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Emulsion click microspheres: Morphology/Shape control by surface cross-linking and porogen

Cite this: DOI: 10.1039/x0xx00000x

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Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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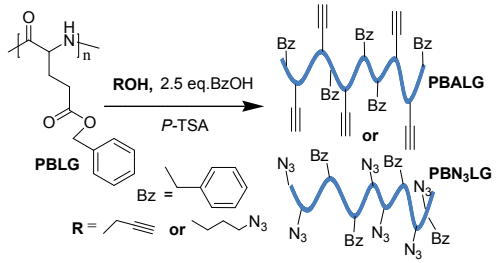
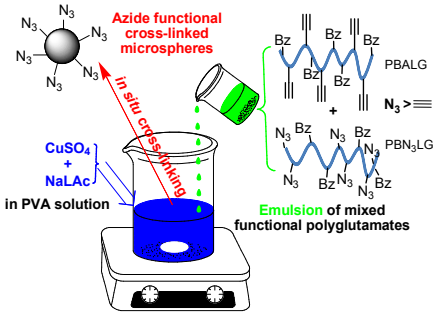
Click chemistry plays a dual role in emulsion microspheres (MS) preparation as both *in situ* cross-linking method and bioconjugation route. The morphology/shape of MS can be adjusted by applying surface click cross-linking and porogen simultaneously. The emulsion click cross-linked MS has the potential to be used in drug delivery, and the preserved azide groups paved the way of further surface conjugation with bioactive molecules.

Biodegradable polymer microspheres have received a lot of attentions since their wide application in drug delivery, biomolecules adsorption/trapping/purification, and endotoxin removal. [1-3] But in drug delivery area, the initial burst release and short duration of loaded drugs released from polymer microspheres have always been the two scabrous problems. [4] To address these problems, cross-linked biodegradable polymer microspheres have been developed. [5, 6] In addition, targeting unit or fluorescent probe conjugation onto microspheres were always needed to realize targeting therapy or bioimaging. [7, 8] As one of the most effective surface/interface reactions that are tolerant to water, oxygen, click chemistry (azide-alkyne cycloaddition, AAC), containing copper-catalyst azide-alkyne cycloaddition (CuAAC) and copper-free click chemistry (such as strain-promoted azide-alkyne cycloaddition, SPAAC), has been widely used in surface/bulk cross-linking of polymeric elastomers/micells/nanogels/miniemulsions, [9-16] and also surface bioconjugation. [7, 17-21] In consideration that click chemistry can play versatile roles in biomaterials design, click chemistry was introduced into emulsion microspheres preparation in this paper, to be used as an *in situ* emulsion surface cross-linking route as well as a surface bioconjugation method.

First, alkyne and azide functionalized poly (γ-benzyl-L-glutamate) (PBLG), abbreviated as PBALG and PBN_3LG separately, were synthesized by ester exchange reactions between PBLG and propargyl alcohol (for PBALG) or 3-azidopropanol (for PBN_3LG) respectively (Scheme 1), according to our previous work. [22]



Scheme 1. Synthesis of alkyne and azide functionalized poly (γ-benzyl-L-glutamate): PBALG and PBN_3LG.



In situ Click cross-linked polyglutamate microspheres by emulsion method

Scheme 2. Preparation of azide-functionalized poly (L-glutamate) microspheres (iECC-PLG-N_3 MS) by *in situ* emulsion click cross-linking of equal-weight mixture of PBALG and PBN_3LG using CuAAC.

Then equal weight of PBALG and PBN_3LG were mixed (the molar content of azide was higher than that of alkyne) and dissolved in organic solvent (mixture of 1,2-dichloroethane and dimethylacetamine (DMAc)) to form oil phase. The oil phase was sheared into O/W emulsion in polyvinyl alcohol (PVA) solution. After addition of copper catalyst ($CuSO_4$-L-ascorbic acid sodium salt (NaLAc)) in water phase, *in situ* emulsion surface CuAAC was promoted simultaneously to form *in situ* emulsion click cross-linked and azide-functionalized poly (L-glutamate) microspheres (iECC-PLG-N_3 MS) (Scheme 2). The remained azide groups could be used for further surface conjugation of targeting units or fluorescent probes. Porogen was also used, and the co-effect of porogen and

emulsion surface click cross-linking on the morphology/shape of microspheres was investigated by adjusting copper-catalyst concentration in water phase and polymer & porogen concentration in oil phase (Table 1). The results are shown in Figure 1.

Table 1. Emulsion click microspheres samples.

Samples	Polymer in oil phase content %(w/v)	Porogen ratio to polymer (mL/g)	Catalyst ratio to alkyne group
1	2	0	0
2	2	1	0
3	5	0	CuSO4 (1.0 eq.), NaLAc (1.0 eq)
4	2	1	CuSO4 (1.0 eq.), NaLAc (1.0 eq)
5	5	1	CuSO4 (1.0 eq.), NaLAc (1.0 eq)
6	2	1	CuSO4 (0.1 eq.), NaLAc (1.0 eq)

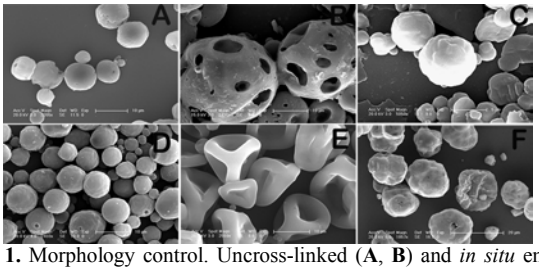
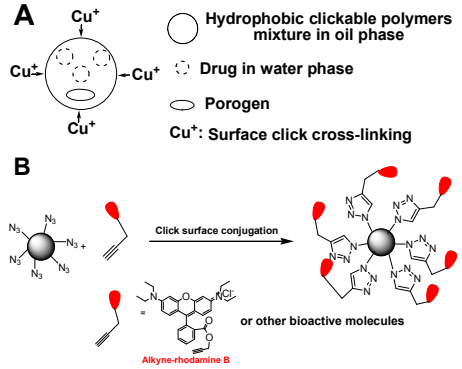


Figure 1. Morphology control. Uncross-linked (**A**, **B**) and *in situ* emulsion click cross-linked azide-functionalized poly (L-glutamate) (iECC PLG-N_3) (**C**, **D**, **E, and F**) microspheres. (**A**) 2% (w/v) of polymer in oil phase, no porogen, no catalyst (sample 1); (**B**) 2% (w/v) of polymer in oil phase, with porogen content of 1mL/g polymer, no catalyst (sample 2); (**C**) polymer: 5% (w/v), no porogen, catalyst: 1.0 eq of $CuSO_4$ to alkyne group, 1.0 eq. of NaLAc (sample 3) (**D**) polymer: 2% (w/v), porogen: 1mL/g, catalyst: 1.0 eq of $CuSO_4$ to alkyne group, 1.0 eq. of NaLAc (sample 4); (**E**) polymer: 5% (w/v), porogen: 1mL/g, $CuSO_4$ (1.0 eq.), NaLAc (1.0 eq.) (sample 5); (**F**) polymer: 2% (w/v), porogen: 1mL/g, $CuSO_4$ (0.1 eq.), NaLAc (1.0 eq.) (sample 6).

It can be seen that without porogen and surface cross-linking, the obtained microsheres are in ordinary round shape with an average diameter around 10 μm (sample 1, Figure 1A). After applying porogen (decahydronaphthalene) but no copper catalyst, the obtained microspheres are also round shape, but with bigger diameter (around 20 μm) and possess a lot of pores (sample 2, Figure 1B). When no porogen but only copper catalyst was used, the obtained MSs are also nonporous as sample 1 (sample 3, Figure 1C). Since the solvent was homogeneously dispersed in the whole microspheres, even after surface cross-linking, it can be evaporated out smoothly. When porogen and copper catalyst were applied simultaneously, the pores formed by porogen decreased a lot, some even disappeared. Changing the concentrations of copper catalyst in water phase and polymer in oil phase brought great effect on the morphology/shape of the obtained microspheres. When the polymer concentration in oil phase was 2% (w/v), porogen was 1 mL/g polymer, and the copper catalyst ratio to the alkyne group was 1/1, the pores were almost sealed by surface click cross-linking. And the obtained microspheres are non-porous spheres with a diameter typically of 5-10 μm (sample 4, Figure 1D). When the polymer concentration in oil phase was increased from 2% (w/v) to 5% (w/v), the shape of the obtained microspheres changed into a strange tetrahedron with all surfaces collapsed into the center, and the size of the microparticles also increased to around 15 μm (sample 5, Figure 1E). It is deemed that, along with the increase of polymer content in oil phase, the size of microspheres increased accordingly, which resulted in an overall increase of solvent and porogen capsuled into each microparticle, because the ratio between progen and polymer was kept at 1 g/mL. Along with the fast evaporation of the organic solvent followed by the slower evaporation of the gathered big porogen liquid drops, collapsed surfaces on the obtained tetrahedral microparticles were left. And since the surface cross-linking speed was fast enough, nearly all pores left were sealed (Figure 1D). When the polymer concentration in oil phase was kept at 2% (w/v), but the copper catalyst ratio to alkyne group decreased from 1/1 to 1/10, the obtained microparticles changed into wrinkled sphere with some small holes (sample 6, Figure 1F). This walnut kernel-like morphology may be caused by the abatement of surface cross-linking, slow surface cross-linking could not seal the holes left by solvent and porogen evaporation, and thinner cross-linked shell collapsed and shrunk together after solvent and porogen evaporation.



Scheme 3. Potential applications of emulsion clicked microspheres: (**A**) In drug delivery system for improving drug loading, controlling MS morphology and drug release; (**B**) Surface conjugation of MSs through click reaction with fluorescent probe or other bioactive molecules.

Emulsion clicked microspheres can be used as cross-linked drug carrier, which may improve drug loading efficiency and reduce initial burst release, as reported before. [5, 6] The emulsion click method can adjust the morphology/shape [23] of microspheres or microparticles (Scheme 3A), which has been reported to be an important design parameter in drug delivery. [24, 25]

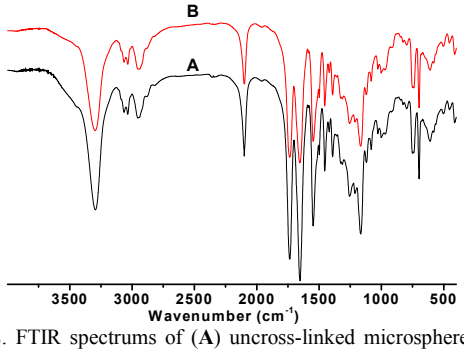


Figure 2. FTIR spectrums of (**A**) uncross-linked microspheres of equal-weight mixture of PBALG and PBN_3LG (sample 1); (**B**) *in situ* emulsion click cross-linked azide-functionalized poly (L-glutamate) microspheres (iECC-PLG-N_3 MS, sample 4) made from equal-weight mixture of PBALG and PBN_3LG.

Since the azide group content is higher than that of alkyne group in the mixed polymer, some azide groups were preserved after surface cross-linking, which can be proved by the FTIR spectrum of iECC-PLG-N_3 MS (around 2100 cm^{-1} in Figure 2). The residual

azide groups could be used for further conjugation (Scheme 3B). As an example, a red fluorescent robe, alkyne-functionalized rhodamine B, [17] was conjugated onto the surface of iECC-PLG-N_3 MS. The fluorescent microscopic image (Figure 3A, from sample 4) clearly showed the red fluorescence covering MS surface, which also proved the reactivity of the azide groups on MS surface, implying the practicability of surface modification on iECC-PLG-N_3 MS by click reaction. To further prove the pore interconnectivity of porous MS (sample 3) and detect the difference between porous MS and nonporous MS, the fluorescent images of the cross-sections of rhodamine B labeled porous and nonporous MS were also observed by confocal laser scanning microscope (CLSM) using Z-section and measurement. The CLSM images showed that the red fluorescence covers most part of the cross-section of rhodamine B loaded porous MS (Figure 3B). As for nonporous MS, only a red ring is observed (Figure 3C, sample 4). These results further confirmed the interconnectivity of the formed pores, which enabled the permeation of alkyne functional fluorescent dye to conduct click reaction with azide groups on the surface of MS surface in the process of surface conjugation.

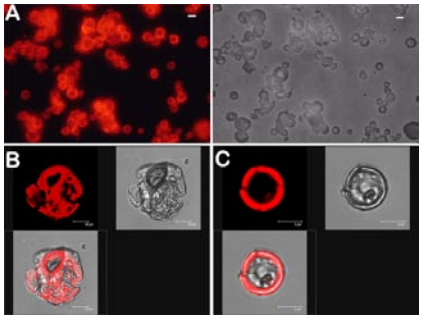


Figure 3. **A**: Fluorescence microscope image (left) and white light control (right) of Rhodamine B loaded iECC-PLG-N_3 MS (sample 3) (bar = 10 μm). **B** and **C**: The fluorescent images of the cross-sections of porous (**B**, sample 2) and nonporous (**C**, sample 4) microspheres were taken by confocal laser scanning microscope (CLSM) using Z-section and measurement.

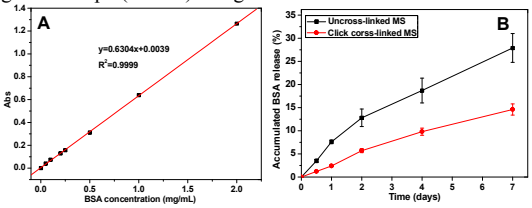


Figure 4. BSA standard curve (A) and the accumulated release of BSA from uncross-linked and click cross-linked MS.

To investigate whether emulsion surface click cross-linking can improve drug loading efficiency and reduce the initial burst release or not, BSA was chosen as model to do the drug loading and release research. The click crosslinked MS do possess higher drug loading efficiency. The BSA encapsulation percentage was determined to be about 64.5% with a drug loading capacity around 19.6%. BSA loaded uncross-linked MS (using no copper catalyst) was also prepared as a control, the BSA encapsulation percentage of it was around 42.4%, and the drug loading capacity was about 13.8%. From figure 4, it can be seen that after applying surface click cross-linking, the BSA release speed decreased, and the total release amount of BSA in 7 days were also much lower than that of BSA released from uncross-linked MS, which agrees with the previous report. [5]

Conclusions

In conclusion, copper-catalyzed azide-alkyne cycloaddition (CuAAC, click chemistry) was applied in the process of microspheres preparation using O/W emulsion method as both surface cross-linking route and surface conjugation method. And the co-effect of surface click cross-linking and porogen on the morphology/shape of the obtained microspheres/ microparticles was investigated. By applying surface click cross-linking and porogen simultaneously, the pores formed by porogen can be sealed to some extent. Microspheres/microparticles with different morphology/ shape were obtained by adjusting polymer concentration and copper catalyst ratios. The emulsion surface click cross-linked microspheres can be used as cross-linked drug carrier to improve drug loading efficiency and reduce the initial burst release. Some azide groups were preserved on the surface of the obtained microspheres, which could be further used for further conjugation with fluorescent probe or targeting unit for the applications of bioimaging or targeting. Click chemistry played a dual role as emulsion surface cross-linking route and surface conjugation method, which not only expanded the application areas of click chemistry in biomaterials design, but also provide an interesting way of surface cross-linking and morphology/shape adjusting method of microspheres made by emulsion method.

Experimental Section

Materials

Alkyne and azide functionalized poly (γ-benzyl-L-glutamate), abbreviated as PBALG and PBN_3LG separately, were synthesized by ester exchange reactions between poly (γ-benzyl-L-glutamate) (PBLG) and propargyl alcohol (for PBALG) or 3-azidopropanol (for PBN_3LG) respectively (Scheme 1), according to our previous work. [22] The average molecular weights of PBALG and PBN_3LG are 13.3 KDa and 14.3 KDa (by ^{1}H-NMR) respectively, and the degrees of functional group substitution of PBALG and PBN_3LG used are 19.8% and 37.5% respectively. L-ascorbic acid sodium salt (NaLAc) was obtained from Acros Organics. Rhodamine B was obtained from Sigma. Bovine serum albumin fraction V (BSA, 99%) was from Sigma-Aldrich. All other reagents were commercially available and used without further purification.

Instrumentation

Fourier transform infrared (FTIR) spectra were measured with a Bruker Vertex 70 spectrometer using KBr pellets. The morphology of microspheres was observed by a field emission scanning electron microscope (SEM) (Model XL 30 ESEM FEG from Micro FEI Philips). Fluorescence microscope pictures were taken at an excitation wavelength of 555 nm (for Rhodamine B), and took a white light graph as comparison. The fluorescent images of the cross-sections of rhodamine B labeled porous and nonporous microspheres were taken by confocal laser scanning microscope (CLSM, TCS Sp2, Germany) using Z-section and measurement. BSA concentration was determined by UV absorbance at 280 nm using a UV-2450 spectrometer (Shimadzu, Japan) with a minimum wavelength resolution of 0.2 nm.

Preparation of microspheres

In situ emulsion click cross-linked azide-functional poly (L-

glutamate) microspheres (iECC-PLG-N_3 MS) was prepared by *in situ* surface cross-linking the O/W emulsion of equal-weight mixture of PBALG and PBN_3LG using copper (I)-catalyzed 1,3-dipolar azide-alkyne cycloaddition (CuAAC) (Scheme 2). Typically, equal-weight mixture (0.1 g) of PBALG (0.05 g) and PBN_3LG (0.05 g) was dissolved in 1, 2-dichloroethane/DMAc (5 mL, v/v=4:1, polymer concentrations in oil phase is 2% (w/v)), 0.1 mL decahydronaphthalene (as porogen) was then added the in the solution. The solution was dispersed in 1.0 wt% of poly (vinyl alcohol) (PVA) solution (100 mL) by high-shear dispersing emulsifier at a speed of 2000 rpm for 2-3 min. Then the O/W emulsion was poured into another 400 mL of PVA solution (1.0 wt%), which contained $CuSO_4$ (1.0eq. to alkyne group). After stirred for 1-2 min, NaLAc (1.0eq. to alkyne group) was added into the system, and the mixture was stirred at 40°C for 12-24 hrs to evaporate the solvent. After that, the microspheres were collected by centrifugation and washed several times with water and hot water, and then freeze-dried. iECC-PLG-N_3 MSs with different polymer concentrations in oil phase, porogen contents, as well as CuAAC catalyst system ($CuSO_4$-NaLAc) contents (shown in Figure 1C, D and E) were prepared. Uncross-linked microspheres made from equal-weight mixture of PBALG and PBN_3LG with or without porogen (A and B in Figure 1) were also prepared as the same method but without CuAAC catalyst addition. And cross-linked microspheres without porogen were also prepared as a control. The microspheres were characterized by SEM and FTIR.

Rhodamine B labeled ISCC-PLG-N_3 microspheres

Rhodamine B was first functionalized with alkyne group to give alkynyl-Rhodamine B using the method described before. [22] Then alkynyl-Rhodamine B was reacted with iECC-PLG-N_3 MS through CuAAC (Scheme 3) to get Rhodamine B labeled iECC-PLG-N_3 MS. The detail was described in the reference, [22] and the MS was characterized by fluorescence microscope with the result shown in Figure 3A. Alkynyl-Rhodamine B loaded porous microspheres (MS) and nonporous MS were also observed by confocal laser scanning microscope (CLSM) using Z-section and measurement, the images are shown in Figure 3B and C.

Drug loading and release of ISCC-PLG-N_3 microspheres

BSA was chosen as the drug model to do the drug loading and release research. Briefly, 1 mL BSA (0.1 g) solution in DI water was dispersed in 5 mL polymer (contains 0.125 g PBALG and 0.125g PBN_3LG, and 0.25 mL decahydronaphthalene) solution in 1, 2-dichloroethane/DMAc (v/v=4:1) to form into W/O emulsion. The W/O emulsion was then dispersed in 100 mL 1.0% PVA solution by by high-shear dispersing emulsifier at a speed of 2000 rpm for 2-3 min to form into W/O/W emulsion, 400 mL of PVA solution (1.0 wt%) was added under stirring. Then calculated amount of $CuSO_4$ (1.0eq. to alkyne group) and NaLAc (1.0eq. to alkyne group) was added. The MS purification process was the same as that of drug-free MS. The supernatant was collected and freeze-dried before re-dissolving in DI water to measure the free BSA amount.

The BSA release study was conducted by putting 100 mg of BSA loaded MS in a dialysis tube with a molecular weight cut-off of 20 KDa, the dialysis tube was immersed in 20 mL of PBS (pH 7.4) at 37°C with shaking. At pre-setted time points, 1 mL of PBS solution was taken out, and 1 mL of fresh PBS was added. The concentration of BSA in PBS was determined by UV-vis, according to the standard curve obtained at the same time (Figure 4A).

Acknowledgements

The authors would like to thank the financial support from the Ministry of Science and Technology of China (863 Project, No. SS2012AA020603), National Natural Science Foundation of China (No. 51321062 and 21174143), "100 Talents Program" of the Chinese Academy of Sciences (No.KGCX2-YW-802), and Jilin Provincial Science and Technology Department (No. 20100588).

Notes and references

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TOC

Emulsion click microspheres: Morphology/Shape control by surface cross-linking and porogen

Jinshan Guo,[a,b] Dongfang Zhou, [a] Jianqing Hu, [b,c] Xuesi Chen,[a] Xiabin Jing,[a] and Yubin Huang [a,*]

Click chemistry was applied as a dual role in emulsion microspheres (MS) preparation to realize *in situ* cross-linking and bioconjugation in the same system. The morphology/shape of the MS can be adjusted by applying surface click cross-linking and porogen simultaneously. This MS has the potentials to be used as cross-linked drug carrier to improve drug loading efficiency and reduce the initial burst release. The residual azide groups on the surface paved the way of further click surface conjugation with bioactive molecules.