We demonstrate that fluorescent, narrow-disperse microspheres are rapidly and readily prepared by photo-clicking pre-functionalised polymers using the highly efficient nitrile-imine mediated tetrazole-ene cycloaddition (NITEC) reaction. Our approach requires no stabilisers, bases or initiators, and proceeds under mild UV irradiation at ambient temperature. The diameter of the microspheres can be tuned in the 0.25 – 0.75 μm range depending on the reaction conditions, including polymer concentration, polymer ratio and the solvent composition. Critically, we explore the application potential of the microspheres, including fluorescence, degradation and swellability for encapsulation.

**Introduction**

Polymer microspheres have a variety of favourable characteristics that have allowed them to find broad application and develop into a multi-billion dollar industry.1-5 These characteristics include a small size and volume, high diffusivity and mobility, and a large specific surface area.6 In materials science, polymer microspheres have found application in toners, chromatography, instrument calibration, photonic crystals, coatings and composite materials.1-7, 13 More specifically, fluorescent microspheres have especially found unique application in the areas of biochemistry, bioanalytics and medicine. Examples include flow cytometry, blood flow determination, immunoassays, in vivo imaging, and they have even been used in drug delivery/release systems and as biological tracers.2, 14-19

**Conceptual Insights**

Polymer microspheres are an important and ubiquitous class of materials. In the current work, we introduce a new class of functional, fluorescent polymer microspheres using a highly efficient, additive-free photochemical ligation technique. For the first time, the utilisation of pre-functionalised polymers for cross-linking under conditions analogous to precipitation polymerisation – a powerful technique for the preparation of stabiliser-free microspheres – is demonstrated. In particle synthesis, many modern chemical reactions have not been explored for use as cross-linking chemistries, and our current report opens the door for the use of such reactions to prepare particles with advanced characteristics and function. The herein described approach enables a powerful new paradigm in particle synthesis and provides a broad platform for future development.

Certainly, the demands of existing and potential applications necessitates continued advances in the design and preparation of polymer microspheres, especially since each application can have very different prerequisites. This has led to the development of several methods for preparing polymer microspheres.6, 20 Still, preparation is limited by the underlying methods of chemical synthesis, and free-radical chain-growth polymerisation is by far the most extensively used and studied.6, 21 However, many desirable substrates – such as biological materials or functional co-monomers – contain functional groups which are sensitive to free radical reactions as well as the high temperatures often employed during particle formation. Thus, incorporation of sensitive substrates generally requires multi-step and post-functionalisation reactions.22-25 In the case of fluorescent microspheres, internal dye entrapment often simply involves solvent swelling, which leaves the particles prone to leaching and subsequent loss of fluorescence.26 For some specific applications such as microcapsules, alternative routes such as polyurea and coacervate particles have been developed.27-29

Thankfully, advances in synthetic chemistry provide one important avenue for constructing materials with increasingly advanced characteristics and functionality. Highlighting how modern, highly efficient reactions can be used to develop new
particle synthesis methods, Bowman and colleagues used thiol-Michael step-growth dispersion polymerisation to prepare microspheres from commercially available thiol and acrylate monomers under ambient conditions. Not only enabling a new paradigm in micro-particle synthesis, the resulting particles had an innovative backbone, stoichiometrically controllable surface functionality and were degradable.

In contrast to other methods of classical heterogeneous polymerization (e.g. dispersion or (mini)emulsion), precipitation polymerisation advantageously forms stabiliser-free microspheres whereby oligomers on the surface provide stabilisation for the growing particles. Typically, the polymerization operates at low concentration and solids content in (near) absence of conventional agitation. Soluble monomer radicals grow until the oligomers reach a critical length and precipitate to form nuclei. Particles subsequently grow by capturing oligomers, leading to particles in the (sub-)micrometre range. Developed extensively by Stöver and coworkers since the early 1990’s, it represents a facile particle synthesis method with a simplified reaction mixture compared to other methods. However, this lack of stabiliser to inhibit aggregation of precipitated polymer chains means even slight changes in the system – physical or chemical – can lead to adverse events such as coalescence and loss of monodispersity. Matching the solubility parameter of the growing polymer network to that of the reaction medium (near-θ conditions) is crucial, and cross-linkable monomers are generally limited to divinylbenzene (DVB), methacrylates and acrylamides where their solubility parameters have well-matched solvent systems. However, many vinyl- and acrylate-based functional comonomers have been successfully incorporated, such as chloromethylstyrene (CMS) and glycidyl methacrylate (GMA). Classical approaches for precipitation polymerisation are well-explored, including thermally induced precipitation polymerisation, photo-induced precipitation polymerisation, and controlled/reversible deactivation radical polymerisation.

Although photo-initiated implementations of precipitation polymerisation succeed in allowing milder temperatures, these reactions still require a radical initiator and thus proceed via free radical polymerisation. Extremely efficient photo-induced reactions with ‘Click’ characteristics – such as the nitrile-imine mediated tetrazole-ene cycloaddition (NITEC) reaction – present as potential candidates for overcoming the aforementioned disadvantages of such reactions. The advantages of the NITEC reaction are numerous and well reported; it is fast, features a high photoefficiency, activates under mild long-wave UV or visible light, yields a highly fluorescent pyrazoline cycloadduct, and finally overcomes the need for any kind of photoactive radical initiator or photocaged base.

The reaction is initiated by generation of a reactive nitrile-imine following release of nitrogen gas from an activated tetrazole. This reactive nitrile-imine can then readily react with a variety of dipolarophiles including alkenes, thiols and acids. Work within our group has, for example, exploited the NITEC reaction to induce the folding of functional single-chain nanoparticles via intra-molecular cross-ligation of linear polymers.

Although never utilised as a cross-linker in particle synthesis, it has been shown to be a powerful ligation tool for advanced macromolecular design. Bowman, Barner-Kowollik and coworkers demonstrated that tetrazole-containing monomer could be copolymerised in the synthesis of thiol-ene microspheres. Subsequent irradiation initiated a NITEC reaction between adjacent residual acrylate and tetrazole groups present on the surface, yielding highly fluorescent particles with interesting application potential. Most recently, our group utilised the NITEC reaction to functionalise the surface of visible and UV-light photo-reactive nanoparticles with a wide range of dipolarophiles. Other highlights include polymer conjugation, detection, surface patterning and network formation. However, expanding the realm of particle synthesis to make possible the utilisation of reactions which are currently only suitable for post-synthesis modification is an exciting pathway which comes with its own very distinct set of challenges, considerations and advantages.

Herein, we report a method of preparing inherently fluorescent microspheres using a precipitation polymerisation technique (Scheme 1). Specifically, well-defined random polystyrene (PST) based co-polymers were synthesised and post-functionalised with either a tetrazole or acrylate moiety.

![Scheme 1: Synthesis of fluorescent microspheres from functionalised cross-linkable polymer precursors](image-url)
Upon exposure to mild UV irradiation, the two precursor copolymers undergo a NITEC cross-linking reaction, yielding fluorescent, spherical, narrow-disperse microparticles. Importantly, the reaction requires no stabilisers, surfactants or initiators of any kind, resulting in unique spherical entities.

**Results and Discussion**

**Polymer Design and Synthesis**

Our approach relies on the use of well-defined functional polymers prepared prior to their use in particle synthesis. Modern reversible deactivation radical polymerisation techniques allow control over critical properties such as size, dispersity and copolymer composition. Thus, precursor polymers were synthesised by nitroxide-mediated polymerisation (NMP) of styrene with CMS as a functional comonomer (see Section 2, ESI). Polymer design was centred on maximising the solubility of the precursor polymers, and molecular weights up to 2600 g mol\(^{-1}\) were targeted (Table 1). A polystyrene backbone was chosen based on its known suitability for use in particle synthesis, with acetonitrile being a commonly used near-\(\theta\) solvent for this system. Subsequently, minimising the molecular weight of the copolymers ensured initial solubility, while still remaining large enough to be isolated and purified for further functionalisation. To minimise the effect on solubility of the functional groups added via post-functionalisation, the functional comonomer composition was also kept to approximately 25% (Scheme 1).

Light plays an important role in the synthetic chemists’ repertoire, and its utilisation in the construction of increasingly advanced materials is an important developmental pathway. Given its well reported benefits, we expected the highly efficient NITEC conjugation protocol to be well suited to the current work. Thus, P(St-co-CMS) copolymers **P1a** and **P1b** were separately functionalised with either cross-linkable tetrazole (Tet) (**P2a**) or acrylate (Acr) (**P3b**) groups (Scheme 1). Both reactions proceeded with almost complete conversion of the chloromethyl functional group as confirmed by \(^1\)H NMR and SEC (Table 1, Fig. S1-S10, ESI), and could be well-isolated and purified prior to particle synthesis reactions. To further probe the conversion, X-ray photoelectron spectroscopy (XPS) analysis before and after functionalisation showed significant reduction in the atom% of chlorine, quantified as 97% conversion for **P2a** and 93% conversion for **P3b** (Table S1, ESI). Tetrazole copolymers having a larger average molecular weight and average number of tetrazole groups per polymer chain (**P2b** ESI) were markedly less-soluble in acetonitrile compared to **P2a**, which could be dissolved at a concentration of 1 mg mL\(^{-1}\) at ambient temperature. Therefore, polymer **P2a** was considered more suitable for subsequent particle synthesis reactions. For the acrylate functionalised copolymer, solubility was higher (10 mg mL\(^{-1}\)) regardless of the size of the precursor polymer.

**Particle Synthesis**

As in classical precipitation polymerisation, ensuring an initially homogeneous reaction medium is critical for the successful preparation of spherical particles from precursor copolymers in the absence of stabiliser. Undissolved copolymer would certainly have negative effects on essential factors such as reproducibility, aggregation and particle dispersity. Thus, to ensure complete dissolution prior to use, solutions of P(St-co-Tet) (**P2a**) and P(St-co-Acr) (**P3b**) were prepared and diluted to concentrations indicated in Tables 2 – 4.

Firstly, particle synthesis reactions were performed with 1:1 mass ratios of each copolymer, although the higher **Mn** and average number of acrylate groups of **P3b** (Table 1) were calculated to give a 2:1 stoichiometric ratio of functional groups. As mentioned for traditional precipitation polymerisation, solid content was low due to the absence of stabiliser. It is worth noting that reactive nitrile-imine formed upon activation of the tetrazole may undergo dimerization, leading to inter or intra-molecular non-fluorescent cross-linking adducts. Consequently, excess nitrile-imine could lead to inter-particle cross-linking and aggregation in the absence of polymer in solution towards the end of the reaction. On the other hand, excess acrylate is expected to be unreactive in the absence of tetrazole and ultimately lead to a functional surface. The

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**Table 1** Physical properties of synthesised copolymers before and after functionalisation, where **Mn** represents the number average molecular weight, **D** represents the polymer dispersity (\(M_w/M_n\)), **Funct** represents the percentage of functional monomer in the polymer backbone, and **Dmax** and **D50** represent the average number of units of functional monomer and styrene, respectively.

| Sample | Polymer composition | **Mn**[^b] | **D**[^b] | **Funct**[^c] | **Dmax**[^e] |
|--------|---------------------|------------|---------|-------------|------------|
| **P1a** | P(St-co-CMS)       | 1350       | 1.1     | 23          | 2          |
| **P1b** | P(St-co-CMS)       | 2050       | 1.2     | 33          | 5          |
| **P2a** | P(St-co-Tet)       | 1900       | 1.1     | 23          | 2          |
| **P3b** | P(St-co-Acr)       | 2500       | 1.1     | 28          | 5          |

[^a]: Combined acrylate and tetrazole copolymer concentrations, at 1:1 mass ratio, calculated from SEC and NMR as 2:1 (Acr:Tet) stoichiometric ratio of functional groups.
[^b]: Determined by SEC in THF with polystyrene calibration.
[^c]: Determined by \(^1\)H NMR.
[^d]: Average number of units per polymer chain, calculated from SEC and NMR; see ESI for details.
[^e]: Calculated from SEC and NMR; see ESI for details.
Particle syntheses were thus performed by exposing the solubilized copolymers under UV-B light (λ = 300 nm) and gentle agitation. Within seconds, the clear solution turned bright yellow – as highlighted by the supplied supplementary video – and the reaction was carried out for 30 or 120 min. Scanning electron microscopy (SEM) images of isolated particles showed that they are spherical with an average diameter of 0.34 μm and a narrow size dispersity (1.04) (Fig. 1a and b). In addition, the particles could be redispersed in solvents such as acetonitrile and tetrahydrofuran (THF) and appear well isolated from each other. A representative diameter distribution obtained by dynamic light scattering (DLS) is also shown in Fig. 1c. Compared to the average diameter obtained by SEM, the larger particle size by DLS (515 nm) is likely due to significant swelling of the particles when dispersed in THF.

With the mass ratio of the copolymers kept at 1:1, increasing the total loading concentration led to an increase in the average diameter of the resulting microspheres, from 0.25 μm up to 0.75 μm (Table 2). This indicates that the size of the microspheres can be controlled by the amount of cross-linkable points present at a given reaction volume. In general, although sphericity was retained in all cases, higher loading concentrations led to slightly more polydisperse particles, a common observation in most particle synthesis methods. Still, for concentrations below 1 mg mL⁻¹, dispersities were lower than 1.1. Commonly, this is considered an indication of monodispersity.⁴⁰

We hypothesise that a critical parameter for preparing particles with low dispersities is the penetration depth and intensity of the light reaching the reaction mixture. Compared to other examples of photo-initiated precipitation polymerisation which proceed via radical chain-growth after initiation, the NITEC reaction proceeds via a step-growth mechanism. Thus, each individual tetrazole requires photon absorption to generate the reactive nitrile-imine for cross-linking. Although this can provide far greater spatio-temporal control and precision, penetration depth becomes important in order to ensure the reaction medium is irradiated as uniformly as possible. Compared to reactions of lower concentration where dispersities remain low, reactions at higher concentrations become noticeably more cloudy, limiting penetration depth of light and thus limiting growth of particles closer to the centre of the reaction mixture. Since stirring and agitation needs to be minimal, simple possibilities for minimising these effects include controlling the diameter of the reaction vessel as well as the intensity of the incident light.

Nonetheless, the advantages of using the highly efficient NITEC reaction are numerous; not only is the reaction fast, activation of the tetrazole by irradiation requires no initiators or bases and has no by-products or contaminants. Thus, the solvent can easily be recovered by evaporation and reused in further reactions, since it lacks volatile reagents or products commonly used in step-growth polymerisation or unreacted monomer present in conventional precipitation polymerisation.

### Off-Stoichiometric Particle Synthesis Reactions

Given the difference in solubility of the copolymers, changing the ratio of acrylate copolymer to tetrazole copolymer is a simple way to facilitate higher loading concentrations. In addition, it should also increase the amount of residual, unreacted acrylate groups present which may be used for post-functionalisation reactions. To assess how this would affect particle formation, experiments were performed with variations in both polymer mass ratio and overall loading concentration (Table 3). Spherical particles were successfully synthesised with a copolymer ratio of 1:2 and a concentration up to 2 mg mL⁻¹. Given the difference in the average number of functional groups per chain (Table 1), the stoichiometric ratio of cross-linking functionality was calculated as approximately 1:4.

| Tet:Acr | C [mg mL⁻¹] | Dn [μm] | Dw [μm] | D [Dw/Dn] | σ [μm] |
|---------|-------------|---------|---------|-----------|--------|
| 1:1     | 1           | 0.487   | 0.551   | 1.13      | 0.10   |
|         | 2           | 0.752   | 0.874   | 1.16      | 0.17   |
| 1:2     | 1           | 0.403   | 0.458   | 1.15      | 0.08   |
|         | 2           | 0.564   | 0.697   | 1.24      | 0.14   |

a Combined acrylate and tetrazole copolymer concentrations b Determined by SEM.

(Tet:Acr). One may expect the cross-linking density of particles prepared by off-stoichiometric reaction to be lower than their

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Fig. 1 Particle synthesis reaction of P(Styrene-co-Tetrazole) P2a and P(Styrene-co-Acrylate) P3b with polymer concentration of 0.67 mg mL⁻¹, irradiated at 320 nm for 30 min at room temperature. Analysed by SEM (A) and (B), and by DLS in THF (C). Scale bar is 1 μm.
stoichiometric counterparts, however sphericity and stability of the particles was retained (Fig. S13, ESI). For an equivalent total polymer loading concentration, particles resulting from a higher ratio of acrylate copolymer had a lower average diameter. This is in line with previous results (Table 2); the diameter appears to be controlled by the amount of reactive tetrazole copolymer, since increasing the ratio of acrylate polymer at a given concentration would mean a lower concentration of tetrazole polymer present for crosslinking.

Particle Synthesis with Co-Solvent

In particle synthesis, the addition of a co-solvent is commonly performed to either increase the initial loading concentration, or to optimise the solubility parameters of the reaction medium for particle formation. Additionally, classical precipitation polymerisation uses non-polar solvents such as toluene to produce porous particles. In our case, we expected that the addition of a solvent which better dissolves the precursor polymers may also increase the critical length at which nucleation occurs. To test whether this would still facilitate particle formation, reactions were performed in a mixture containing 10 – 20% THF (Table 4). In each case, spherical particles were successfully prepared, and particle sizes (0.33 – 0.61 μm) and dispersities (1.10 – 1.24) increased with increasing concentration, consistent with reactions performed in acetonitrile. However, the average diameter of the microspheres was smaller compared to reactions performed in acetonitrile. Microspheres prepared at a concentration of 3 mg mL\(^{-1}\) in 10% THF had an average diameter of 0.61 μm, compared to the larger diameter (0.75 μm) of microspheres prepared in acetonitrile at a lower concentration of 2 mg mL\(^{-1}\). Increasing the proportion of THF to 20% led to microspheres with an even lower diameter (0.42 μm). Nonetheless, tailoring the solubility parameter of the reaction medium – especially for alternative cross-linking chemistries or polymer backbones – should certainly be considered a viable option for optimising particle formation in this system.

Particle Fluorescence

One of the most attractive features of our method is the generation of a fluorescent pyrazoline cycloadduct upon cross-linking, which renders the resultant particles inherently and highly fluorescent. The appearance of the reaction mixture before and after irradiation is shown in Fig. 2a (under white light) and Fig. 2b (under 365 nm UV lamp). Imaging via confocal fluorescence microscopy allowed confirmation of the uniform and inherent fluorescence of synthesised microspheres (Fig. 2d). Furthermore, fluorescence spectroscopy was used to quantify the emission and excitation spectra of the isolated particles, with an excitation centred around 400 nm and an emission peak at 540 nm (Fig. 2c).

### Kinetic Analysis of Particle Formation

Given the aforementioned generation of fluorescence upon cross-linking, particle formation would be expected to correlate with the observed increase in fluorescence over time. In order to explore this, samples for both fluorescence and SEM analysis

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**Table 4** Synthesis of fluorescent microspheres with THF as co-solvent where \(C\) represents initial polymer concentration, \(D_0\) represents the number average particle diameter, \(D_w\) represents the weight-average particle diameter, \(D_\sigma\) represents particle dispersity (\(D_w/D_0\)), and \(\sigma\) represents the standard deviation.

| THF [%] | \(C\) [mg mL\(^{-1}\)] | \(D_0\) [μm] | \(D_w\) [μm] | \(D_\sigma\) [\(D_w/D_0\)] | \(\sigma\) [μm] |
|--------|----------------|-------------|-------------|----------------|-------------|
| 10     | 1.5            | 0.335       | 0.375       | 1.12           | 0.06        |
|        | 3              | 0.606       | 0.699       | 1.15           | 0.14        |
| 20     | 1.5            | 0.330       | 0.363       | 1.10           | 0.06        |
|        | 3              | 0.420       | 0.520       | 1.24           | 0.11        |

\(a\) Combined acrylate and tetrazole copolymer concentrations, at 1:1 mass ratio, calculated from SEC and NMR as 2:1 [Acr:Tet] stoichiometric ratio of functional groups. \(b\) Determined by SEM.

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were taken at times ranging from 20 seconds to 60 minutes (Fig. 3). As shown in Fig. 3a, the reaction proceeds rapidly, with fluorescence observed almost immediately. Fluorescence continues to increase rapidly over the first 5 minutes, peaking at approximately 15 minutes. The slight decrease in fluorescence observed after 30 minutes is likely due to re-aromatisation of the fluorescent pyrazoline into a non-fluorescent adduct, as well as overlapping of polymer chains during particle growth.

In SEM analysis, particle droplets are observed in the sample after 5 minutes, and well-isolated spherical particles are observed in all samples after 15 minutes (Fig. 3b). Compared to classical precipitation polymerisation, which generally requires reaction times of 24 hours or more, our platform technology yields narrow-disperse, spherical particles with a greatly reduced reaction time of minutes.

**Particle Encapsulation and Degradation**

Highly swellable particles are of broad interest, with applications including encapsulation, chemical sensing to solid-phase synthetic supports. With around 20% cross-linking functionality of the precursor polymers (Table 1), we tested the suitability of encapsulation using the highly swellable microspheres prepared in this work. Since particles prepared via NITEC cross-linking are inherently fluorescent, it is therefore possible to incorporate other desirable substrates while retaining fluorescence. Thus, 4-bromothiophenol – possessing multiple functionalities not present in the initial microspheres – was introduced to a solution of swollen fluorescent microspheres. After extensive washing, analysis of the microspheres (using XPS) showed incorporation of the substrate with the presence of both sulfur and bromine quantified at 0.2 – 0.3 atom% (Table 5, Fig. S16 ESI). Although an illustrative small molecule was used in this case, incorporation of stimuli responsive functionalities or biomolecules are possible applications. Finally, to highlight how additional functionality present in the precursor polymers – and thus the final microspheres – can be utilised depending on the application, our particles contain labile ester groups that have been highlighted for use in controlled release and degradation.

| Atom [%] | C 1s | N 1s | O 1s | Br 3d | S 2p |
|---------|------|------|------|-------|------|
| Before  | 92.14 ± 0.16 | 1.75 ± 0.02 | 6.14 ± 0.15 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| After   | 91.77 ± 0.41 | 1.92 ± 0.12 | 5.84 ± 0.30 | 0.21 ± 0.01 | 0.28 ± 0.01 |

* Analysis performed at room temperature on carbon tape. Average values of two different locations.

![Fluorescence spectra](image)

**Fig. 4** Fluorescence spectra ($\lambda_{exc}$ = 400 nm; $\lambda_{em}$ = 540 nm) of the solution containing fluorescent polymer from the degradation of fluorescent microspheres over time.
NaOH solution, SEM analysis showed significant degradation after 10 min and complete degradation after 3 h (Fig. S17 ESI). In addition, the release of fluorescent polymeric material could also be used to monitor degradation, with fluorescence intensity increasing as a function of time (Fig. 4). Although a strong base was used here, it is certainly possible to use milder conditions to control degradation. A much more interesting possibility – which highlights the power of incorporating functionality prior to particle synthesis – is the incorporation of more labile or alternative stimuli-responsive functional groups.

Conclusions

We exploit pre-functionalised photoactive polymers for advanced fluorescent microsphere formation at ambient temperature. Highlighting the power of our technique is the utilisation of the rapid, photochemically induced NITEC reaction to prepare narrow-disperse microspheres in the absence of stabiliser or initiator. Ultimately, the power of this system is wide-ranging with broad application potential, especially in bioanalysis and imaging applications. Critically, the power of adding functionality to precursor polymers prior to particle synthesis adds an entirely new paradigm for the synthesis of cross-linked polymer microspheres. We envision a variety of avenues for future development, including polymer backbone/solvent combinations, alternative cross-linking chemistries, or the incorporation of additional functionality with orthogonal reactivity for increasingly advanced material design (e.g. stimuli responsive functionality).

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Conflicts of interest

There are no conflicts to declare.

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