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Modified chitosan emulsifiers: small compositional changes produce vastly different high internal phase emulsion types

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Abstract

High internal phase emulsions (HIPEs) are indisputably a core technology for various industries involving pharmaceuticals, food, cosmetics, biologics but they usually require surfactants/co-surfactants to form, which is often undesired. More specifically, micro-HIPEs are thermodynamically stable, optically clear emulsions with droplet sizes in the range of around 1 - 100 nm that form spontaneously with little energy input but are rare. Mini-/macro-HIPEs have larger droplet sizes in the range of 50 - 500 nm and > 500nm, respectively, and typically require high energy input for emulsification. We have synthesized a series of <u>chito</u><u>s</u>an-graft-oligo<u>N</u>-isopropylacrylamide-graft-oligo<u>Lys</u>ine (CSNLYS) copolymers that act as both emulsifiers of HIPEs without needing extraneous surfactants as well as the matrix material of the resulting porous solid polyHIPE. By merely adjusting the length of the oligoLysine graft from relatively long to medium to short, we can form either a micro-, mini- or macro-HIPE, respectively. These emulsions can then be solidified into porous polymers, polyHIPEs, by merely increasing the temperature by exploiting the copolymer thermo-responsiveness and then removing the solvents. These porous polyHIPE, particularly the ones from micro-HIPEs, have surface areas as high as 988 m^2/g and pore sizes below 200 nm.

Microemulsions with droplet sizes in the range of 1 - 100 nm[1] and miniemulsions with droplet sizes of 50 - 500 nm[2] are used in a myriad of products including pharmaceuticals, food, cosmetics, *etc* due to their ultra-high interfacial area and ability to solubilize both hydrophilic and hydrophobic molecules[3]. These emulsions are typically formed using high concentrations of surfactants and co-surfactants[4]. Microemulsions are particularly attractive also because they are thermodynamically stable and high-intensity emulsification is not needed for their formation so that large fragile biomacromolecules such as proteins and DNA, can be encapsulated without damage[5]. Further, emulsions with dispersed phase volume fractions greater than 74%, known commonly as <u>h</u>igh <u>i</u>nternal <u>p</u>hase <u>e</u>mulsions (HIPE), are particularly attractive because of their high interfacial area. Thus far, there is
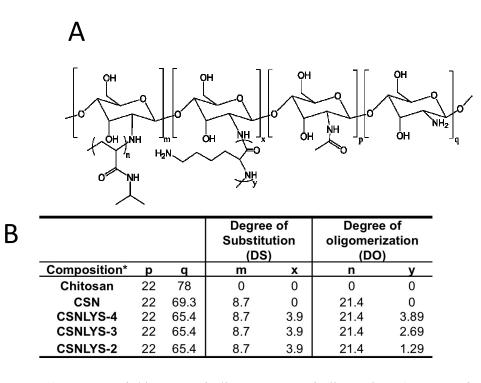
only one report on high internal phase microemulsions,[6] because of the challenge of designing novel surfactant/co-surfactants that will sufficiently reduce the interfacial tension.

Emulsion technology offers a convenient route for the synthesis of porous polymer solid foams. Common methods to produce porous polymers include chemical and physical blowing or porogen leaching, which often allow only limited control over pore structure and content[7]. While particle templating allows for the production of highly porous polymers, the removal of inorganic templates often requires harsh conditions, which might affect the integrity of the structure[8]. Hence, HIPE templating has emerged as a facile method for producing high porosity polymer foams by solidification of the continuous minority emulsion phase and subsequent removal of the dispersed phase[9]. The foam properties such as porosity, pore structure and degree of pore interconnectivity, can usually be easily controlled[10]. However, a key challenge in the application of microemulsion templates to create porous solid foams with small pore sizes less than 100 nm is the destabilization of the emulsion during the polymerization/hardening process because of changing emulsion composition[11].

Herein, we show that graft-copolymers of chitosan-*graft*-oligo(*N*-isopropylacrylamide)-*graft*-oligoLysine (CSNLYS) (**Figure 1A**) display multi-functionality so that these polymers alone when added to water and oil solvents can form HIPEs as well as polyHIPEs. Firstly, the graft copolymers act as emulsifiers for HIPEs without needing extraneous surfactant. Secondly, we discovered that by tuning the chain length of the grafted oligoLysine from a small (about 1.3) to a medium (2.7) to a many repeat units (3.9) (**Figure 1B**), the HIPE type produced can be varied from macro- to mini- to micro-emulsion types, respectively. Remarkably, when the oligoLysine chain length is the longest, we achieve optically clear oil-in-water (o/w) micro-HIPEs mixing oil and aqueous phase – without any high energy input. Thirdly, these copolymers also dissolve in the aqueous phases of the emulsion templates and solidify to form high porosity porous polymer foams as the temperature is raised above the copolymer's cloud point and with subsequent removal of solvent from the initial internal emulsion phases by freeze-drying. Various types of porous polymer foams, *i.e.* macro-/mini-/micro-polyHIPEs can be formed by solidifying the macro-/mini-/micro-emulsions stabilised by the CSNLYS derivatives having the shortest/medium/longest chain lengths of oligoLysine above its cloud point through the temperature-induced aggregation of the grafted oligoNIPAM[12]. Particularly, the micro-polyHIPE has sub-micron sized pores below 200 nm and a huge surface area. This quick and simple solidification method allows us to easily lock the emulsion template structure, removes the need for additional crosslinking agents and requires only relatively low temperatures, exceeding 34°C - the copolymer's cloud point (**Table 1**). The ability to solidify emulsion templates at such a low temperature as compared to conventional polymerization methods, which require the use of elevated temperatures or UV radiation, provides a route for co-encapsulation of cells or temperature-sensitive proteins or drugs and is therefore suitable for tissue engineering, encapsulation of biological ingredients or for drug and gene delivery applications.

A

B

Composition*	p	q	Degree of Substitution (DS)		Degree of oligomerization (DO)	
			m	x	n	y
Chitosan	22	78	0	0	0	0
CSN	22	69.3	8.7	0	21.4	0
CSNLYS-4	22	65.4	8.7	3.9	21.4	3.89
CSNLYS-3	22	65.4	8.7	3.9	21.4	2.69
CSNLYS-2	22	65.4	8.7	3.9	21.4	1.29

*Figure 1*A) Structure of chitosan-*graft*-oligoNIPAM-*graft*-oligoLysine, B) Degree of substitution (DS) and degree of oligomerization (DO) as determined from NMR integrals. *p+q+m+x =100 whereby m=DS of oligoNIPAM (%), x=DS of oligoLysine (%), n=DO of grafted oligoNIPAM and y=DO of grafted oligoLysine.

The chitosan-*graft*-oligo(*N*-isopropyacrylamide)-*graft*-oligoLysine (CSNLYS) was synthesized via a 2-step reaction: firstly, by graft radical copolymerization of *N*-isopropylacrylamide (NIPAM) onto a chitosan macroradical formed by ceric mediated redox initiation (**Figure S1**). Secondly, the polycondensation of the residual amine groups in chitosan-*graft*-oligoNIPAM (CSN) with lysine monomers in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC.HCl) and *N*-hydroxysuccinimide (NHS) produces CSNLYS (Figure 1A). By adjusting the ratio of lysine to the amino groups of CSN from 2:1 to 3:1 and 4:1, we synthesized three graft copolymers - CSNLYS-2, CSNLYS-3 and CSNLYS-4 - with different oligoLysine chain lengths (Figure 1B). The synthesis of these copolymers was confirmed by ^{1}H-NMR analysis (**Figure S2**). The molecular weights of CS, CSN, CSNLYS-2, CSNLYS-3 and CSNLYS-4 were determined using GPC to be 1.80, 2.25, 2.32, 2.34 and 2.35 x10^6 Daltons, respectively (**Figure S3**).

The degree of substitution (DS) and the degree of oligomerization (DO) of the two grafts – oligolysine and oligoNIPAM - were determined from ^{1}H-NMR integrals of the modified chitosan derivatives; DS and DO were denoted by m or x (based on 100 repeat units) and n or y (based on 1 glucosamine unit), respectively, as shown in Figure 1A-B. For the oligoNIPAM grafts, the DS and DO are similar for all the three CSNLYS derivatives (m=8.7, n=21.4). For the oligoLysine grafts, the three copolymers CSNLYS-2, CSNLYS-3 and CSNLYS-4 yielded similar DS values (x=3.9) while differing in their DO (CSNLYS-L, y=1.29; CSNLYS-M, y=2.69; CSNLYS-H, y=3.89) (**Figure 1B**). The amount of residual unreacted –NH$_2$ on the graft copolymers was 65.4 mol%, which indicated that a significantly large fraction of the glucosamine monomers remained unmodified by lysine condensation,

suggesting that grafting of lysine onto the chitosan backbone may not be as favourable as condensation of oligoLysine in free solution, possibly because of steric hindrance.

Unlike native chitosan which only dissolves in water with a pH below 6.4[13], all CSNLYS derivatives are water-soluble over the whole pH range from 1-12 and have a solubility of up to 40%w/v at pH 7 (Table 1). They also have a cloud point of 34°C (Figure S4). The differences between CSNLYS-2, CSNLYS-3 and CSNLYS-4 are evident in their solution and interfacial properties (Table 1). ζ-potentials of CSNLYS measured at pH 7 increased from 0.1±0.1 mV for CSNLYS-2 to 1.5±1.0 mV for CSNLYS-3 and to 7.5±1.5 mV for CSNLYS-4 because of the increasing length of the positively charged oligoLysine grafts. The interfacial tensions of aqueous solutions of the three copolymers measured against air (γ_{wa}) at a concentration of 1%w/v were similar corroborating the similar oligoNIPAM grafts (with similar m and n, Figure 1B). On the other hand, stark differences were observed in interfacial tensions γ_{wo} of these solutions measured against *p*-xylene (oil) (Table 1) corroborating the different DO values of different copolymers (y in Figure 1B); γ_{wo} was 36.8 ± 0.1 mN/m (without any polymer) while CSN in water lowered γ_{wo} significantly to 11.7 ± 0.2 mN/m; on the other hand, CSNLYS-2 lowered γ_{wo} to 12.6 ± 0.1 mN/m and CSNLYS-3 to 6.0 ± 0.1 mN/m and that of CSNLYS-4 is zero or very close to zero as the aqueous droplet was seen solubilizing instantly in the oil phase, resulting in the emulsion seen in Figure 2C1.
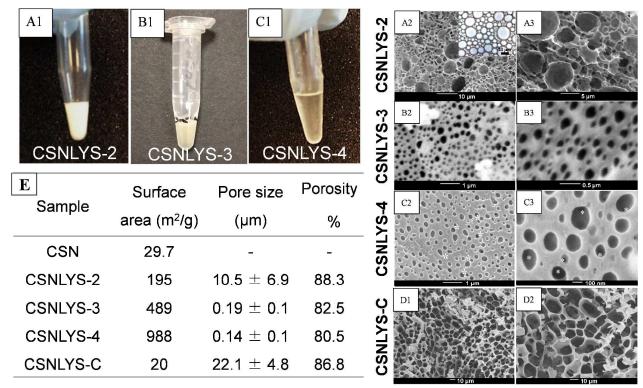
Table 1. Physical properties of CSNLYS-2, -3 and -4: Maximum solubility, ζ-potential, cloud point and interfacial tensions γ_{wa} and γ_{wo}. All measurements were made at pH 7, the pH during emulsification, except for CS, which had to be dissolved at pH 6. [a] Interfacial tension of aqueous sample in air. [b] Interfacial tension of aqueous sample in *p*-xylene.

Sample	Max solubility (%w/v)	ζ (mV)	Cloud point (°C)	γ_{wa} (mN/m) [a]	γ_{wo} (mN/m)[b]
CS	1	1.5 ± 0.4	-	-	-
CSN	2.5	0.8 ± 0.4	35	44.0 ± 0.4	11.7 ± 0.2
CSNLYS-2	40	0.1 ± 0.1	34	44.6 ± 0.2	12.6 ± 0.1
CSNLYS-3	40	1.5 ± 1.0	34	40.3 ± 0.4	6.0 ± 0.1
CSNLYS-4	40	7.5 ±1.5	34	40.9 ± 0.8	~ 0

Emulsions were prepared at room temperature by mixing 125 µl of either 20%w/v CSNLYS-2, CSNLYS-3 or CSNLYS-4 dissolved in water at pH 7 as continuous aqueous phase and 375 µl of *p*-xylene as internal oil phase to give an oil-in-water (o/w) HIPE with φ = 0.75. (The emulsions should be more accurately called inverse-or o/w HIPEs but for simplicity sake, we just call them HIPE.) When mixing CSNLYS-2 in a vortex mixer at 2500 rpm, a milky emulsion resulted (**Figure 2A1**), while CSNLYS-3 produced a translucent emulsion (**Figure 2B1**). A clear transparent one-phase emulsion spontaneously formed upon the addition of oil to the aqueous solution containing CSNLYS-4 (**Figure 2C1**). The emulsion stabilized by CSNLYS-2 was a macro-HIPE with droplet sizes of 13.6 ± 6.0 µm (measured by optical microscopy, see inset in Figure 2A2) possibly because of the higher γ_{wo} requiring more energy to form. The emulsion type was investigated using the drop test (Figure S5) to be an o/w emulsion at both room temperature and the solidification temperature (40°C). When CSNLYS-3 was investigated as the

emulsifier, the lower γ_{wo} allowed a mini-HIPE to form after vortex agitation, which was rather translucent in appearance. Remarkably, when CSNLYS-4 was used, a clear mixture was formed spontaneously when *p*-xylene was added to the aqueous phase (Figure 2C1). This phenomenon suggests the formation of micro-HIPEs[14] in which the diameter of the emulsion droplets is smaller than ¼ wavelength of visible light. The low interfacial tension of near zero results in the spontaneous formation of a HIPE with nano-sized droplets when CSNLYS-4 was present in the aqueous phase. The stability of HIPEs stabilized by the CSNLYS derivatives can be attributed to the fact that CSNLYS is surface active due to its overall amphiphilicity. The polysaccharide backbone and grafted oligoLysine side chains of CSNLYS form the hydrophilic component. On the other hand, the oligoNIPAM graft chains could be present in both oil and water phases due to the isopropyl groups and vinyl backbone that are lipophilic[15] and the amide linkages (*i.e.*- C(O)NH-) that are hydrophilic and participate in hydrogen bonding at temperatures below the copolymer's cloud point. We postulate that within the water phase, smaller oil droplets co-exist.

Sample	Surface area (m²/g)	Pore size (µm)	Porosity %
CSN	29.7	-	-
CSNLYS-2	195	10.5 ± 6.9	88.3
CSNLYS-3	489	0.19 ± 0.1	82.5
CSNLYS-4	988	0.14 ± 0.1	80.5
CSNLYS-C	20	22.1 ± 4.8	86.8

Figure 2. **(A1) – (C1)** Photographs of HIPEs formed by A1) CSNLYS-2, B1) CSNLYS-3 and C1) CSNLYS-4. **(A2) – (C3)** SEM images of polyHIPEs produced by solidifying the HIPEs after removal of solvent and internal phase by lyophilization. (A2) – (A3) CSNLYS-2 (inset shows an optical micrograph of HIPE) (B2)-(B3) CSNLYS-3; and (C2)-(C3) CSNLYS-4 *Asterisks show positions of some of the open pores. **(D1)-(D2)** Control foam (CSNLYS-C) using CSNLys-2 with no *p*-xylene added. Please note that the scale bars are different because of the pore size. **(E)** PolyHIPE properties: Surface area, average pore size and porosity.

The HIPEs were solidified by raising the temperature above the copolymers' cloud points, which were determined using cloud point measurements to be 34°C for the three CSNLYS copolymers (Table 1), so that phase separation and aggregation of the thermoresponsive oligoNIPAM side chains occurred in the aqueous phase to reject water molecules[12a]. These HIPEs remained stable with increasing temperature as the oligoNIPAM grafts in the oil phase still remain soluble[16]. Furthermore the high molecular weight chitosan increased the viscosity of the continuous aqueous phase, which contributes to the kinetic stabilization of the HIPEs[17].

After removing the template, *i.e.* both the dispersed oil phase and water from the continuous phase by freeze-drying, porous solid polyHIPEs were obtained. The resultant polyHIPEs inherited the thermo-responsiveness: they retained their form above the cloud point and dissolved completely in water below the cloud point **(Figure S6)**. PolyHIPEs were characterized by SEM and BET (**Figure 2E**). The polyHIPE porosity was generally high (>80%). However, the pore structure and sizes differed greatly (Figures 2A2-A3, B2-B3 and C2-C3). Using CSNLYS-2, a control foam without *p*-xylene was also prepared by thermally induced phase separation (CSNLYS-C, Figure 2, D1-D2). The surface areas of the polyHIPEs formed by CSNLYS-2, CSNLYS-3 and CSNLYS-4 (Figure 2E) were much higher (195, 489 and 988 m^2/g, respectively) as compared to the control foam CSNLYS-C (20 m^2/g): additional interface was created during emulsification, which resulted in a greater surface area after removal of both oil and water by freeze-drying. Also, secondary pores formed in the polymer walls of the polyHIPEs also contribute towards the large surface area.

With CSNLYS-4, the polyHIPE formed had the highest surface area because of the much smaller interconnected pores (indicated by asterisks in Figure 2C2-C3) as compared to CSNLYS-C and CSNLYS-2/3 polyHIPEs; the pores in CSNLYS-4 polyHIPEs were interconnected (Figure 2C3). From the SEM images, it is seen that the pore structure does not show the typical closed packed pore structure of polyHIPEs nor the high porosity measured. We hypothesized that this is attributed to the presence of even smaller secondary pores that cannot be visualized by SEM but is apparent in their contribution to the increased surface area (**Figure S7**). These smaller pores are formed possibly due to the hydrophobic character of the oligoNIPAM side chains which participated in the formation of smaller nano-droplets of oil in the water phase, which in turn contributed to the porosity and high surface area of the CSNLYS-4 polyHIPE.

Our results show that various chitosan-*graft*-oligoNIPAM-*graft*-oligoLysine derivatives with varying oligoLysine chain lengths can stabilize respective oil-water emulsions to form HIPEs without the need for extraneous surfactant. We postulate that a portion of the oligoNIPAM side chains extended into the oil phase while the oligoLysine side chains were compatible with the water phase. Also, the relatively rigid polysaccharide backbone forms extended segments at the o/w interface minimizing the total energy of the system. While keeping the DS and DO of oligoNIPAM constant, a shorter chain length of grafted oligoLysine resulted in the formation of a macro-HIPE. However, a longer chain length of oligoLysine resulted in γ_{wo} being zero and the formation of micro-HIPE without the need for additional surfactants, which are typically required for most conventional macro-/mini-/micro-emulsions[6, 14, 18]. With the amount of hydrophobic hydrocarbon moieties being constant, a larger hydrophilic group contributed by longer oligoLysine chain increases the hydrophilicity and also the hydrophilic-lipophilic balance of the CSNLYS-4 derivative[19], thereby increasing solubilisation of the aqueous phase and the
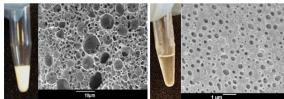
oil/water miscibility. Indeed, without oligoLysine side chains, CSN is only soluble up to 2%w/v in water and does not stabilize (o/w) HIPEs (Figure S8) as the lower polymer content compromises emulsion stability.

In summary, we reported the synthesis of multifunctional thermoresponsive water-soluble chitosan derivatives that are able to 1) stabilize high internal phase oil-in-water emulsions, 2) allow the solidification of HIPE templates with temperature increase and without chemical crosslinking and 3) act as the polyHIPE matrix itself. By varying the chain length of oligoLysine grafts on CSNLYS, we found that very different emulsion types formed, namely micro- to mini- to macro-HIPEs. Interestingly, micro-HIPEs can be formed with just mixing CSNLYS-4 with water and *p*-xylene by hand. We also showed that the formation of highly porous polyHIPEs with high surface areas (up to 988 m^2/g) is also possible without using additional surfactant to stabilize the HIPEs and crosslinking agents with our chitosan-graft-oligoNIPAM-graft-oligoLysine derivatives.

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HIPE → polyHIPE   microHIPE → polymicroHIPE!

By varying the oligolysine units of chitosan-*graft*-oligoNIPAM-*graft*-oligoLysine, high internal phase emulsions of different droplet sizes can be stabilized which can subsequently serve as template for macroporous polymers.