Novel hybrid reversed phase packing material for high productivity of insulin purification

Hiroki Kanezaki, Chiaki Iwata, Moemi Miyashita, Hiroyuki Tanaka, Takashi Sato, Noriko Shoji, and Naohiro Kuriyama

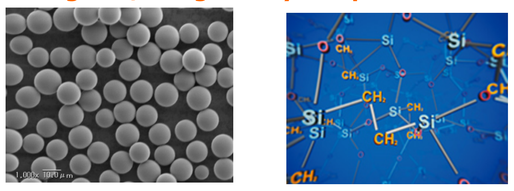
YMC Co., Ltd.

Introduction

Reversed phase chromatography plays an important role in the purification of high value–added insulin pharmaceuticals. However it is very important to improve the productivity in the industrial purification of insulin. Therefore, packing materials which have long lifetimes and high separation performances are constantly required. In order to meet such demands, YMC has developed a novel reversed phase C8 material based on a novel hybrid silica, "Triart-SIL". It's widely known that the choice of the appropriate pore size and hydrophobicity is important for purification of peptides and proteins, and the packing materials with bonded C8 ligands and 200 Å pore size increase the efficiency for insulin purification. To enhance the separation performance and stability for large scale production of insulin, we have optimized the pore size distribution, the density of C8 functional groups and the chemical bonding method. In this poster, we will show characteristics and improvements of this novel hybrid based packing C8 material, and the chromatographic performance and advantages for use in purification of insulin.

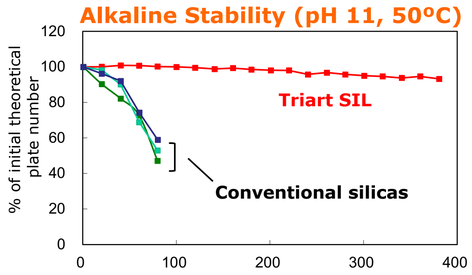
Features and benefits of hybrid silica, "Triart-SIL" base material

Organic/inorganic hybrid particle



- Triart-SIL is an organic/inorganic hybrid silica. Its structure contributes towards the physical and chemical stability and to the high chromatographic performance.
  In particular, it has much greater stability under alkaline conditions than conventional silica.

Alkaline Stability (pH 11, 50ºC)



Alkaline durability test conditions

Column:	(5~10 µm, 10~12 nm) 150 x 4.6 mm ID
Eluent:	acetonitrile/water/NH_3 (90/10/0.1) [0.1% NH_3 (pH 11.3) in acetonitrile/water (90/10)]
Temperature:	50 ℃
Flow rate:	1.0 mL/min
Sample:	cytosine

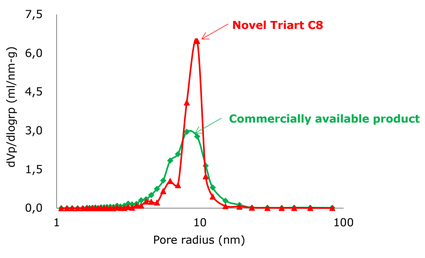
C8 Packing materials based on hybrid silica to enhance productivity of insulin purification

- In this study, we compare various packing materials with Novel Triart C8 which has an optimized pore size distribution and bonding chemistry.

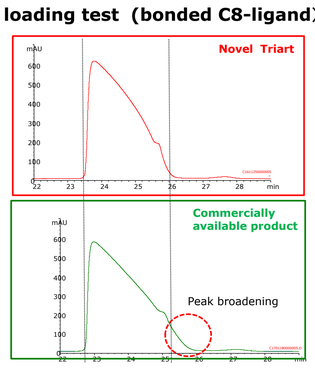
	Novel Triart C8	Prototype C8	Commercially available product
Base material	Triart-SIL (Hybrid silica)	Triart-SIL (Hybrid silica)	Hybrid silica
Particle size (µm)	10	10	10
Pore size (Å)	200 (Optimized pore distribution)	200 (Optimized pore distribution)	200
Bonding chemistry	Tri-functional C8	Tri-functional C8	Tri-functional C8
	Ligand density: High (Optimized modification)	Ligand density: Low	Ligand density: Low

Optimal pore distribution

200 Å pore size distribution



loading test (bonded C8-ligand)



HPLC conditions

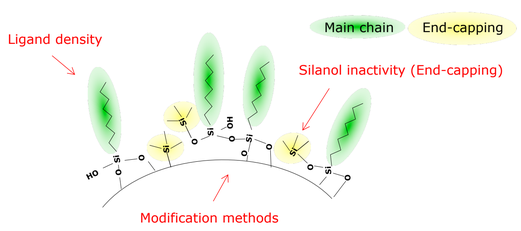
Column Size:	150 x 3 mm ID
Eluent:	A) buffer＊/acetonitrile (90/10) B) buffer＊/acetonitrile (10/90)
Gradient:	0%B (0-3 min) 0-20% (3-6 min) 20-27.5%B (6-33 min) 50%B (33-38 min) 0%B (38-88 min)
Flow rate:	0.43 mL/min
Temperature:	25 ℃
Detection:	UV at 295 nm
Sample:	10 mg/mL human recombinant insulin
Injection:	1000 µL

＊buffer:20 mM CH_3COONH_4-CH_3COOH, 50 mM NH_4Cl (pH4.45)

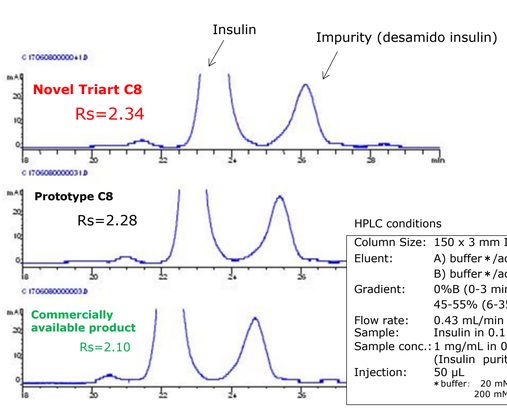
- Packing materials which have different pore size distributions were tested under conditions of insulin overload (estimated loading: about 20 g/kg gel). Although the mean of pore sizes were the same 200 Å, uniformity affects the insulin loading performance. This means that the number of pores which interact with insulin has increased, and the C8 ligand is bonded uniformly in pore. In order to obtain high loadability and high productivity, we needed to optimize pore the size distribution.

Optimal bonding chemistry

Surface modification image



Separation test (bonded C8-ligand)



HPLC conditions

Column Size:	150 x 3 mm ID
Eluent:	A) buffer＊/acetonitrile (90/10) B) buffer＊/acetonitrile (60/40)
Gradient:	0%B (0-3 min) 0-45% (3-6 min) 45-55% (6-35 min) 0%B (35-60 min)
Flow rate:	0.43 mL/min
Sample:	Insulin in 0.1 N HCl left at 40℃ for 16 h
Sample conc.:	1 mg/mL in 0.1% CH_3COOH (Insulin purity about 75%)
Injection:	50 µL

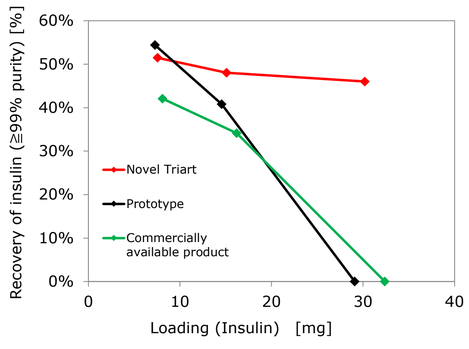
＊buffer: 20 mM CH_3COONH_4-CH_3COOH, 200 mM $(NH_4)_2SO_4$ (pH4.50)

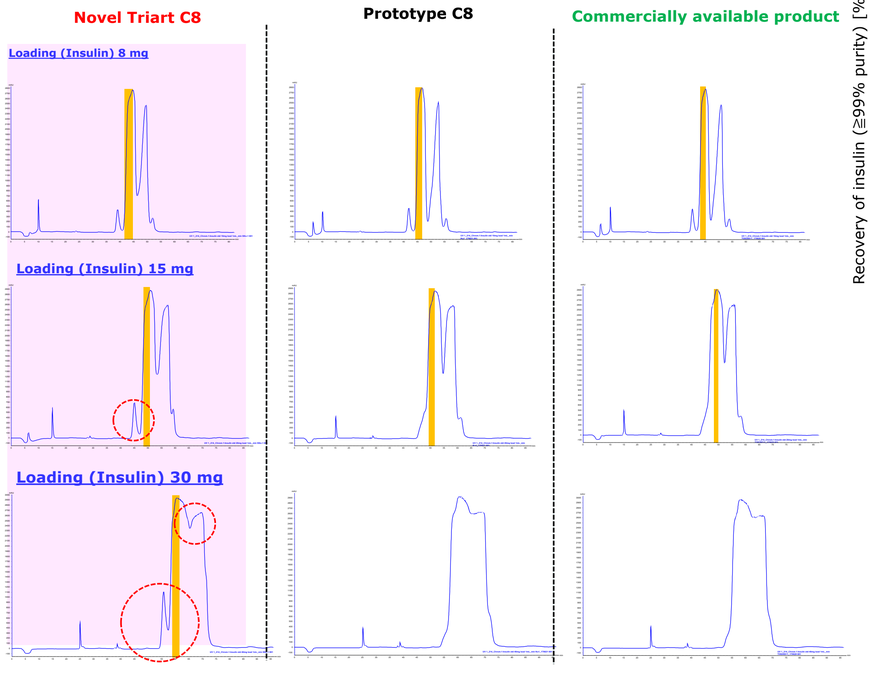
- The efficiency of preparative purification depends on the ability to separate impurities (such as desamido insulin) from insulin. This study evaluated the resolution between insulin and impurities for several packing materials which differed in modification method and density of C8 ligands and end-capping groups. The improved resolution is achieved on the novel Triart C8, which is based on a new hybrid silica with extremely uniform 200 Å pore size and bonded with high-coverage C8 ligands. In addition the novel Triart C8 phase is fully end-capped.

Loadability study on insulin purification

Fraction with ≧99% purity of insulin

Loading and recovery





Preparative conditions

Column Size:	250 x 4.6 mm ID
Eluent:	A) buffer＊/acetonitrile (90/10) B) buffer＊/acetonitrile (60/40)
Gradient:	0%B (0-5 min) 0-45% (5-10 min) 45-55% (10-58 min) 100%B (58-100 min)
Flow rate:	1.0 mL/min
Sample:	Insulin in 0.1 N HCl left at 40℃ for 16 h
Sample conc.:	2 mg/mL in 0.1% CH_3COOH (Insulin purity about 75%)

＊buffer:20 mM CH_3COONH_4-CH_3COOH, 200 mM $(NH_4)_2SO_4$ (pH 4.5)

Fraction analysis

Column:	YMC-Triart C18 (5 µm, 12 nm) 250 x 4.6 mm ID
Eluent:	A)*0.2 M Na_2SO_4, 40 mM H_3PO_4 (pH 2.3) / ACN (82/18) B)*0.2 M Na_2SO_4, 40 mM H_3PO_4 (pH 2.3) / ACN (50/50)
	*pH is adjusted with 2-aminoethanol
	30%B (Isocratic)
Detection:	UV at 230 nm
Flow:	1.0 mL/min
Temperature:	40 ℃

- The novel Triart C8 material maintains superior resolution and recovery at a loading of more than 30 mg insulin with the desired purity (in this case ≧99% insulin) being obtained. In contrast both the prototype and commercially available product failed to meet these criteria.

Effect of pH on stability of Novel Triart C8 material

Acidic stability (pH 1, 70 ºC)

Test procedure

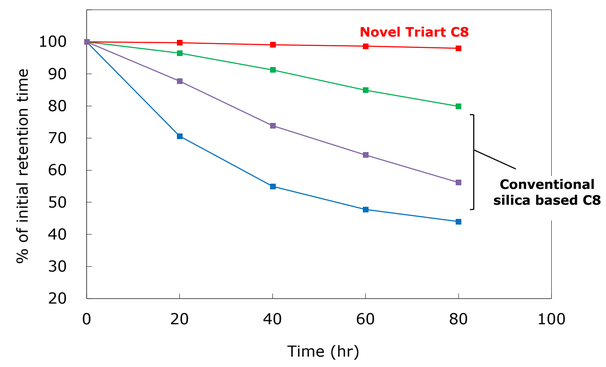
① Inspection of column (HPLC conditions)
Column: 10 µm, 20 nm, 50 × 2.0 mm ID
Eluent: acetonitrile/water (60/40)
Flow rate: 0.2 mL/min
Temperature: 37 ℃
Detection: UV at 254 nm
Sample: *n*-Butylbenzoate

② Purging low pH mobile phase
acetonitrile/water/TFA (10/90/1: pH 1) (0.2 mL/min) for 10 column volumes

③ Storage
20 hr storage at pH 1, 70 ℃

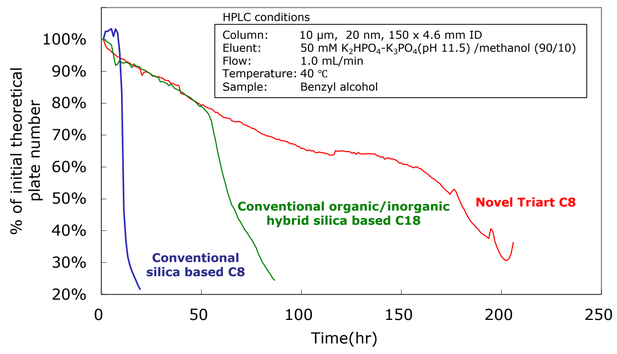
④ Removal of eliminated ligand
acetonitrile/water (60/40) (0.2 mL/min) for 10 column volume,
acetonitrile (1.0 mL/min) for 10 column volumes

Repeat for 4 cycles



Alkaline stability (pH 11.5, 40 ºC)

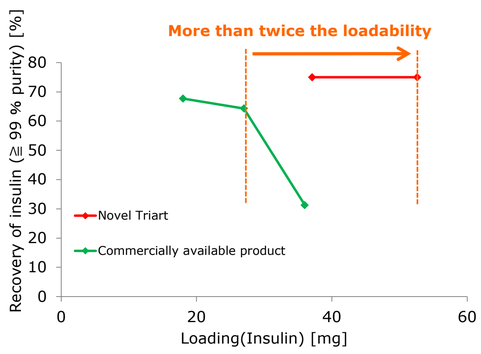
- In addition to using hybrid silica as the base material, further optimization of pore size distribution and bonding chemistry provides high chemical stability at both low and high pH. This provides greater flexibility in method development for preparative separations, and also allows the effective cleaning of the gel with alkaline solutions which is often required in purification processes for peptides.



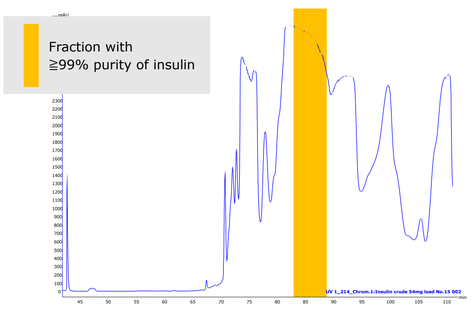
Effect of productivity using the Novel Triart C8 material

Purification of crude insulin human recombinant

Loading and recovery



52.6 mg load chromatograms (Novel Triart C8)



- In crude insulin purification, Novel Triart C8 would produce more than twice as much purified insulin as commercially available product, and with remarkably reduced costs.

Preparative conditions

Column:	250 x 4.6 mm ID
Eluent:	A) 20 mM CH_3COONH_4-CH_3COOH, 200 mM NH_4Cl (pH 4.33) /ACN (90/10) B) 20 mM CH_3COONH_4-CH_3COOH, 200 mM NH_4Cl (pH 4.33) /ACN (10/90)
Gradient:	0%B (0-10 min), 0-20%B (10-15 min), 20-25%B (15-45 min)
Detection:	UV at 214 nm
Flow:	0.74 mL/min
Temperature:	ambient
Sample:	Crude human recombinant insulin (Purity 75.8%)

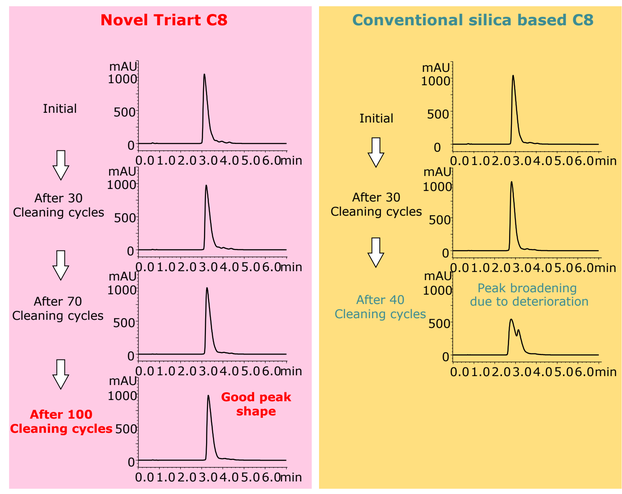
Regeneration of packing materials with alkaline solution

Test procedure

Insulin sample injection ※

Cleaning with alkaline solution
0.1 M NaOH/acetonitrile (50/50)
10 column volumes (CV)

Neutralization and cleaning with organic solvent
①acetonitrile/water (20/80) for 30 CV
②acetonitrile/water (90/10) for 5 CV

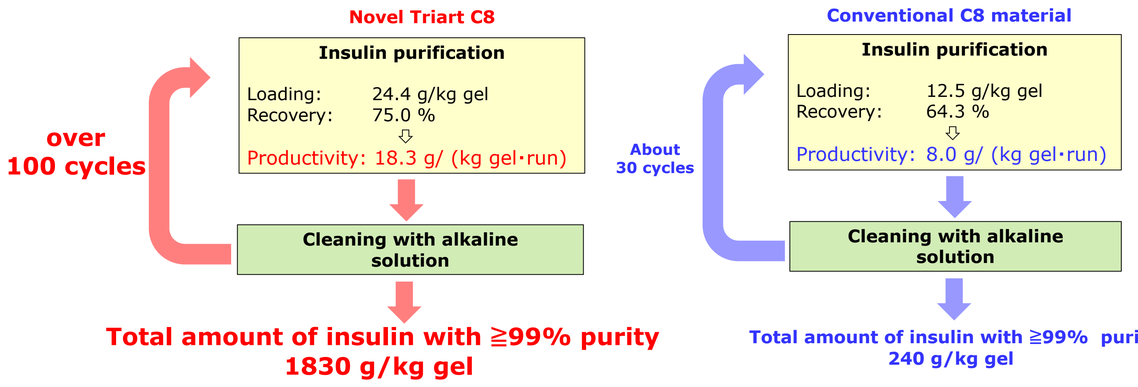


- After repeated injections of a crude peptide sample from actual production, absorption of protein and other impurities on the surface of packing materials degrades the retention capacity and resolution. In such cases cleaning with alkaline solution can have the effect of regeneration of the packing material. The Novel Triart-based C8 phase which has increased stability towards high pH offers more than twice the lifetime compared to conventional silica based materials under conditions of alkaline cleaning. This provides increased cost-effective purifications for crude peptides samples.

※Conditions of insulin sample injection

Column:	(10 µm) 20 nm, 50 x 4.6 mm ID
Eluent:	A) water/TFA (100/0.1) B) acetonitrile
	26-36%B (0-3 min) 36%B (3-4 min) 26%B (4-7 min)
Flow rate:	1.0 mL/min
Temperature:	25 ℃
Detection:	UV at 280 nm
Sample:	10 mg/mL human recombinant insulin
Injection:	30 µL

Productivity comparison in model protocol



- In the above test system, the productivity per run of insulin with ≧99% purity was increased by more than a factor of two and the packing material consumption was reduced by about 70% for the novel Triart-based C8 phase compared to conventional a C8 packing material.

Conclusions

- **YMC has developed a novel packing material based on hybrid silica, which is specially optimized for highly efficient purification of insulin. The novel Triart-based C8 material exhibits high loadability and chemical stability, and offers the most cost-effective production process. This novel packing material will provide efficient purification of insulin and other peptides.**