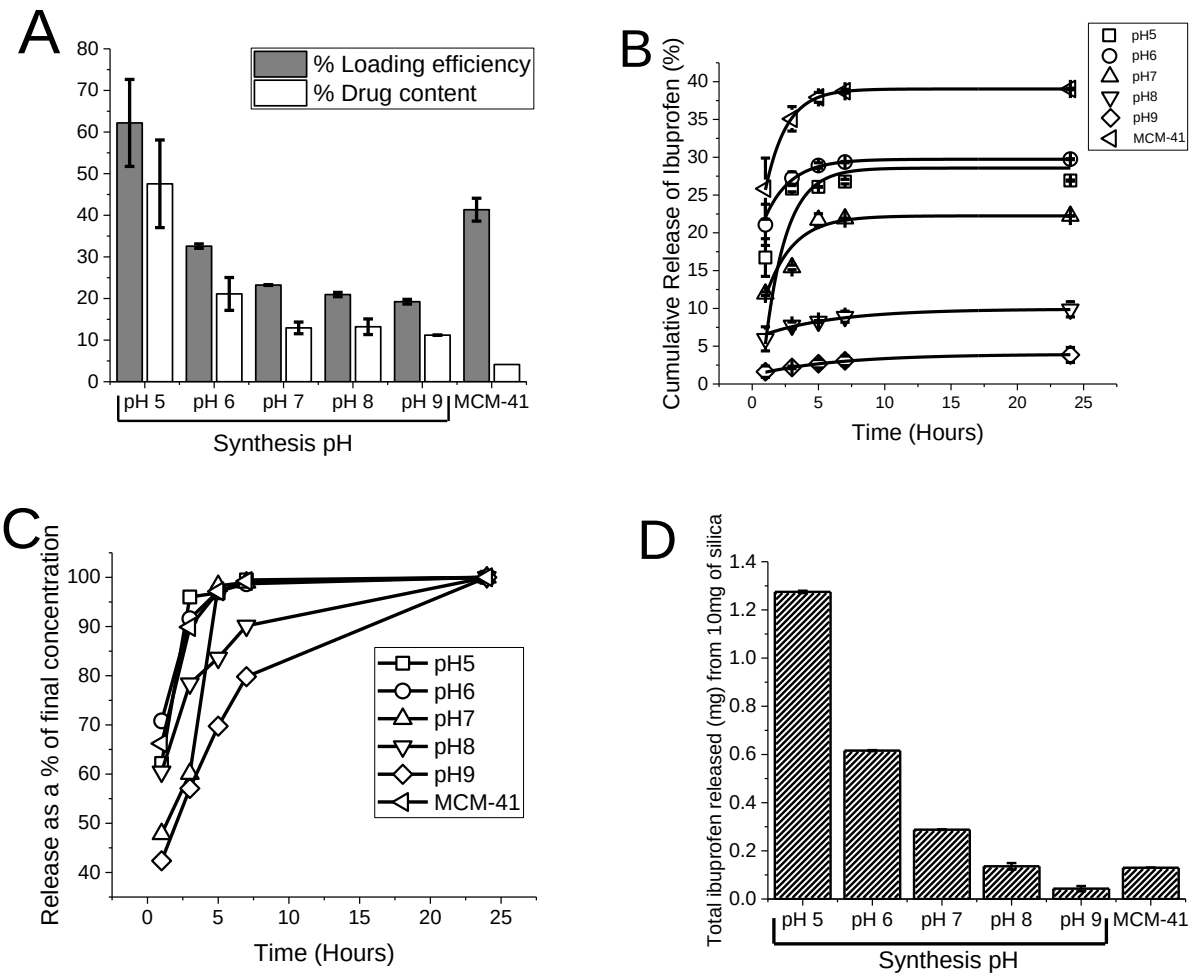






D	**Surface area (m^2/g)**	**Pore volume (cm^3/g)**	**Pore size (nm)**
2:2	157	0.73	15.9
2:1	180	0.531	11.3
1:1	129	0.91	23
1:2	76	0.69	28.94
MCM-41	983	0.74	2

Figure 2: - Effect of reactant concentrations on the loading and release profiles of ibuprofen (A) % loading efficiency and % drug content (wt/wt) of ibuprofen laoded into BIS synthesised with different reactant ratios and MCM-41 (B) The % release of loaded ibuprofen from four different BIS and MCM-41, (C) Total mass of ibuprofen (mg) released from 10mg of silica sample, (D) Surface area, pore volume and pore size figures for BIS synthesised with different reactant concentrations and MCM-41. For A-C, n=3, error bars represent one standard deviation. For D, n=1.



E	**Surface area** (m^2/g)	**Pore volume** (cm^3/g)	**Pore size** (nm)
5	142	0.60	21
6	149	0.67	21
7	129	0.91	23
8	161	0.84	25
9	140	0.68	25

Figure 3: - Effect of reaction pH on the loading and release profiles of ibuprofen (A) % loading efficiency and % drug content (wt/wt) of ibuprofen loaded into BIS synthesised at different pH and MCM-41, (B) The % release of loaded ibuprofen from silica, (C) Release of loaded ibuprofen expressed as a % of final concentration released from BIS synthesised at a range of pH, (D) Total mass of ibuprofen (mg) released from 10mg of silica sample, (E)) Surface area, pore volume and pore size figures for BIS synthesised at different pH and MCM-41. For A, B and D, n=3, error bars represent one standard deviation. For C and E, n=1.

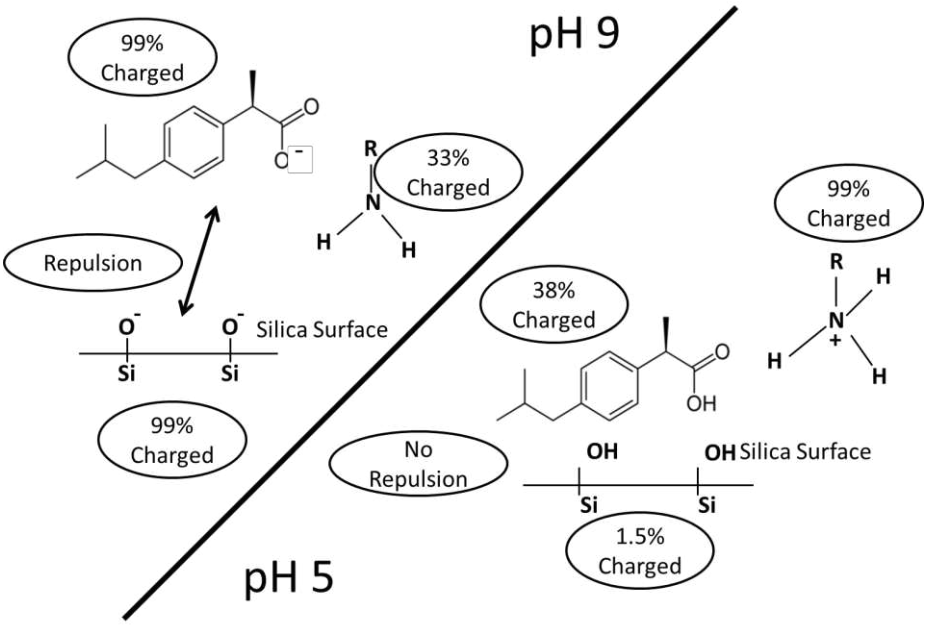


Figure 4:- Scheme to illustrate the differences in charge of silica, amine and ibuprofen during synthesis at pH ranging from 9 to 5.

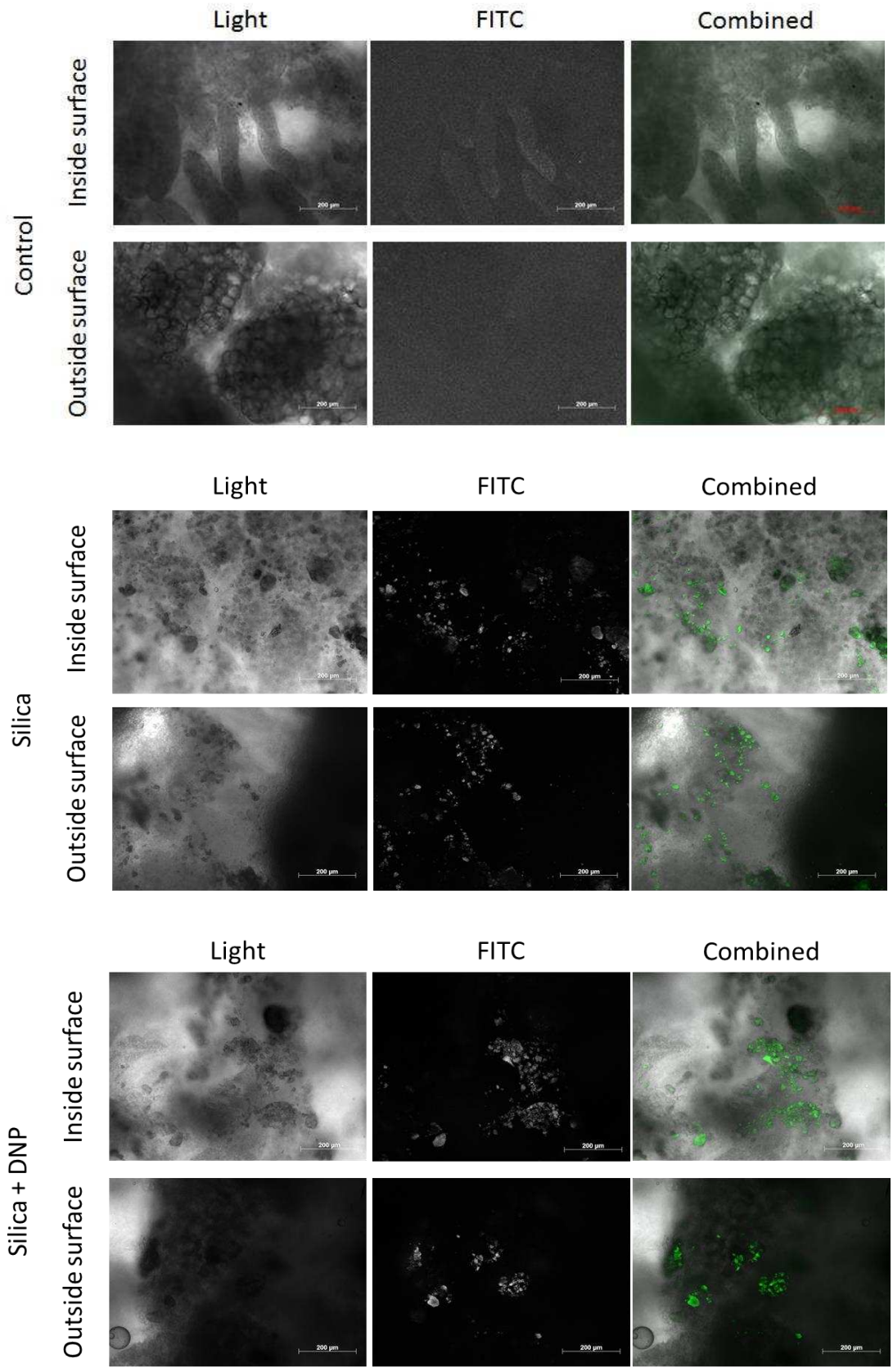


Figure 5:- Light and FITC microscopy images of the inside and outside surfaces of rat gut incubated with no silica (control), fluorescent silica or fluorescent silica and DNP. Images taken using Carl Zeiss Axio Imager Z1 with 10x/0.30 lens

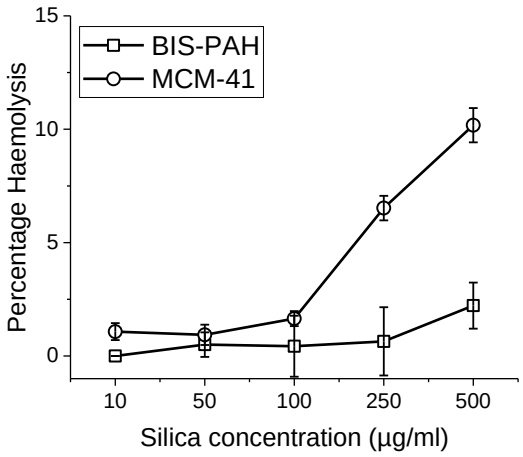


Figure 6:– Percentage haemolysis induced by varying concentrations of BIS-PAH and MCM-41 after 1 hour incubation with red blood cells. n=3, error bars represent one standard deviation

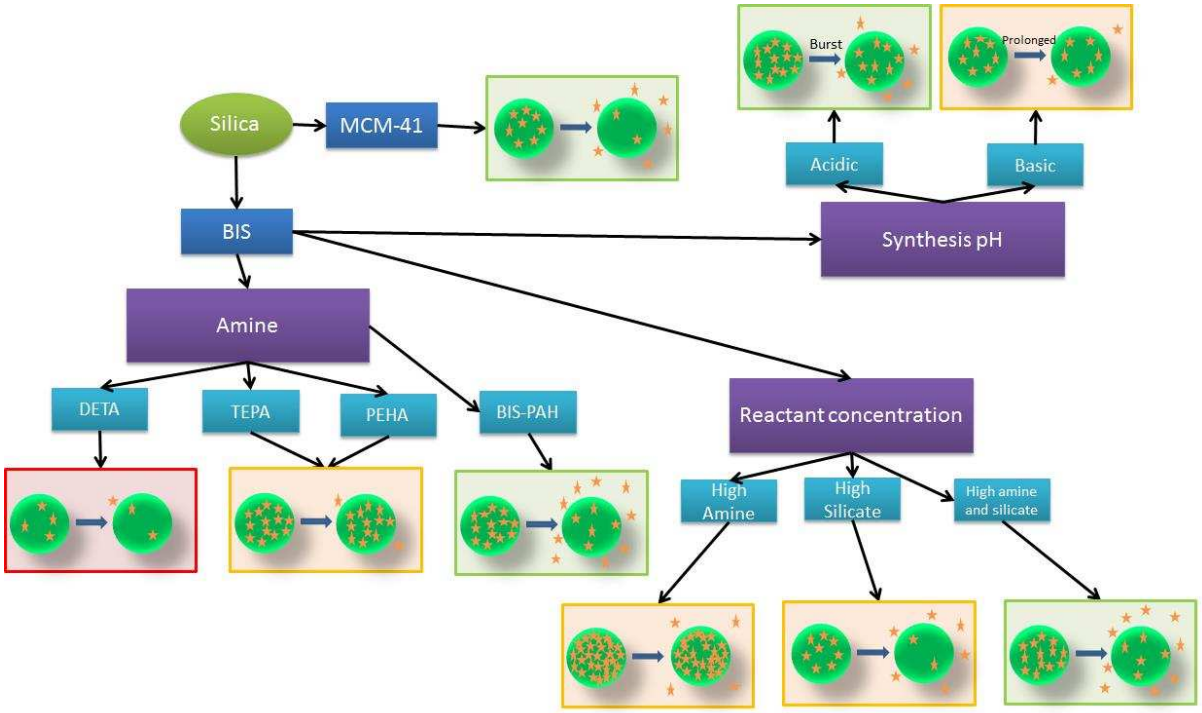


Figure 7:- Schematic summary of results presented in this paper.

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