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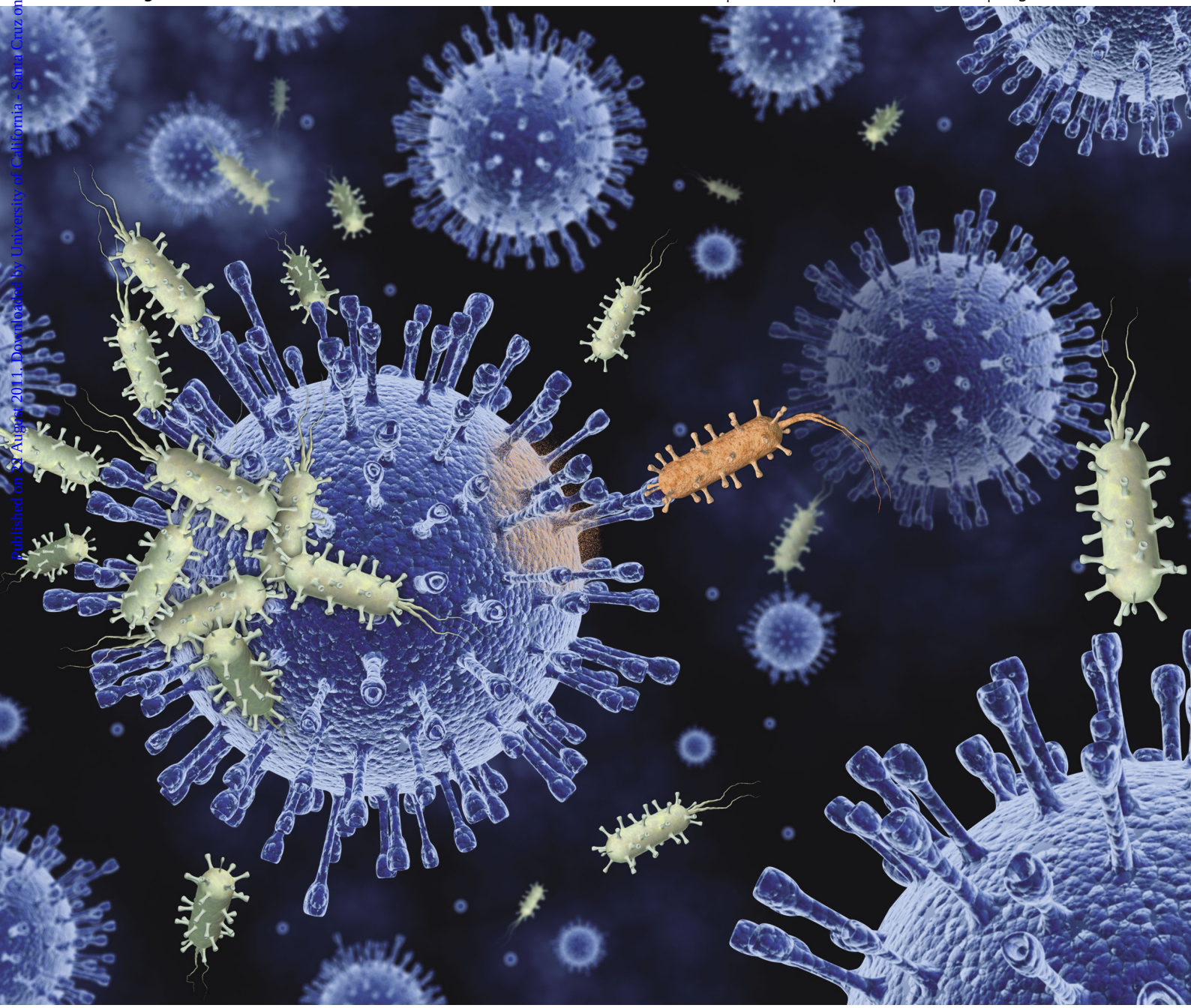

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COMMUNICATION

A facile one-pot sonochemical synthesis of surface-coated mannosyl protein microspheres for detection and killing of bacteria†

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We report a remarkably facile one-pot sonochemical approach to prepare protein microspheres whose shells are covalently decorated with a mannosyl derivative to target *Escherichia coli* (*E. coli*), while their cores are encapsulated with tetracycline. Conjugated microspheres induced the agglutination of *E. coli* and increased the anti-bacterial activity of the encapsulated tetracycline by five fold.

Protein microspheres have attracted increasing interest in recent years due to their potential applications in different bio-medical imaging modalities and drug delivery systems. Methods commonly used to prepare protein microspheres include spray- and freeze-drying, crystallization, calcium carbonate templating and emulsion polymerization.[1] Suslick and Grinstaff have developed a remarkably facile sonochemical technique to prepare gas- or liquid-filled protein microspheres from Cys-containing proteins, including bovine serum albumin (BSA), human serum albumin and hemoglobin.[2] Oxidation of the intermolecular protein Cys residues to disulfide bonds by the $HO_2^{\bullet}$ generated during sonochemical irradiation[3] was suggested to be responsible for holding the microsphere shells together.[4] Similar procedures have been used to prepare microspheres from Cys-less proteins[5] or even from the polysaccharide, chitosan.[6] Intermolecular interactions between the protein molecules and intermolecular imine formation between the β-D-glucosamine subunits in chitosan were suggested to assist the microsphere formation.[6]

Protein microspheres are highly biocompatible and non-immunogenic.[7] Thus, liquid-filled serum albumin microspheres have been used for various *in vivo* imaging applications[8] whereas air-filled microspheres have been approved for clinical use as contrasting agents (*e.g.*, Albunex) in echocardiography by FDA. In addition, the capability of these microspheres to encapsulate different therapeutics has been utilized for drug delivery to organs, such as lung and liver, where the microspheres tend to accumulate.[9] However, in order to be effective and selective as potential drug delivery carriers, the surface of the microspheres should be modified with vectors to target a specific cell type or a cell component. Surface-modified BSA microspheres have been previously prepared by a non-covalent electrostatic layer-by-layer (LBL) method.[10] The overall negative charge of the BSA surface, at physiological pH, was used as a template for electrostatic deposition of the positively charged polyelectrolyte, RGDKKKKKK (R = Arg, G = Gly, D = Asp, K = Lys), to target colon tumors over-expressing integrin receptors.[10]

Protein–carbohydrate interactions regulate many vital biological processes.[11] These interactions are however characterized by intrinsically low binding affinity that hampers the development of new therapeutics. To compensate for the low binding affinity of these interactions, nature usually uses a polyvalent presentation of the ligands and receptors on the cell surface.[12] Therefore, there is a growing interest in neoglycoconjugates as multivalent ligands for diverse biological applications.[13] Multivalent carbohydrate systems including polymers, dendrimers, nanoparticles and self-assembled supermolecular polymers have been demonstrated to bind and detect pathogens such as *Escherichia coli* (*E. coli*) that express the corresponding receptors.[14]

Targeting and detection of bacteria by surface-modified protein microspheres has not been reported yet. Here, we report on a versatile one-pot sonochemical method to covalently modify the surface of protein microspheres with a thiol-containing mannosyl derivative **3** *via* thiol/disulfide exchange reaction. The conjugated microspheres (CM) are formed through intermolecular disulfide exchange reaction between the mannosyl derivative **3** containing a thiol group and the BSA shell. These microspheres have a shell composed of mannosyl conjugated BSA that can bind and detect *E. coli* expressing mannose-binding lectin, and a core that can encapsulate different compounds—including tetracycline as a broad-spectrum antibiotic. The main advantages of this method over the LBL method are that it does not require the preparation of a charged targeting vector that may exhibit reduced biological activity. This is especially true in the case of a small molecule, such as mannose. Moreover, surface modification in this method is unrelated to the net charge of the protein and can be applied to any protein expressing either Cys or disulfide bonds. Most importantly, in contrast to the LBL method, the modification of the protein by the sonochemical approach is carried out under "one-pot" conditions.

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† Electronic supplementary information (ESI) available: Detailed experimental details, Table S1 and Fig. S1. See DOI: 10.1039/c1cc13518j

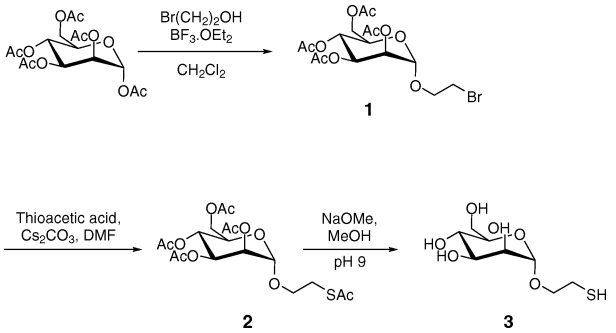

Fig. 1 Synthesis of thiol-containing mannosyl derivative.

The requisite *O*-linked mannosyl mercaptoethanol **3** was easily synthesized by glycosylation of 2-bromoethanol with peracylated mannose, using $BF_3{\cdot}OEt_2$, followed by reaction with thioacetic acid. The acetyl protecting groups of **2** were then removed by NaOMe in MeOH (Fig. 1).[15]

To generate the protein microspheres coated with the mannose derivative, an aqueous solution of **3** was mixed with different amounts of BSA (mole ratio BSA : **3** 1 : 1 to 1 : 1000) and over-layered by a vegetable oil or dodecane. The mixture was then subjected to a sonochemical irradiation for three minutes while cooling to produce microspheres filled with the organic co-solvent. The HPLC analyses of the aqueous phase before and after sonication suggest that more than 95% of **3** was incorporated into the microspheres in each preparation.

The optical microscopy and SEM images show that the microspheres are spherical with a smooth outer surface (Fig. 2a and b). The average diameter size of the conjugated microspheres measured by optical microscopy was 1.80 ± 0.5 μm (Fig. 2c). However, the SEM analysis of the microspheres (Fig. 2b) clearly indicated the presence of smaller particles that could not be observed by optical microscopy. The size of these microspheres is therefore somewhat smaller than those reported for the naked BSA microspheres and is expected to be suitable for *in vivo* applications.[2a,8] This is especially true for the small 100–200 nm

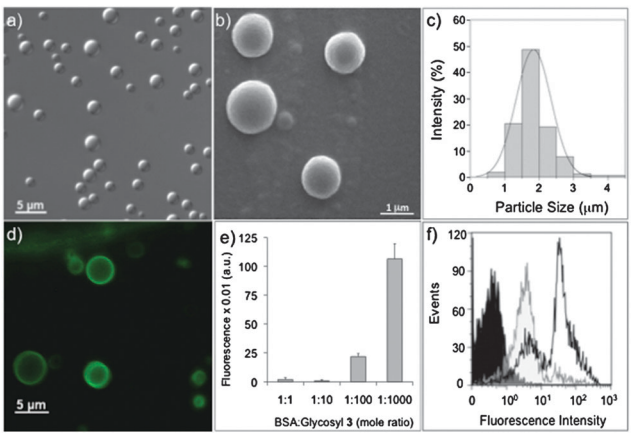

Fig. 2 Physical properties of mannosyl-CM. (a) Optical micrograph of the mannosyl-CM and (b) the corresponding SEM. (c) Size distribution analysis of the mannosyl-CM as measured with an optical microscope. (d) Fluorescence-microscopy images of the mannosyl-CM incubated with FITC-Con A. (e) Dose-dependent binding of FITC-Con A to mannosyl-CM generated from increasing amount of **3**. (f) The extent of binding of FITC-Con A to mannosyl-CM (black) and naked BSA microspheres (tinted gray) as studied by flow cytometry.

spheres detected in Fig. 2b. The diameter size and spherical shape of these microspheres may be advantageous over the linear or nano-sized structures as they mimic more closely the surface of the cells with which the bacteria interact.[16] Moreover, the microspheres exhibited extended stability in aqueous solutions over several months at 4 °C as their size and content did not change over the time.

Support for the core–shell structure of the microspheres and the presence of the bioactive mannosyl ligands on their surface was provided by fluorescent-microscopy studies. Conjugated microspheres were incubated with fluorescein labeled mannose-binding protein-concanavalin A (FITC-Con A) and visualized by confocal microscopy. The data demonstrated that FITC-Con A is evenly associated on the surface of the microspheres (Fig. 2d). This suggests that the mannosyl moieties present on the surface of the BSA microspheres retained their ability to interact with the carbohydrate-binding protein, Con A. Moreover, the binding of the FITC-Con A to the microspheres was found to increase with increasing amount of the mannosyl precursor **3** used for the microspheres preparation (Fig. 2e). The presence of mannosyl derivative on the microspheres surface and their bioactivity were further confirmed by fluorescence-activated cell sorting (FACS). Fig. 2f suggests that although FITC-Con A can bind non-selectively to the surface of the BSA-microspheres (tinted gray), it accumulates to an approximately 10 fold greater extent on the surface of mannosyl-CM (black line).

Having established that the mannosyl moieties on the BSA microspheres are capable of binding Con A, we have microscopically examined whether they can bind to the *E. coli* bacteria and cause their agglutination. Microspheres were incubated with either an *E. coli* strain expressing FimH mannose-binding protein (K-12) or with the *E. coli* isolate that is deficient of FimH (1313). Incubation of the mannosyl-CM with K-12 resulted in the formation of large bacterial clusters and their agglutination that could be easily detected by optical microscopy (Fig. 3 and Fig. S1, ESI†). In contrast, only negligible bacterial binding or aggregation was induced when the *E. coli* strain (1313) was incubated with the conjugated microspheres (Fig. 3b). This suggested that the mannosyl moiety **3** and its corresponding lectin are responsible for the specific binding of the bacteria to the microspheres and the eventual agglutination.

The selective binding of the mannosyl-CM with the bacteria encouraged us to evaluate the capability of these microspheres for targeted delivery of tetracycline (TTCL) as a broad-spectrum antibiotic. Among the promising new therapeutic approaches developed in recent years, targeted drug carriers have received increasing interest.[17] These carriers aim to release their payloads at the affected site, protecting the active substance from fast degradation and elimination, which leads to dose reduction and avoids side effects. In order to encapsulate TTCL within the mannosyl-CM, a solution of BSA and **3** in molar ratio of 1 : 1000 was over-layered with a solution of dodecane containing different amounts of TTCL (10 and 60 mg), and sonochemically irradiated in a one-pot reaction.[9a] Spectroscopic examination of the loaded TTCL suggested that about 60% of TTCL was encapsulated inside the mannosyl-CM—similar to that of BSA microspheres (Table S1, ESI†).[9a] Higher amounts of TTCL may be encapsulated within the BSA microspheres by dissolving increasing amounts of TTCL in the organic phase.[9a]

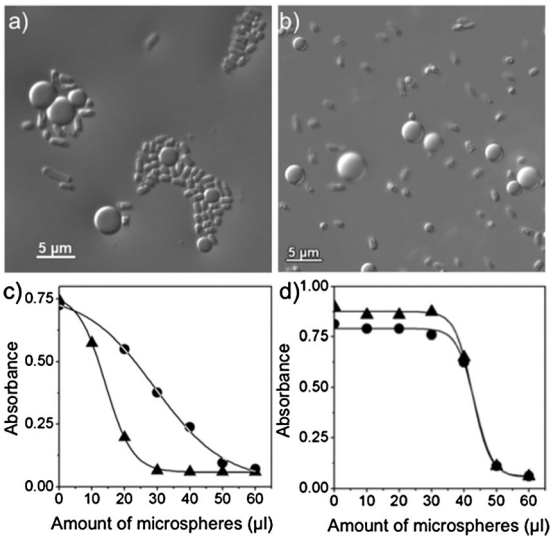

Fig. 3 Association of the mannosyl-CM with the *E. coli* strains and the corresponding antibacterial activity of the TTCL encapsulated microspheres. Optical micrographs of mannosyl-CM incubated with (a) FimH expressing *E. coli* strain K-12 and (b) the strain 1313 that is deficient of FimH protein. Antibacterial activity of TTCL-encapsulated mannosyl-CM (▲) and TTCL-encapsulated naked BSA microspheres (●) toward the *E. coli* strains (c) K-12 and (d) 1313. The concentration of TTCL in the mannosyl-CM and BSA microspheres was 0.11 and 0.12 mg ml^{-1}, respectively.

The antimicrobial activity of the TTCL-loaded BSA microspheres and loaded mannosyl-CM was then tested on the two *E. coli* strains, K-12 and 1313, using the broth microdilution assay.[18] The bacteria (1×10^5 cfu ml^{-1}) were incubated for 20 hours with different amounts of TTCL-encapsulated microspheres, and the bacterial growth was then monitored spectroscopically at 595 nm. The unconjugated BSA microspheres loaded with TTCL showed similar antibacterial activity (MIC, minimal inhibitory concentration of 7.2 μg ml^{-1}) against both the bacterial strains. This suggests that the BSA microspheres induce low selectivity to TTCL activity. In contrast, mannosyl-CM that selectively binds the *E. coli* strain K-12 (Fig. 3a) significantly increased the antibacterial activity of TTCL to the K-12 strain (MIC of 3.3 μg ml^{-1}), most likely due to the selective interaction of the microspheres with this strain. In the control experiments, TTCL-loaded mannosyl-CM failed to exhibit selective antibacterial activity toward the *E. coli* strain 1313 (Fig. 3d), confirming that the mannosyl derivative **3** is responsible for the selectivity. Interestingly, the MIC of free TTCL to both strains was found to be about 17 μg ml^{-1}. This indicates that TTCL alone is unable to differentiate between the two strains and encapsulation of TTCL to the microspheres preserved or even increased its antibacterial activity. Moreover, these data imply that the content of the microspheres is stable toward sonochemical irradiation and is likely to be fully released upon interaction with the bacteria.[9a]

In summary, sonochemical irradiation of an aqueous mixture of BSA and a thiolated mannosyl derivative can generate proteinaceous core–shells expressing the mannosyl groups covalently on their shells. The microspheres can easily encapsulate different compounds that are dissolved or suspended in the organic phase prior to the sonochemical process. The conjugated mannosyl groups preserved their biological activity as the microspheres could effectively interact with Con A protein. Moreover, the mannosyl-CM selectively bind and cluster *E. coli* strain containing the corresponding lectin. We have also shown that encapsulation of TTCL by the targeted microspheres significantly increased the toxicity of the drug to bacteria that express mannose-binding protein. This method could be easily generalized for the preparation of other microspheres containing different targetable ligands on their surface that can be applicable for selective drug delivery and imaging purposes. However, further experiments are needed to probe the toxicity, immunogenicity and pharmacokinetics of the conjugated microspheres in related animal models.

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