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Exploiting the structural metamorphosis of polymers to 'wrap' micron-sized spherical objects

Patrick L. Higgs,[a] Jordan L. Appleton,[a] W. Bruce Turnbull[b] and David A. Fulton*[a]

[a] Dr. P. L. Higgs, J. L. Appleton, Dr D. A. Fulton
Chemistry-School of Natural and Environmental Sciences,
Bedson Building,
Newcastle University, Newcastle upon Tyne,
NE1 7RU (UK).
E-mail: david.fulton@ncl.ac.uk

[b] Prof. Dr. W. B. Turnbull
School of Chemistry and Astbury Centre for Structural MolecularBiology,
University of Leeds,
Leeds,
LS2 9JT (UK).

Abstract

There is growing interest in developing methods to 'wrap' nano- and micron-sized biological objects within films that may offer protection, enhance their stability or improve performance. We describe the successful 'wrapping' of lectin-decorated microspheres, which serve as appealing model micron-sized objects, within cross-linked polymer film. This approach utilizes polymer chains able to undergo a structural metamorphosis, from being intramolecularly cross-linked to intermoleculary cross-linked, a process that is triggered by polymer concentration upon the particle surface. Experiments demonstrate that both complementary molecular recognition and the dynamic covalent nature of the crosslinker are required for successful 'wrapping' to occur. This work is significant as it suggests that nano- and micron-sized biological objects such as virus-like particles, bacteria or mammalian cells—all of which may benefit from additional environmental protection or stabilization in emerging applications—may also be 'wrapped' by this approach.

Keywords: dynamic covalent polymers, structural metamorphosis, supramolecular, polymer wrapper

Introduction

Crosslinked polymer films are ubiquitous in the modern world, acting as barriers to protect objects from their environments, providing stabilization or improving performance.[1] Scientific advances are continually leading to the development of new nano- and micron-sized biological objects that may also benefit from their inclusion within crosslinked polymer films able to act as a protective 'wrapper'. For example, virus-like particles (VLPs) are nanoscale components of vaccines[2] that often lack robustness towards temperature, pressure and humidity,[3] and methods to 'wrap' them may improve their stabilities in hot or dehydrating conditions.[2b] Engineered bacteria, such as those able to produce biofuels, can also suffer from stability issues, and their inclusion within a 'wrapper' that helps to maintain membrane integrity may also improve their performance.[4]

There are numerous existing approaches to wrapping objects in polymer chains. Electrostatic layer-by-layer approaches in particular are a well-established approach to wrap objects within polymer films.[5] This process exploits electrostatic interactions between a charged surface and an oppositely charged polyelectrolyte; further layers can then be built up by alternating the charge of the polyelectrolyte. Issues with this approach can be the destabilizing effect of polyelectrolytes upon biological surfaces, especially lipid membranes, and can lead to issues of cytotoxicity when applied to live cells. Nevertheless, the approach has been successfully applied, by e.g. being used[6] to coat

platlets (red blood cells), showing that the inclusion of cells within a coating can be harnessed to present novel approaches to manipulate their biological functions. Covalent grafting approaches, where polymer chains are either grafted onto or grown from surfaces has also become an established approach to stabilize particles and bioobjects.[7] Although effective, this approach does rely on the application of high yield and biorthogonal chemical transformations to be effective. Furthermore, the irreversible nature of chemical conjugation can result in an undesired structural change that may alter the fundamental properties of an object.

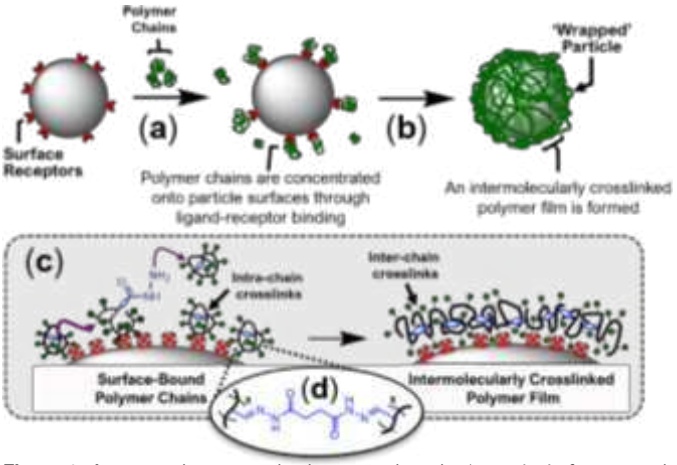


Figure 1: A structural metamorphosis approach to the 'wrapping' of nano- and micron-sized objects. (**a**) Polymer chains bind through specific molecular recognition to receptors located upon the object surface. (**b**) Now spatially close to one another, polymer chains undergo intra- to intermolecular crosslinking with their neighbouring chains to form a crosslinked polymer film upon the particle surface. (**c**) Film formation is driven by the conversion of intra-chain crosslinks into inter-chain crosslinks. This process relies upon the ability of dynamic covalent acyl hydrazone bonds (**d**) to undergo component exchange process.

To address the challenge of 'wrapping' nano- or micron-sized objects, we propose a structural metamorphosis approach. A structural metamorphosis can be defined as a transition between two discrete structures where components are neither added or taken away; only the interconnectivity of the building blocks is changed at the molecular/supramolecular level, facilitating a switch from one architecture to another. Work in this area is relatively new, and examples of metamorphosis that exploit non-covalent or dynamic covalent bonds have been reported.[8]

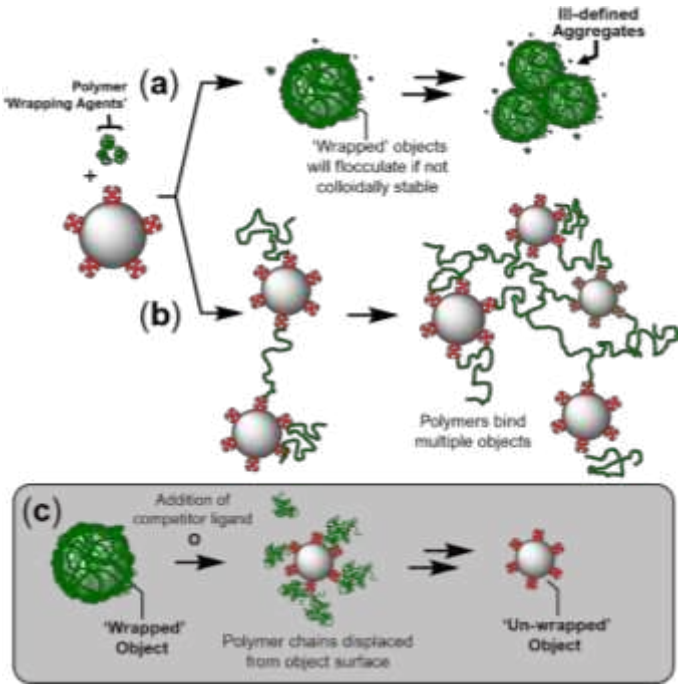
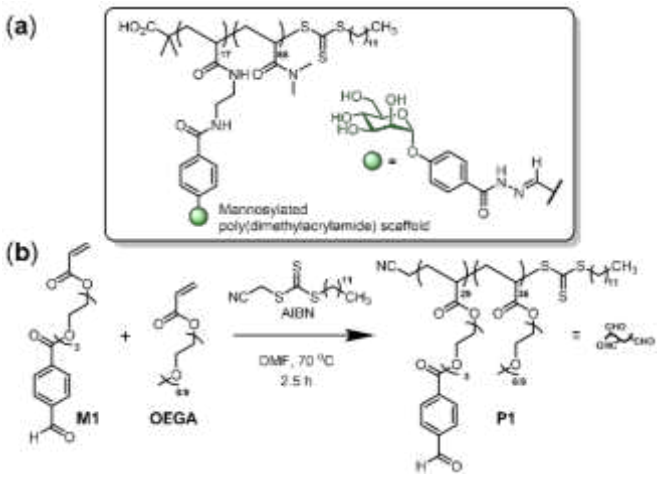


Figure 2: The challenges of 'wrapping' nano- and micron-sized objects. (**a**) The 'wrapped' objects flocculate to form unwanted crosslinked aggregates. (**b**) Polymer chains bind to multiple objects, again leading to the unwanted formation of aggregates. (**c**) Control experiment in which the polymer 'wrapping' is displaced from the particle by addition of a competitor ligand which binds receptors upon the object surface.

In our approach to wrapping, the intramolecularly cross-linked polymer chains have been designed to be kinetically stable at relatively low concentration *i.e* there is a kinetic barrier that presents their aggregation and subsequent cross-linking. When concentrated upon a surface (Figure 1a), however, they are spatially close to one another and the kinetic barrier is eliminated, thus allowing the polymers to undergo intra- to inter-chain crosslinking. Thus, in our approach the trigger for the metamorphosis is a change in concentration. Surface concentration can be driven by molecular recognition between ligands displayed upon the polymer and complementary surface receptors. The structural metamorphosis involves intramolecularly crosslinked polymers transforming into intermolecularly cross-linked polymer film (Figure 1b). The change in interconnectivity exploits (Figure 1c) the component exchange properties of dynamic covalent acyl hydrazone bonds.[9] These well-known bonds can exchange their components (Figure 1d) to form new bonds, and this molecular-level process facilitates intra- to inter-polymer chain crosslinking, driving polymer film formation.

The successful application of this metamorphosis approach to 'wrap' a dispersion of nano- or micron-sized objects, however, presents some considerable challenges. After successful 'wrapping', it is important that the objects do not subsequently flocculate as this would likely lead to further component exchange process involving the dynamic covalent linkers, leading to the formation of an ill-defined aggregate bound together by crosslinked polymer chains (Figure 2a). Polymer chains must also not bind multiple objects, otherwise the outcome of the experiment may also tend towards the formation of aggregates (Figure 2b). This unwanted outcome can be minimized by the elimination of non-specific interactions between polymer and object and the careful choice of experimental conditions, in particular the polymer: object stoichiometries and concentrations. The elimination of non-specific aggregation between polymers and objects is also required for what we consider to be the key experiment (Figure 2c) to prove that successful 'wrapping' has occurred. This experiment requires that excess small molecule competing ligand be added in an attempt to displace the bound polymer chains from the surface of the object. If the polymer chains cannot not be displaced then it suggests they have successfully crosslinked with one another; if they are displaced, then crosslinking between chains has not occurred, and the 'wrapping' process has therefore not been successful. Unwanted non-specific interactions between polymer and object may lead to polymers remaining bound to the object even if they have not crosslinked with one another to form a film, thus making it difficult to ascertain if the intra- to interpolymer crosslinker rearrangement has occurred successfully.

Here, we describe a polymer 'wrapping agent' that lowers non-specific binding effects and thus successfully 'wrap' a micron-sized object. This polymer was based upon a poly(oligoethylene glycol acrylate) (p(OEGA)) scaffold, that on account of its outstanding hydrophilicity does not participate in non-specific binding, minimizing the unwanted outcomes described in Figure 2 and thus helping to ensure effective 'wrapping' occurs.



Scheme 1: (**a**) Mannose-decorated polyacrylamide used in unsuccessful preliminary studies, presumably on account of being overly hydrophobic. (**b**) Preparation of hydrophilic aldehyde-functionalized copolymer scaffold **P1**. The oligo(ethylene glycol) side chains provide exceptional hydrophilicity.

Results and Discussion

As a stepping stone towards the 'wrapping' of nano- and micron-sized biological objects we focused on 'wrapping' spherical silica microparticles with diameters of approximately 5 µm, as these provided a readily-available low-cost target with which to develop the 'wrapping' chemistry. Silica microparticles can be easily functionalized with protein receptors that provide a means for polymer chains decorated with complementary ligands to concentrate upon their surfaces. Their relatively large size also means they can be visualised easily by fluorescence and optical microscopy. This feature circumvents a reliance upon dynamic light scattering techniques, where the formation of even small quantities of multiparticle aggregates can skew measured hydrodynamic radii and polydispersities, rendering the technique unable to provide convincing evidence for the formation of 'wrapped' objects. We chose to coat silica microparticles with the mannose-binding lectin concanavalin A (ConA)[10] (MP-ConA) (see ESI). Lectins are particularly relevant receptors as many viruses[11] and bacteria[11c] are decorated with lectins whose purpose is to bind complementary carbohydrates upon the surfaces of mammalian cells.[11a]

Preliminary attempts at 'wrapping' microparticles using mannose-decorated polymers based upon poly(dimethylacrylamide) (Scheme 1a) were unsuccessful, with the vast majority of 'wrapped' species present as multiparticle aggregates. We speculate that on account of their utilization of relatively hydrophobic aromatic aldehyde-functionalized co-monomers, which are required as 'anchor points' for dynamic covalent crosslinkers, these copolymers display a propensity to engage in non-specific interactions that lead to the unsuccessful outcomes described in Figure 2. Furthermore, it is likely that the hydrophilicity imparted by the dimethyl acrylamide co-monomers does not sufficiently mask this hydrophobicity, further compounding the problem.

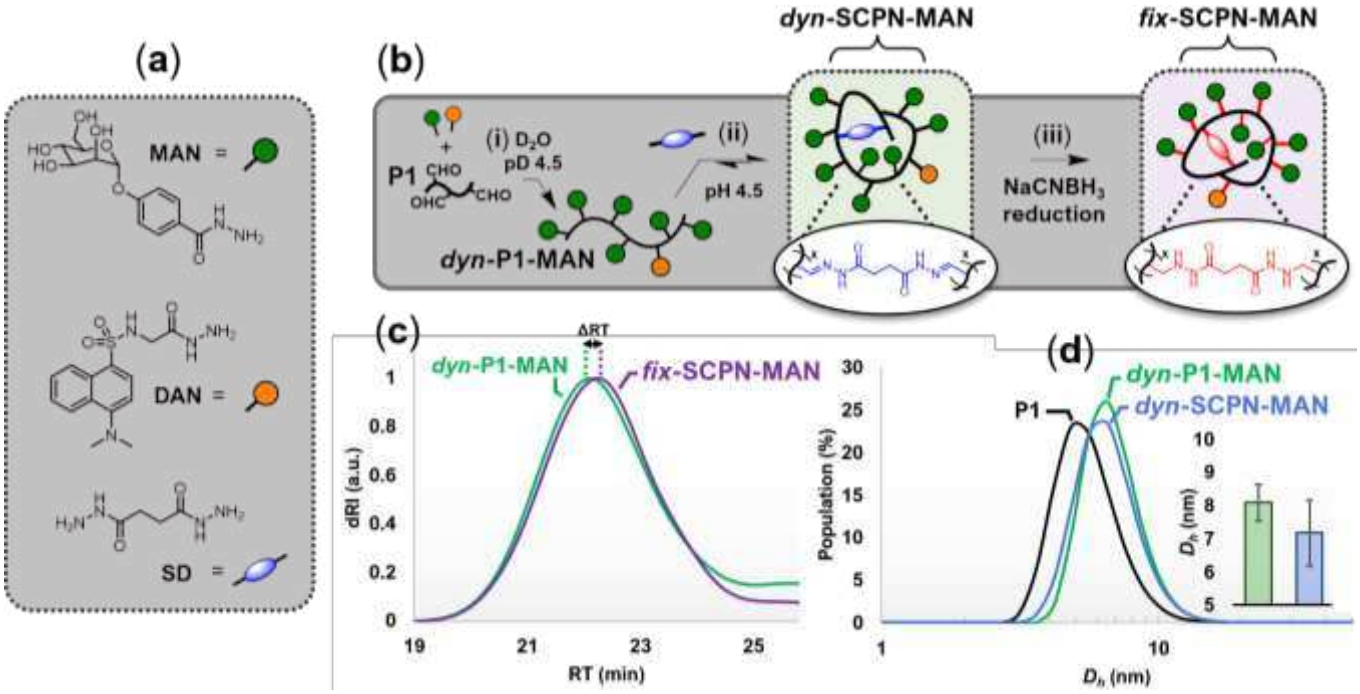


Figure 3: Synthesis and characterization of mannosylated polymer species; ***dyn*-P1-MAN**, ***dyn*-SCPN-MAN** and ***fix*-SCPN-MAN**. (**a**) Structures of mannose hydrazide (**MAN**) ligand, dansyl hydrazide (**DAN**) fluorescent label and succinic dihydrazide (**SD**) crosslinker. (**b**) **P1** was 'decorated' (step **i**) with hydrazide residues **MAN** and **DAN** to afford mannosylated polymer scaffold ***dyn*-P1-MAN**. Addition of dihydrazide **SD** (1 eqv) (step **ii**) afforded intramolecularly crosslinked polymer ***dyn*-SCPN-MAN**. Reduction of ***dyn*-SCPN-MAN** (step **iii**) afforded ***fix*-SCPN-MAN**, whose crosslinks were no longer dynamic. (**c**) GPC analysis of conversion of ***dyn*-P1-MAN** into ***dyn*-SCNP-MAN**. Normalized differential refractive index (dRI) traces revealed a small increase in retention time (ΔRT) for ***fix*-SCPN-MAN** (**purple**) relative to ***dyn*-P1-MAN** (**green**), consistent with chain collapse. (**d**) DLS (D_h) particle size distributions of **P1** (**black**), ***dyn*-P1-MAN** (**green**) and ***dyn*-SCPN-MAN** (**blue**) at [polymer] = 1 mg mL^{-1}. **Inset:** Mean D_h values of ***dyn*-P1-MAN** (**green**) and ***dyn*-SCPN-MAN** (**blue**). Error bars indicate standard deviation.

Synthesis of hydrophilic polymer 'wrapping'agent

We thus chose to base our next generation of polymer 'wrapping agents' upon p(OEGA) copolymers, where we anticipated that the superior solubilizing effects of the OEGA moieties meant these copolymers could comfortably accommodate poorly water-soluble benzaldehyde units, and thus would be less likely to participate in non-specific binding. PEGylation is a well-established strategy in drug-development,[12] where the incorporation of non-toxic, non-immunogenic, highly hydrophilic PEG fragments onto relatively hydrophobic pharmaceutical 'cores' has afforded therapeutic agents with enhanced aqueous solubility, biodistribution and pharmacokinetic profiles, lower levels of hydrophobic aggregation,[13] less off-target toxicity and has brought substantial improvements to therapeutic index.[14] Inspired by this tried-and-tested approach, our decision to improve hydrophilicity and eliminate non-specific binding by inclusion of PEG units within copolymer scaffolds was thus anticipated to also enhance biocompatibility of the 'wrapping' agents.

The synthesis of the polymer 'wrapping' agent was based upon the linear aldehyde-functionalized copolymer **P1** (Scheme 1b), which was prepared by the RAFT-copolymerization of aldehyde-containing acrylate monomer **M1** (supporting information) with the commercially-available hydrophilic comonomer OEGA. Analysis by gel permeation chromatography (GPC) revealed (supporting information) a monomodal distribution of reasonably low polydispersity (PDI = 1.4),

consistent with a controlled polymerization process. The monomer composition (**M1**:OEGA of 1:1.24) and the number-average molecular weight (M_n = 27.4 kDa) were determined by ^{1}H NMR spectroscopy. The reaction[15] (Figure 3b, step i) of **P1** with excess mannosyl hydrazide (**MAN**) and dansyl hydrazide (**DAN**) afforded the mannosylated copolymer ***dyn*-P1-MAN**. The mannose residues are complementary ligands for the ConA receptors upon the peripheries of target microsphere **MP-ConA**, and the fluorophore **DAN**, which was appended in small quantities, allows visualization of polymer films by microscopy. Purification by dialysis against water and subsequent characterization by ^{1}H NMR spectroscopy indicated that all aldehyde functions had reacted and unconjugated **MAN**/**DAN** had been successfully removed. We chose to intramolecularly crosslink **P1** with dynamic covalent acyl hydrazone bonds, which we have previously[16] shown are able to undergo intra- to interchain crosslinking when concentrated. Thus, treatment of ***dyn*-P1-MAN** with succinic dihydrazide (**SD**) (Figure 3b, step ii) induces[17] component exchange through a transimination-type process, resulting in intra-polymer chain crosslinking to yield the desired polymer 'wrapping agent' ***dyn*-SCNP-MAN**. The crosslinking process was monitored (Figure 3d) by GPC analysis of aliquots of reactions which were treated with $NaBH_3CN$ to reduce the dynamic hydrazone bonds (Figure 3b, step iii); this 'fixing' of all dynamic bonds was required to ensure the species cannot undergo any component exchange process during analysis. This revealed an increase in retention time of ***fix*-SCPN-MAN** (purple) compared to ***dyn*-P1-MAN** (green), an observation that indicates collapse of polymer chains to form species of decreased volume,[18] and is consistent[18-19] with successful intramolecular crosslinking. DLS analysis (Figure 3d) revealed a subtle decrease in the hydrodynamic diameter (D_h) of ***dyn*-SCNP-MAN** (blue) relative to that of the linear (non-crosslinked) ***dyn*-P1-MAN** (green), an observation that was also suggestive of successful intramolecular crosslinking. The intramolecular nature of the crosslinking means ***dyn*-SCNP-MAN** possesses the so-called single chain polymer nanoparticle (SCNP) architecture,[19] an emerging class of polymer nanoparticle which has received much attention from researchers over the last decade or so.[20]

It was anticipated that after binding to **MP-ConA**, the polymer chains would then crosslink with one another to afford a crosslinked polymer 'wrapper' (Figure 4a). For control experiments (Figure 4b), we used mannose-decorated polymers able to bind onto the microsphere but unable to then crosslink with one another as they do not possess crosslinks (***dyn*-P1-MAN** and ***fix*-P1-MAN**), or their dynamic covalent crosslinks had been kinetically fixed (***fix*-SCNP-MAN**). ***fix*-P1-MAN** was prepared by the reduction of ***dyn*-P1-MAN** with $NaBH_3CN$. It was anticipated that these controls would be displaced from the particle surface by the addition of excess α-D-methyl mannopyranoside (**αMM**), a small molecule ligand that binds ConA.[21]

Microparticles are successfully 'wrapped' when treated with the polymer 'wrapping' agent

In 'wrapping' experiments, microparticles **MP-ConA** (1 mg/mL) were incubated with dansyl-labelled ***dyn*-SCNP-MAN** or control polymers (***dyn*-P1-MAN**, ***fix*-P1-MAN** or ***fix*-SCNP-MAN**) (2.5 mg/mL) in 100 mM acetate buffer (pH 4.5) for 18 h and unbound polymers were removed from the particle surfaces by centrifugal washing steps (supporting information). Aliquots of each sample (3 x 5 µL) were then deposited onto a clean glass slide, dried, and then carefully imaged by fluorescence microscopy (Figure 5a-d). As anticipated, fluorescence was observed around the microparticles (Figure 5, columns C1, C2), indicating all polymers were able to successfully bind onto the surfaces of the microparticles. These images also show individual microparticles or relatively low-number aggregates, suggesting the effectiveness of the oligoethylene glycol appendages of the polymers in preventing unwanted aggregation. Excess **αMM** was then added and each sample left to incubate at 37 °C for 24 h then purified by centrifugation to remove unbound polymer, and the samples imaged by microscopy (Figure 5, columns C3, C4). Significant fluorescence was still observed upon the microparticles originally treated with ***dyn*-SCNP-MAN** (Figure 5d), an observation indicating that the polymer coating was not displaced from the microparticle surface upon treatment with an excess of **αMM**. The images

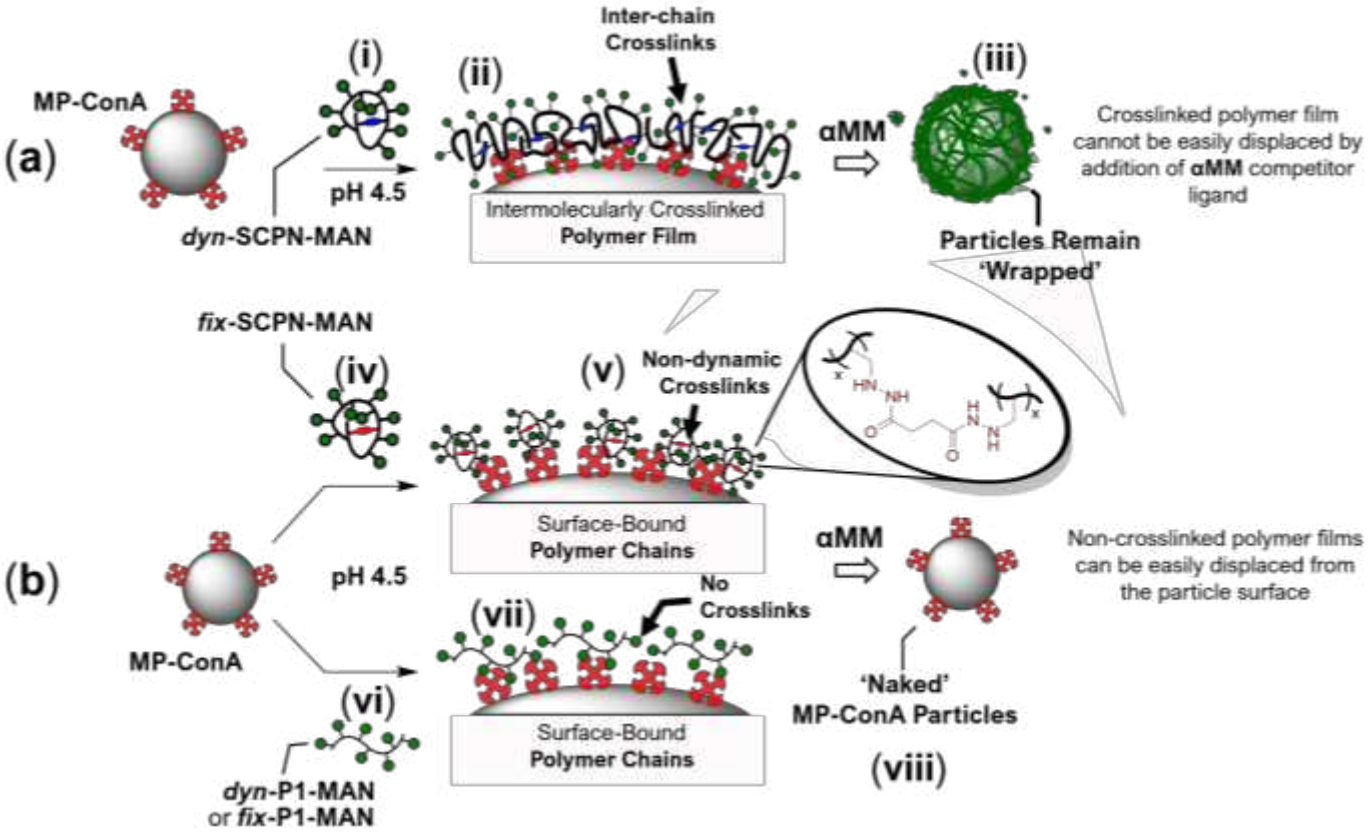


Figure 4: (**a**) Treatment of microparticles (**MP-ConA**) with ***dyn*-SCPN-MAN** (step **i**) leads to the successful 'wrapping' of the microparticle within a polymer film (**ii**) where the polymers are intermolecularly crosslinked with one another. This film cannot be displaced from the particle surface by incubation with competitor ligand ***α*MM** (**iii**). (**b**) Control experiments performed with ***fix*-SCPN-MAN** (non-dynamic crosslinks) (**iv**) and ***dyn*-P1-MAN** (no crosslinks)(**vi**) lead to non-crosslinked particle surfaces (**v** and **vii**), and thus these polymer layers are readily displaced by incubation with ***α*MM** to reveal the 'naked' ConA microparticles (**MP-ConA**)(**viii**). These control experiments aim to demonstrate that (1) 'shrink-wrapping' introduces additional stability to polymer films generated upon particle surfaces and (2) the crosslinks must have a dynamic covalent character in order for 'shrink-wrapping' to successfully occur.

of the microparticles originally treated with controls (***fix*-SCNP-MAN**, ***dyn*-P1-MAN** and ***fix*-P1-MAN**) (Figure 5a-c) show a significant reduction in the fluorescence associated with the microparticles, indicating that excess **αMM** has been able to successfully displace these polymers from the surfaces of the microparticles. Taken together, these observations suggest that upon their binding to the microparticle surface, polymer 'wrapping agent' ***dyn*-SCNP-MAN** had successfully undergone dynamic intra- to interpolymer chain linking to form a crosslinked polymer 'wrapping' around the microparticle which could not be displaced upon the addition of excess **αMM**. Control polymers (***fix*-SCNP1-MAN**, ***dyn*-P1-MAN** and ***fix*-P1-MAN**), on the other hand, were not able to form crosslinked coatings, and thus were displaced from the microparticle surface upon the addition of excess **αMM**.

The relative levels of binding of polymers to the microspheres were estimated by measuring the magnitude of the fluorescence for each sphere using image analysis software which calculates mean gray values (MGVs) for each sphere (see supporting information for details). Comparison of MGV values after treatment with polymers indicate (Figure 5e) that all microparticles are of similar brightness (MGV values ~ 37 – 51), except the sample treated with ***dyn*-SCNP-MAN** (pink bars), which is considerably brighter (MGV ~ 116 ± 21). Previous work has shown[16] that crosslinked films prepared by molecular recognition-mediated film formation are often thicker than a single layer of polymer chains because the initially formed layer is able to recruit further chains from solution into the crosslinked network. This possibility is not available to the control polymers as they lack dynamic crosslinkers, and hence there is less fluorescence associated with their coated microparticles. After treatment with excess **αMM**, there was a fall in MGV from 116 to 74 (a 36% drop) for the microparticle treated with ***dyn*-SCNP-MAN**, suggesting that some polymer was only weakly bound to the microparticle surface. This observation was rationalized by the hypothesis that not all ***dyn*-SCNP-MAN** chains will successfully crosslink upon the microparticle surface because of the relatively low

intramolecular crosslinking density (each chain has on average only one crosslinker, and thus it is likely that a portion of the population contains no crosslinker and cannot incorporate into the film).

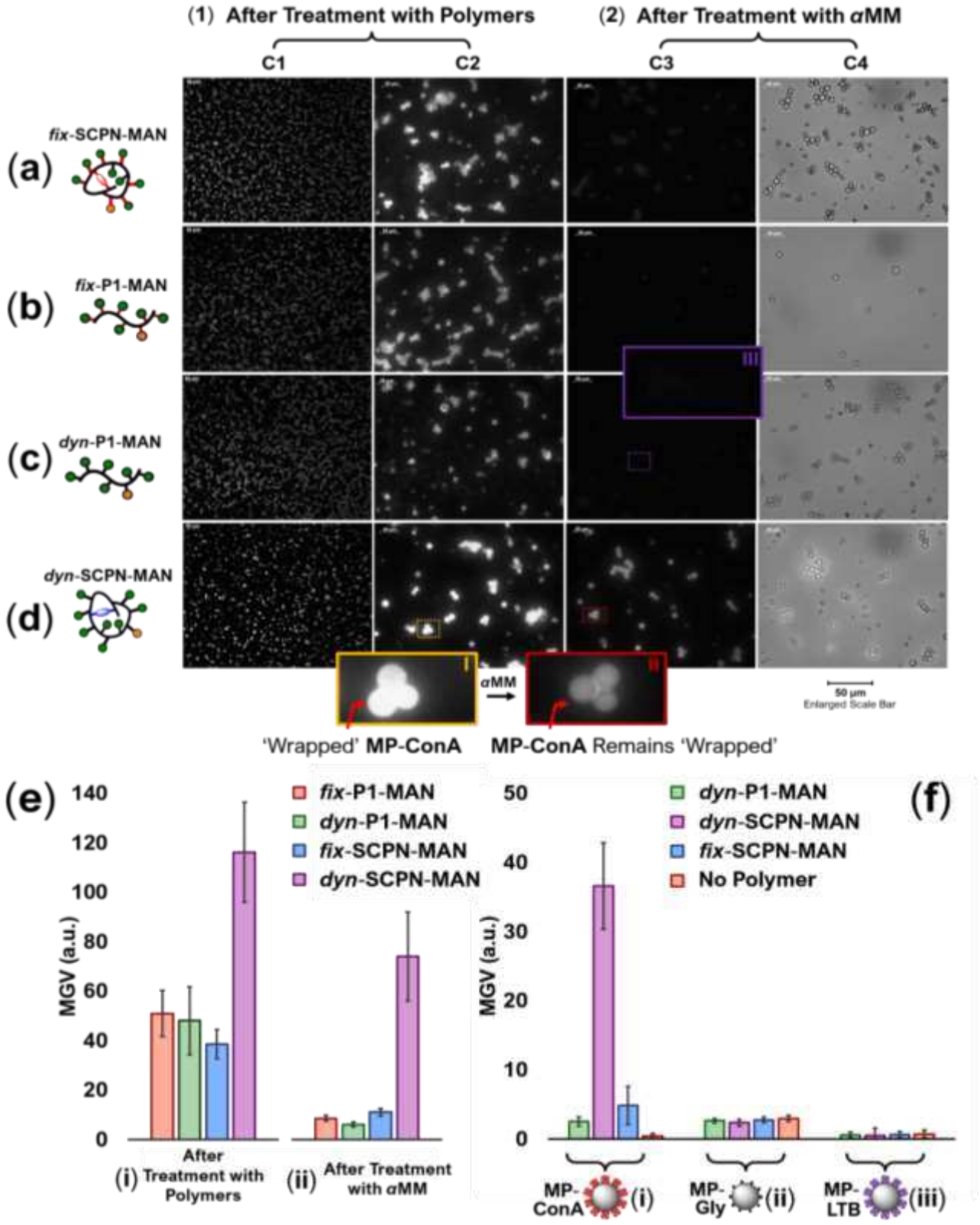


Figure 5: Representative fluorescence microscope images of 'wrapping' experiments. Images show microparticles (**MP-ConA**) after treatment with polymers (Image group (**1**), column **C2** is a magnification of column **C1**) and then after incubation with ***α*MM** (Image group (**2**) shows the same particles both under normal light (**C4**) and under UV irradiation (**C3**); this comparison was required to demonstrate the presence of non-emissive particles). After treatment of microparticles with control polymers (**a**) ***fix*-SCPN-MAN** (non-dynamic), (**b**) ***fix*-P1-MAN** (non-dynamic) and (**c**) ***dyn*-P1-MAN** (no crosslinks,) fluorescence was observed around all microparticles, indicating polymers have bound to the microparticle surfaces. After treatment with ***α*MM**, however, all polymers were fully displaced from the surfaces, indicating polymer crosslinking has not occurred. (**d**) After microparticle treatment with polymer 'wrapping agent' ***dyn*-SCPN-MAN** fluorescence was observed around all microparticles. This fluorescence remains after treatment with ***α*MM**, indicating the polymer chains have successfully crosslinked and thus cannot be displaced. Insets **I**-**III**: Further magnified images produced for regions of interest (see highlighted areas) within selected micrographs. (**e**) Mean gray-values (MGVs) obtained for 'wrapped' **MP-ConA** (**i**) before and after (**ii**) incubation with ***α*MM** to displace polymer 'wrappings'. (**f**) MGVs obtained from 'wrapping' and control experiments with three microparticle types: (**a**) **MP-ConA,** (**b**) **MP-Gly** and (**c**) **MP-LTB**. MGVs were determined from images after **αMM** incubation to attempt to displace the polymers from the particle surfaces. Bars represent the average MGVs were measured for > 100 individual particles. Error bars indicate standard deviation. A no polymer control experiment was included (**orange bars**), which reveal the 'background' brightness of 'naked' microparticles in the absence of polymer. Bars in (**e**-**f**) represent average MGVs determined for < 100 particles. Error bars indicate standard deviation.

The control polymers experience a significant decrease in MGV (~80%) upon treatment with excess **αMM**, suggesting that most, but not all, of the polymers are displaced from the microparticle surfaces. This observation suggests the possibility of a small degree of non-specific binding between the polymers and microparticle surfaces, perhaps also involving the relatively hydrophobic dansyl residues appended to the polymer chains.

Specific molecular recognition events are required for 'wrapping' to occur

To confirm that specific molecular recognition events are required to drive the successful 'wrapping' of the microsphere, 5 μm microparticles (**MP**-**LTB**) decorated with heat-labile toxin (LTB),[22] a lectin that does not bind mannose, and a glycine-capped microparticle (**MP**-**Gly**), which is absent in any carbohydrate receptors, were prepared (supporting information). These microparticles were incubated with solutions of polymers and centrifuged to remove unbound polymers. Samples were then treated with **αMM** and reanalysed by fluorescence microscopy (images not shown) and MGVs calculated (Figure 5f). There was no significant fluorescence observed around microparticles **MP**-**LTB** and **MP**-**Gly**, indicating that the polymers do not bind to microparticles unable to engage in complementary molecular recognition, thus confirming complementary molecular recognition between polymer and microparticles is required to drive 'wrapping'.

Conclusion

We have successfully demonstrated the structural metamorphosis approach to 'wrapping' of lectin-functionalized silica microspheres that serve as low-cost and readily-available models for developing 'wrapping' chemistry. This goal was accomplished using polymers displaying mannose residues that drive their concentration upon the microparticle surfaces, and dynamic covalent crosslinkers able to undergo intra- to interpolymer interconversion to form a crosslinked polymer film. Crucial to the successful outcome was the use of a p(OEGA)-based polymer 'wrapping agent' that eliminated non-specific interactions that can drive unwanted aggregation processes and also make 'wrapping' difficult to prove. As 'wrapping' only occurs in the presence of complementary recognition between the polymer 'wrapping agent' and the object, it may be possible to selectively 'wrap' target objects within mixtures.

This work is significant because it demonstrates that small objects can be 'wrapped' within crosslinked polymer films, thus suggesting that smaller and biologically-relevant objects such as VLPs and bacteria may also be successfully 'wrapped'. 'Wrapping' may stabilize these species, increase their shelf-lives and improve their performance, in much the same way that plastic wrappers and films do for everyday objects. Indeed, recent work[2b] has shown the encapsulation of brome mosaic virus, a small icosahedral plant virus, within a layer of tannic acid crosslinked with Fe^{3+} ions was stabilized towards dehydrating conditions that would otherwise lead to virus disassembly, and suggests that 'wrapping' VLPs can indeed improve stability. We envisage that the structural metamorphosis approach described here will help address stability issues associated with nano- and micron-sized biological objects, and we are working towards applying this method to 'wrap' VLPs.

Conflicts of interest

There are no conflicts to declare.

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Keywords: adaptive materials, dynamic covalent chemistry, polymer films, structural metamorphosis, supramolecular chemistry

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Exploiting structural metamorphosis to wrap microspheres: The ‘wrapping’ of lectin-decorated microspheres, which serve as appealing model micron-sized objects, within cross-linked polymer film is described. This approach utilizes dynamic covalent polymer chains able to undergo a structural metamorphosis, from being intramolecularly cross-linked to intermoleculary cross-linked, a process that is triggered by polymer concentration upon the particle surface. Experiments demonstrate that both complementary molecular recognition and the dynamic covalent nature of the crosslinker are required for successful ‘wrapping’ to occur.