

Self-Healing Microencapsulation of Biomacromolecules without Organic Solvents**

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Modern synthetic polymeric biomaterials are widely used to slowly release medicines over days to years after administration to the body.^[1] These polymers are configured in numerous biomedical and pharmaceutical forms (spheres, rods, coatings, porous matrices), including micrometer-to-millimeter-scale injectable depots,^[2] drug-eluting stents,^[3] scaffolds for engineering tissues,^[4] and blood-circulating nanometer-scale particles^[5] and can be made biodegradable or nondegradable. Until now, drugs, particularly peptides and proteins, have most commonly been microencapsulated by first combining the drug with a polymer dissolved in an organic solvent.^[6] Before or after this combination step, the drug is either micronized (e.g., by homogenization, sonication, or grinding) or molecularly dissolved in the solvent to yield drug domains that later become dispersed in the final polymer matrix (see Figure S1a in the Supporting Information).^[6] Both steps can compromise the stability of encapsulated proteins^[6] and other biomacromolecules.^[7] The organic solvent is removed to clinically acceptable levels and the polymer dried before use.

Described herein is a novel microencapsulation paradigm (see Figure S1b in the Supporting Information) for controlled release based on the spontaneous capacity of the polymer to undergo self-assembly of its chains to heal tiny polymer holes or defects in aqueous media.^[8] Key features of this new approach include a simple mixing process (the mixing of naked DNA with lipofectin gene delivery vector),^[9] lack of exposure of the biomacromolecule to an organic solvent during encapsulation (supercritical-fluid polymer process-

ing),^[10] and mild processing conditions (spray congealing for the manufacture of PLGA-encapsulated growth hormone (PLGA = poly(lactic-co-glycolic acid))).^[11]

To test the concept, we prepared self-healing microencapsulating (SM) depots of biocompatible copolymers of lactic and glycolic acids (PLGA) by a standard emulsion-based method with leachable trehalose to create an interconnected porous network in the polymer, but without a drug (SM-1, Table S1 in the Supporting Information). After sugar leaching, pores of 30–3000 nm in diameter were visible (Figure 1a). The dry microspheres were incubated at 4°C (which is lower than the glass transition temperature of the hydrated polymer ($T_g \approx 30^\circ\text{C}$))^[12] in a concentrated lysozyme solution to enable protein entry into the open polymer pores. Pore healing was initiated without an organic solvent by raising the temperature above the T_g value (SM-1, Table S2 in the Supporting Information). The resulting lysozyme-encapsulated microspheres had a protein loading of $3.8 \pm 0.1\%$ (w/w protein/polymer matrix) and a nonporous polymer surface (Figure 1b).

Biomacromolecules penetrate deep within the polymer matrix, as observed in confocal micrographs of healed SM microspheres prepared with fluorescent coumarin–bovine serum albumin (BSA) (Figure 1c). Dextrans as large as 2 MDa were encapsulated (see the Supporting Information). Protein loading, which was determined after extensive washing of the healed polymer, was readily adjustable, as seen by the sensitivity of the BSA (Figure 1d) and lysozyme loading (Figure 1e), respectively, to the initial concentration of the protein-loading solution (SM-2, Tables S1 and S2) and polymer porosity (SM-3, Tables S1 and S2). To test the encapsulation quality, SM microspheres prepared under several different sets of conditions were loaded with protein and incubated under physiological conditions for 48 h to investigate the “initial burst release” of protein (Figure 1f), which is undesirably high if encapsulation is incomplete.^[2b] SM microspheres with an elevated protein loading of between 1.2 ± 0.1 and $9.8 \pm 0.3\%$ and an optimal porosity loading ($1.5\text{--}4.5\%$ (w/w magnesium carbonate/polymer matrix)) typically exhibited an initial burst release of protein below 20%. Importantly, the loading and initial-burst values were within the desirable range as established by clinically used PLGA depots,^[2b] and the required loading time was approximately 12 h (Figure 2a).

Spontaneous self-healing in homogenous polymer systems has been described in nanoscale cracks of solid rocket propellants, following the creation of bullet holes in plastic plates, during film formation from latex particles, and across lap joints of polymer films.^[8a,13] The process mechanism,

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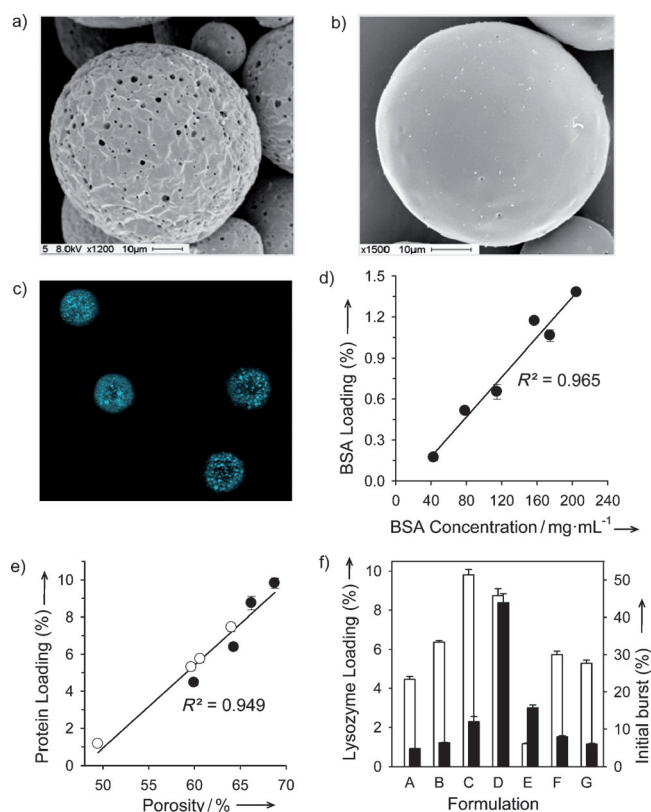


Figure 1. Self-healing microencapsulation (SM) of biomacromolecules in PLGA microspheres (SM-1, SM-2, and SM-3 in Tables S1 and S2 in the Supporting Information). a,b) Scanning electron micrographs of microspheres (SM-1) before (a) and after SM (b). c) Laser confocal fluorescence micrograph of the cross-sectional distribution of BSA–coumarin microencapsulated microspheres (in the white domains; 20–63 μm microsphere diameter; SM-2). d) Graph showing the increase in polymer protein loading at increasing concentrations of the protein in the loading solution (SM-2). e) Graph showing the increase in polymer protein loading at increasing microsphere porosity due to an increasing volume of the inner water phase (WP; 25, 100, 200, and 350 μL , open circles) or an increasing porosigen content (0, 1.5, 4.3, and 11 % w/w MgCO_3 , closed circles; SM-3). f) Lysozyme loading (white bars) and initial burst of the enzyme (black bars) as a function of porosigen loading (0 (A), 1.5 (B), 4.3 (C), and 11 % w/w MgCO_3 (D)) and WP volume (25 (E), 100 (F), and 350 μL (G)). Values in (d–f) are the mean $\pm$ standard error of the mean (s.e.m.); $n=5$.

which is common to polymers in the vicinity of their T_g value or above,^[8a] has been analyzed in detail and found to involve multiple elements,^[8a,13] including polymer-chain interdiffusion driven by minimization of the energetically unfavorable interfacial area and/or transfer of potential energy stored in the defect. We first observed spontaneous pore closing on the surface of peptide-containing PLGA microspheres during the initial peptide release shortly after exposure of the polymer to physiological conditions;^[8b] the resulting closure of the pores and peptide release route initiated a lag phase in release characteristic of this polymer above a critical molecular weight.^[14] Consistent with previous mechanistic analysis, the healing of PLGA pores requires a minimum temperature ($T > T_g$) for polymer-chain mobility to occur over reasonable

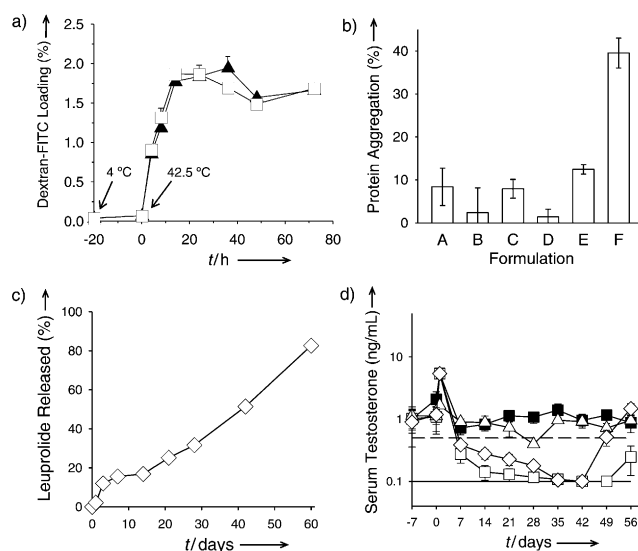


Figure 2. PLGA self-healing microencapsulation (SM) minimizes bio-macromolecule instability and enables long-term slow release in vivo. a) Kinetics of SM (initiation of self-healing at $T > T_g$) and effect of the prehydration of microspheres (SM-3; incubation at 4 $^{\circ}\text{C}$ for 20 h and then at 42.5 $^{\circ}\text{C}$ ($t=0$ h) with dextran–FITC (65 mg mL^{-1})). Squares and diamonds represent results from two separate batches of identical SM microsphere formulations. b) Extent of the aggregation of the protein into insoluble aggregates after SM of encapsulation-labile lysozyme (formulations A–D) and after standard solvent-evaporation microencapsulation (formulations E and F). Microspheres were prepared from PLGA with an M_w value of 11 (A,B) or 51 kDa (C–F) in the presence (B,D,F) and absence (A,C,E) of 0.45 M sucrose in the aqueous lysozyme solution (A and B: SM-4; C and D: SM-3; E and F: TM-1; Tables S1 and S2 in the Supporting Information). c) In vitro release characteristics of leuprolide acetate (LA) from SM microspheres. d) Ability of LA-containing SM microspheres to suppress serum testosterone in vivo (SM-5). Filled squares, open diamonds, open squares, and open triangles correspond to soluble leuprolide (1-month dose at day 0), LA-containing SM microspheres (2-month dose at day 0), commercial Lupron Depot (1-month dose at days 0 and 28), and SM microspheres without LA (once at day 0), respectively. LA dose was 100 $\mu\text{g/kg/day}$. Solid and dashed lines indicate the lower limit for the detection of testosterone (0.1 ng mL^{-1}) and the castration level (0.5 ng mL^{-1}), respectively. All values are the mean $\pm$ s.e.m.; $n=3$ (a), 5 (b and c), or 6 (d). FITC = fluorescein isothiocyanate.

time scales, and the high PLGA/water interfacial tension^[15] provides a driving force for polymer-chain self-assembly.

As expected on account of the mild SM conditions (37–43 $^{\circ}\text{C}$, no harsh mixing or exposure to an organic solvent), protein stability was also improved with SM microspheres relative to that observed with microspheres prepared by traditional emulsion-based solvent evaporation.^[2b] We used lysozyme, which is well-established to undergo aggregation during solvent evaporation,^[16] to evaluate the potential improvement in enzyme stability in SM microspheres relative to that observed for solvent-evaporation control groups. In formulations of two polymers with different molecular weights with and without protein-stabilizing sucrose, the stability of lysozyme was improved with SM microspheres in each case (Figure 2b). Furthermore, when the proteins were loaded by healing with sucrose, negligible aggregation or

activity loss of the enzyme was detected (see the Supporting Information).

To demonstrate *in vivo* controlled release, we loaded leuprolide acetate (which is used to suppress testosterone in prostate-cancer patients to inhibit growth of the hormone-dependent cancer) into SM PLGA microspheres and used ZnCO_3 to create pores for healing and to facilitate continuous release of the peptide.^[17] The resulting SM microspheres (see Figure S2 in the Supporting Information) encapsulated the peptide with a loading of $3.0 \pm 0.2\%$ (w/w peptide/polymer matrix) and exhibited continuous controlled release *in vitro* for 2 months (Figure 2c). After the administration of a single injection of the formulation in rats, steady suppression of testosterone was observed (Figure 2d), owing to down-regulation of the LHRH receptors,^[18] until the concentration of testosterone rose above castration levels after 6 weeks. Similar behavior was seen after two monthly doses of Lupron Depot formulation, whereas SM microspheres without leuprolide acetate and a 1 month dose of leuprolide solution were ineffective in suppressing testosterone. The common proteins BSA and lysozyme were also released slowly (see Figure S3 in the Supporting Information), without the classic acid-induced aggregation of BSA^[19] and with full recovery of the monomeric and enzymatic activity of lysozyme in the polymer after release incubation for 1 month (see the Supporting Information).

The ultimate success of the microencapsulation of expensive biotechnological drugs requires minimal drug loss during encapsulation. In a single-batch process with SM microspheres, the encapsulation efficiency (EE) was low (ca. 1.5–13%) for the passive process. However, as minimal or no peptide or protein damage occurs upon polymer self-healing, the loading solution could reasonably be recycled multiple times with concentration adjustment. A similar issue was resolved in the marketed Doxil stealth liposomes by the active (or remote) loading of doxorubicin through precipitation of the drug with ammonium sulfate as it diffused into the empty liposome.^[20]

We investigated similar active-loading strategies with two vaccine antigens, ovalbumin (OVA) and tetanus toxoid (TT). OVA or TT protein antigens were loaded into SM PLGA containing lyophilization-stabilized $\text{Al}(\text{OH})_3$ adjuvant^[21] (ASM, Table S1 in the Supporting Information). The antigens were absorbed from the surrounding solution ($0.5\text{--}1\text{ mg mL}^{-1}$ in the antigen) and stabilized in the polymer matrix, with up to 87–98% EE and a loading of 1.0–1.6% OVA (ASM-3, Tables S3 and S4 in the Supporting Information) or TT (ASM-2, Table S5 in the Supporting Information). The extent of self-healing of the polymer pores was also readily enhanced by the addition of common plasticizers, such as diethyl phthalate (DEP; see Figure S4 in the Supporting Information). After self-healing, the surface porosity of the microspheres was visibly decreased in the presence of a plasticizer (Figure 3b). Vaccine antigens were microencapsulated effectively in both preparations with the active-loading strategy, as indicated by the slow release of the antigens relative to that of their unencapsulated counterparts on $\text{Al}(\text{OH})_3$ in aluminum-gel-dissolving (190 mM sodium citrate; see Figure S5 in the Supporting Information) and release buffers (phosphate-

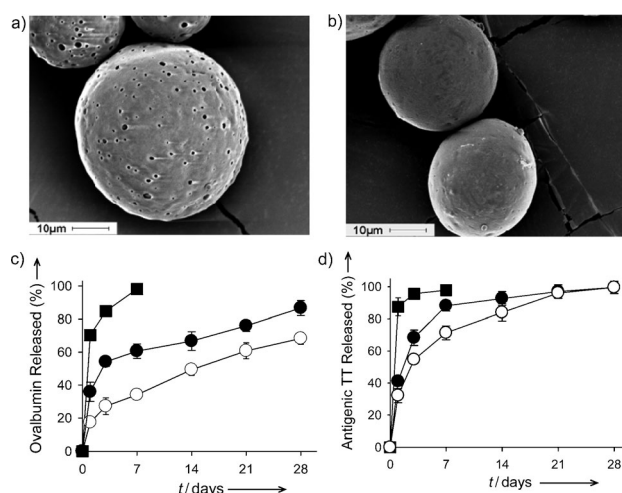


Figure 3. Efficient active self-healing microencapsulation of ovalbumin (OVA) and tetanus toxoid (TT) in $\text{Al}(\text{OH})_3$ -PLGA microspheres (ASM, Tables S1–S5 in the Supporting Information) enables long-term protein-antigen stabilization and release. a,b) Scanning electron micrographs of microspheres loaded with 0 (a; ASM-1) and 5% (b; ASM-3) diethyl phthalate (DEP) after active microencapsulation of OVA. c,d) Controlled release of monomeric OVA (c) or antigenic TT (d) from self-healed microspheres relative to release from OVA- or TT-loaded $\text{Al}(\text{OH})_3$ without PLGA (filled squares, $\text{Al}(\text{OH})_3$ without PLGA; filled circles, DEP-free microspheres (ASM-1); open circles, microspheres loaded with 5% DEP). Values are the mean $\pm$ s.e.m.; $n = 3$.

buffered saline, PBS; Figure 3c), and in a buffer composed of PBS with 0.02% Tween 80 and 0.2% BSA (Figure 3d). Hence, it is now possible to microencapsulate in biodegradable polymers essentially the entire mass of bioactive macromolecules from a low-concentration aqueous solution by simple mixing and heating of the solution with an SM-polymer matrix. Also significant were a) the lack of requirement of a drying step after the microencapsulation of OVA and TT, as drying can cause irreversible damage to proteins,^[6] and b) the complete release of antigenically active TT without commonly observed formaldehyde- or acid-induced antigen instability.^[6]

The potential of the SM paradigm is far-reaching. Imagine, for example, that a clinician in developing countries could mix sterile SM microspheres with an injectable solution of a vaccine (e.g., tetanus toxoid) before injecting them into women of child-bearing age to provide improved immunity for their unborn children against neonatal infection.^[22] Consider the potential for new biomaterial architectures (e.g., drug-eluting stent coatings) that release process-sensitive large molecules: previously uncharted formulation conditions (e.g., high temperature, reactive molecules, organic solvent) could now be used to create the SM-polymer delivery system without concerns about damaging the encapsulated macromolecule. For manufacturing, the rules would also be very different. It is fascinating to consider the possibility of combining a mixture of several different SM-microsphere formulations, each with distinct design characteristics (release kinetics, size, surface biofunctional groups), for the drug of interest in a single sterile mixing step. Owing to the absence of aseptic processing of organic solvents, this strategy could have

significant cost savings. This expense was a significant factor in halting the production of the Nutropin Depot, the first and only FDA-approved injectable controlled-release protein depot.^[23] It is also conceivable that the simplicity of self-healing microencapsulation may significantly facilitate the study of controlled-release approaches by researchers who are not formulation scientists and thus enable the more rapid advancement of controlled-release technology.

Further necessary studies are in progress, in particular a) to develop additional active microencapsulation approaches for important therapeutic proteins, b) to increase loading capacity in active SM microspheres and minimize initial burst release, c) to expand the technology to additional delivery platforms, polymers, and biomacromolecule classes, and d) to optimize stability and long-term release kinetics. Through these initiatives, a new class of self-healing microencapsulating polymers can indeed be envisaged.

In conclusion, biomacromolecules up to 2 MDa in size were microencapsulated in PLGA by placing the aqueous biomacromolecule solution in contact with a solid polymer that had been preformed with an interconnected pore network at below the T_g value and then healing the pores at $T > T_g$. Our results demonstrate that the healing of PLGA depots obviates micronization- and organic-solvent-induced protein damage, leads to the stabilization and slow release of the labile tetanus protein antigen, and enables the long-term in vivo release of encapsulated leuprolide. We also found that the introduction of protein-binding stabilizers in the PLGA pores enabled microencapsulation of the entire protein in a single aqueous mixing step. Self-healing microencapsulation could be reasonably applied to additional delivery platforms (drug-eluting stents, tissue-engineering scaffolds, nanoparticles that circulate in the blood), biomacromolecules, and biodegradable polymers.

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