Cholesterol extraction from ghee using glass beads functionalized with beta cyclodextrin

Muhammad Nazir Tahir · Saleem Ahmed Bokhari · Ahmad Adnan

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Abstract Beta-cyclodextrin (β -CD) was covalently immobilized on glass surface. Functionalized glass surface was characterized by X-ray photoelectron spectroscopy (XPS) and elemental analysis. Glass surface functionalized with β -CD was used to remove cholesterol from ghee (clarified butter fat). 78.8 % cholesterol was reduced in 2 h at 25 °C and 170 rpm. Same surface was used repeatedly for 10 cycles and no reduction in cholesterol removal efficiency was observed. Modified glass surface showed almost no degradation in repeated use in cholesterol reduction experiments.

Keywords Cholesterol reduction · Ghee · β -cyclodextrin · Glass immobilization

Abbreviations

DMF	<i>N,N</i> -Dimethylformamide
DMSO	Dimethyl sulfoxide
Et ₂ O	Diehtyl ether
EtOAc	Ethyl acetate
HCl	Hydrochloric acid
i-pr-OH	Isopropyl alcohol
KOH	Potassium hydroxide

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M. N. Tahir
Department of Bioscience and Biotechnology, Konkuk University,
Seoul 143-701, Republic of Korea

S. A. Bokhari
Department of Biosciences, COMSATS Institute of Information
Technology, Islamabad 44000, Pakistan

A. Adnan (✉)
Department of Chemistry, GC University, Lahore 54000, Pakistan
e-mail: dr.ahmadadnan@gcgu.edu.pk

NaH	Sodium hydride
NH ₄ Cl	Ammonium chloride
THF	Tetrahydrofuran
TLC	Thin layer chromatography
XPS	X-ray photoelectron spectroscopy
β -CD	Beta-cyclodextrin
β -CD-decyne	Mono-6-decyne-cyclodextrin (2)
β -CD-OTs	Mono-6-(<i>p</i> -toluenesulfonyl)-6-deoxy-cyclodextrin (1)

Introduction

Ghee (clarified butter fat) is commonly used in south Asian (Indian, Pakistani, Bangladeshi, Neplai and Iranian) cuisine. Ghee is prepared by heating butter at high temperatures. It is considered an important source of fat in the Indian diet (Kumar et al. 2000). Usually it is prepared from cow or buffalo milk or combination thereof. Ghee is most widely used milk product in the Indian sub-continent. It is consumed as such along with rice or other food preparations. It is also used for frying and seasoning of some spices. Ghee is served in hot melted form and used for garnishing rice or spreading lightly on *chapattis* (local form of wheat bread). Ghee contributes significantly towards nourishment of people of all age groups. It is a good source of fat-soluble vitamins (A, D, E and K) and essential fatty acids (Chand et al. 1986; Rangappa and Achaya 1974; Kumar et al. 2010). Along with these advantages, concerns about its high cholesterol content (0.2–0.4 %) and its relation with cardiovascular diseases is also increasing.

Statistically, there is a strong relation between higher serum cholesterol level and heart diseases (Gurr 1992; Kim et al. 2004). Based on these studies, different physical, chemical, and biological methods to reduce cholesterol from food products have been developed (Kwak et al. 2004; Hansel et al.

2007; Dias et al. 2010). The methods to reduce cholesterol from dairy products include: blending with vegetable oils, adsorption with saponin and digitonin to form cholesterol complexes (Micich 1990), degradation of cholesterol by cholesterol oxidizers, removal by supercritical fluid extraction (Arul et al. 1988), extraction with high-methoxyl pectins (Rojas et al. 2007), and organic solvents (Larsen and Froning 1981). A number of studies have indicated that reducing the cholesterol from dairy products does not show significant effects on their chemical, rheological, and sensory properties (Dias et al. 2010). However, most of these methods are relatively nonselective and along with cholesterol, other lipid-soluble components including flavor and nutritional components may also be extracted.

Beta-cyclodextrin (β -CD) is a cyclic heptamer composed of seven glucose units joined “head to tail” by an α -(1–4) glucopyranose unit link. It has a cavity at the centre of its molecular arrangement, which can form a non-covalent bonding with many molecules having phenyl ring by formation of a reversible host-guest complex (Reddy et al. 2001; Han et al. 2007). β -CD is non-toxic, edible, non-hygroscopic and chemically stable. In previous years, studies have indicated that β -CD can be used to reduce cholesterol effectively from food products (Kwak et al. 2002; Shim et al. 2003; Hwang et al. 2005; Lee et al. 2007). In most of these reports, powdered β -CD was used and it allows about 90 % of the cholesterol to be removed but separation and collection of β -CD after completion of process is a time consuming and laborious task. Therefore, it is required to investigate simpler and economically favorable methods to reduce cholesterol from food products.

Due to biocompatibility, surface immobilization of carbohydrates is considered a very important step in the manufacturing of biosensors and microreactors used in pharmaceutical and food industries. The key requirements for such processes are the preservation of biochemical and/or catalytic properties of immobilized substrates and stability of functional layers on solid surface under various conditions used for its applications (Kuzmin et al. 2010). The trend of surface-modified materials is increasing rapidly in modern science and technology. Applications of such modified surfaces have many advantages over traditional chemical or physical methods e.g. low amount of reagents needed and financial savings due to easy separation after completion of process (Manz and Becker 1998). Different materials e.g. silicon, glass, quartz and organic polymers are being used to manufacture such functional surfaces. Among these materials, glass is more interesting because it is readily available at low cost, has high mechanical stability, and easy surface modification techniques are known for this material (Henry 2006; Kuzmin et al. 2010). Here we report a simple method for functionalization of glass beads surface with β -CD for cholesterol reduction from ghee. These modified surfaces can be separated easily and can be applied repeatedly for several cycles without losing efficiency.

Experimental

Materials and methods

Ghee was purchased from a south Asian based local store. β -CD (97 %), borosilicate glass beads (diam. 2 mm), 1-(*p*-toluenesulfonyl)-imidazole, 10-undecyn-1-ol (≥ 95 %), acetone (99.9 %), hydrochloric acid (HCl, 37 %), dimethyl sulfoxide (DMSO, 99.9 %), acetic acid (99.7 %), tetrahydrofuran (THF), *N,N*-diisopropylethylamine (99.5 %), and copper(I) iodide (99.5 %) were purchased from Sigma-Aldrich. *N,N*-dimethyl formamide (DMF) was from Fluka. Sodium hydride (NaH) and 1,2-epoxy-9-decene (96 %) were from Tokyo Chemical Industry. Methanol (99.9 %), *n*-hexane (99.9 %), isopropanol (99.9 %), ethyl alcohol (99.9 %), acetonitrile (99.9 %), diethyl ether (99.9 %), ethyl acetate (99.9 %) and sodium hydroxide were from Duksan. Sodium azide was from Junsei Chemical Co., Ltd. Korea and DMSO-*d*₆ was purchased from Cambridge Isotope Laboratories, Inc., USA.

Water was purified using Direct-Q Millipore water purification system from SAM WOO S&T Co., Ltd. Korea. β -CD was dried under vacuum until constant weight, 1,2-epoxy-9-decene was further purified by column chromatography (EtOAc/Et₂O 3:1), solvents used for column chromatography were distilled while other chemicals were used without further purification.

NMR spectra were recorded on a Bruker AMX spectrometer at 500 MHz. Thin layer chromatography (TLC) was carried out on Merck Kieselgel 60 F254 plates. TLC spots were visualized by dipping in 5 % sulfuric acid-ethanol and heating at 140 °C. X-ray Photoelectron Spectroscopic (XPS) spectra were recorded by using a Sigma Probe (ThermoVG, UK) photoelectron spectrometer. High-resolution spectra were obtained using monochromatic *Al-K α* X-ray radiation at 15 kV and 100 W and an analyzer pass energy of 50 eV (1.0 eV step size) for wide-scan and 20 eV (0.1 eV step size) for narrow-scan. All high-resolution spectra were corrected with a linear background before fitting.

Synthesis of mono-6-decyne-cyclodextrin (β -CD-decyne, **2**)

β -CD was converted into mono-6-(*p*-toluenesulfonyl)-6-deoxy-cyclodextrin (**1**, β -CD-OTs) as described in (Wang et al. 2011) which in next step was converted into mono-6-decyne-cyclodextrin (**2**, β -CD-decyne). For this purpose, NaH (200 mg of 60 % dispersion in mineral oil, 5 mmol) was cleaned several times with pentane and dry DMF (8 mL) were added in a dry round bottom flask under N₂ atmosphere. 10-Undecyn-1-ol (4.4 mmol) in dry THF (10 mL) was added dropwise while keeping the flask at 0 °C (Fig. 1). After 4 h, hydrogen evolution was finished and CD-OTs (**1**, 1.54 mmol)

Fig. 1 Immobilization of beta-cyclodextrin (β -CD) on glass surface by click reaction

dissolved in dry DMF (10 mL) was added slowly. The reaction was left stirring under N_2 for 48 h before quenching with water (20 mL). Reaction mixture was concentrated under vacuum and product was purified by flash column chromatography (i-Pr-OH: H_2O :EtOAc: NH_4OH :5:5:1:1), 1.32 g, 1.04 mmol, 67 %. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ =1.28 (s, 10H), 1.43 (m, 2H), 1.51 (m, 2H), 2.16 (m, 2H), 3.33–3.61 (m, 40H), 3.37 (m, 2H), 4.13 (m, 1H), 4.27–4.30 (m, 1H), 4.32 (t, 1H), 4.39 (m, 2H), 4.48 (t, 3H), 4.70 (d, 2H), 4.80 (m, 5H), 5.01 (m, 2H), 5.64–5.71 (m, 14H), 5.80 (m, 1H).

Modification of glass surface with epoxide-terminated monolayer (**3**)

Modification was carried out as described in (Nguyen et al. 2011). Glass beads (20 g) were washed with acetone, n-hexane, methanol and ethanol followed by sonication for 5 min per solvent and etched for 30 min in a freshly prepared solution of HCl (37 %) and ethanol (1:1 v/v). After etching,

the samples were rinsed with ultrapure water and methanol for 5 min per solvent, and dried under nitrogen stream. Immediately after cleaning and drying, the glass beads were transferred into a dried glass flask. Glass beads were coated with 1,2-epoxy-9-decene (64.8 mmol) while keeping under nitrogen atmosphere before closing tightly. To remove the traces of oxygen and moisture that might enter into the reaction flask during coating, it was dipped into liquid nitrogen to freeze the coated 1,2-epoxy-9-decene on glass beads (while keeping under nitrogen atmosphere) and allowed to liquefy again at room temperature under vacuum. This freeze-thaw cycle was repeated three times. Finally, the reaction flask was backfilled with nitrogen, dipped into silicon oil bath and heated at 130°C for 24 h while keeping under slight nitrogen pressure. The modified glass beads (**3**) were removed from the reaction flask and washed thoroughly with deionized water, n-hexane and acetone followed by sonication for 5 min per solvent and dried under nitrogen stream.

Azide terminated monolayer on glass surface (4)

Azidation of epoxide terminated monolayer on glass surface was carried out according to the method described by Chini et al. (1990) with some modifications. NaN_3 (30.76 mmol) and NH_4Cl (13.54 mmol) were dissolved in methanol: water (8:1, 70 mL) mixture. Epoxide functionalized glass beads were added in the mixture and refluxed. After 24 h, glass beads were taken out of reaction mixture and followed by washing and drying as described in previous step.

Immobilization of β -CD on glass surface by click chemistry (5)

β -CD-decyne (2, 0.78 mmol) was dissolved in DMSO/ H_2O (8:1, 50 mL) followed by addition of *N,N*-diisopropylethylamine (2.34 mmol, 3 eq./azide group) and CuI (0.2 mmol, 0.25 eq./azide group). Azide functionalized glass beads (4) were incubated in the reaction mixture and heated at 40 °C for 24 h. After which, glass beads were taken out of the reaction flask followed by cleaning and drying as described for previous steps. All modified glass surfaces were characterized by XPS and elemental analysis.

Cholesterol extraction from ghee and analysis by HPLC

Cholesterol extraction and analysis was carried out according to the procedure described in (Han et al. 2005) with some modifications. For this purpose, ghee (g) was added in a screw-capped glass tube followed by the addition of β -CD functionalized glass beads (g, equal to weight of ghee), closed tightly and placed in shaker at 25 °C and 170 rpm. As a control, same treatment was done using unmodified glass beads. Samples were withdrawn after 2, 4, 6 and 8 h. For cholesterol determination, 1 g of β -CD treated ghee was transferred into a test tube followed by addition of 10 mL of 10 % KOH (ethanolic) in it. Test tube was closed tightly and heated at 70 °C for 30 min to complete the saponification. After cooling back to room temperature, 2 mL water was added in it and unsaponified fraction was

extracted with *n*-hexane (3×5 mL). Organic layers were collected and evaporated under vacuum. Residue was redissolved in methanol (1 mL) and analyzed by HPLC. As a control, 1 g of untreated ghee was used and same process was carried out in parallel.

A Jupiter C18 column (5 μM , 250×4.60 mm) HPLC (Shimadzu, Japan) was used for quantitative analysis of cholesterol by the method developed by Oh et al. (2001). The analysis was carried out at 30 °C and a flow rate of 1.5 mL/min with the mobile phase (acetonitrile:isopropanol=9:1 v/v). Elution was monitored at 205 nm. For quantitative analysis, cholesterol solution of different concentrations was run in HPLC under the same conditions and a straight line between concentration and peak area was drawn ($R^2=0.992$). Cholesterol reduction from ghee was calculated using Eq. (1).

Cholesterol reduction (%)

$$= 100 - \left[\frac{\text{Cholesterol in } \beta\text{-CD treated ghee}}{\text{Cholesterol in un - treated ghee}} \times 100 \right] \quad (1)$$

Results and discussion

Functionalization of glass surface with β -CD

Surface modification of glass beads was started with epoxide terminated monolayer (Fig. 1) as described in “Experimental” section (Nguyen et al. 2011). Elemental composition of modified surface shows a significant increase in C and a decrease in O and Si content (Table 1), indicating successful modification of glass surface. Wide-scan XPS spectrum (Fig. 2a) displayed signals for C_{1s} (285.0 eV), O_{1s} (533.0 eV) and Si_{2p} (104.0 eV). The narrow-scan C_{1s} region of XPS displayed a signal at 285.0 eV corresponding to C–C bonds resulting from alkyl chain and a peak at 286.81 eV (Fig. 2b) corresponding to C–O bonds (Nguyen et al. 2011). The ratio of (C–O)/(C–C) is 0.41 which is in good agreement

through click reaction, after repeated use for cholesterol reduction from ghee and relative proportion of different bond linkages

Table 1 Elemental composition of: blank clean glass, after functionalization with epoxide and azide terminated monolayers, after beta-cyclodextrin (β -CD) immobilization on glass surface (fresh)

Substrate	Elemental Comp. Atomic (%)				C–C bond		C–X ^a bond	
	Si	O	C	N	Ther.	Expt.	Ther.	Expt.
Blank glass (before modification)	32.96	67.04	0.00	0.00				
Epoxide terminated monolayer (3)	17.24	29.45	53.31	–	7	7.07	3	2.93
Azide terminated monolayer (4)	14.05	29.79	50.25	5.91	7	7.14	3	2.86
Glass modified with β -CD (5)—Fresh surface	3.08	16.58	76.17	4.18	14	20.84	48	41.16
5, after 10 cycles of repeated use	4.63	16.22	74.98	4.17	14	20.95	48	41.05

^a C–X=(C–O)+(C–N) bond linkages in the alkyl chain immobilized on glass surface (see compound 5)

Fig. 2 Wide-scan X-ray photoelectron spectroscopic (XPS) spectrum of epoxide terminated monolayer (**a**, **3**), narrow-scan C_{1s} XPS spectra of epoxide terminated layer (**b**, **3**), azide terminated layer (**c**, **4**) and after immobilization of beta-cyclodextrin-decyne (**2**) on glass surface (**d**, **5**)

with the theoretical value of 0.43. All these results indicate that high-quality epoxide monolayers on glass surface were obtained. The thickness of the formed epoxide layer, calculated using Eq. (2), was 1.12 nm and is slightly higher than the length of 1,2-epoxy-9-decene (1.11 nm, as calculated with Chem3D), confirming that the formed layer was still a monolayer.

$$I_{si} = I_{si}^{\infty} \exp\left(\frac{-d}{\lambda_{si,c} \cos \theta}\right) \quad (2)$$

Variables, I_{si} (absolute silicon peak intensity), I_{si}^{∞} (absolute silicon peak intensity of unmodified glass), d (thickness of the adsorbed layer), $\lambda_{si,c}$ (attenuation length of Si_{2p} electrons in the hydrocarbon layer), and θ (electron takeoff angle), were calculated from XPS data as described in (ter Maat et al. 2009).

The epoxide-terminated glass surface was subsequently converted into an azide terminated surface via a reaction with sodium azide (Fig. 1). In XPS spectrum (Fig. 2c), three signals corresponding to C–C (285.0 eV), C–O (286.32 eV) and C–N (287.72 eV) appeared. Excellent agreement between measured (0.41) and theoretical (0.43) value for $(C-X)/(C-C)$ (where $C-X = C-O + C-N$) indicate successful reaction corresponding to 94 % conversion of epoxide into azide-terminated surface. Appearance of a signal for organic nitrogen at 400.0 eV (not shown here, see [electronic supplementary material](#)) from the resultant monolayer also indicates the successful reaction between NaN_3 and epoxide-terminated layer on glass surface. Presence of 5.91 % N along with C, O and Si content in

elemental composition (Table 1) also confirm that epoxide terminated surface (**3**) was successfully converted into azide terminated layer (**4**).

In the last step of this reaction, β -CD-decyne (**2**) was clicked with azide terminated layer (**4**) on glass surface. In XPS spectrum, due to overlapping of C–O and C–N signals, only two signals corresponding to C–C (285 eV) and C–X (C–O+C–N, 285.87 eV) appeared (Fig. 2d). Ratio of $(C-X)/(C-C)$ (theoretical 3.43, experimental 1.97) indicate that

Fig. 3 Narrow-scan C_{1s} X-ray photoelectron spectroscopic (XPS) spectrum of beta-cyclodextrin (β -CD) functionalized glass surface (**5**) after 10 cycles of repeated use in cholesterol extraction from ghee

58 % β -CD-decyne (**2**) were clicked with azide terminated monolayer (**4**) on glass surface. Relatively low conversion was probably due to steric hindrance of bigger sized β -CD derivative. Increase in C content from 50.25 % to 76.17 % and decrease in O (29.79 to 16.58 %) and Si (14.05 to 3.08 %) content after click reaction of β -CD-decyne (**2**) to azide terminated layer (**4**) (Table 1) also indicate successful immobilization of β -CD on glass surface.

Application of functional surfaces for cholesterol reduction from ghee

Application of β -CD covalently attached on glass surface for cholesterol reduction from ghee is not common and up to our knowledge, no such example exists in literature. For cholesterol extraction, ghee was incubated in glass beads functionalized with β -CD at 25 °C and 170 rpm. Samples withdrawn after 2, 4, 6 and 8 h show respectively 78.8, 78.8, 79.0 and 78.9 % of cholesterol reduction. These results indicate that there was almost no change in cholesterol reduction after 2 h. As a control, un-modified glass beads were also used in parallel under the same conditions but no reduction in cholesterol from ghee was observed. Therefore, in detailed studies, samples were incubated with modified glass beads only for 2 h.

Stability and repeated use of β -CD functionalized glass surface

For implementation of such process at industrial scale, where many cycles of high yield are required, the stability of β -CD immobilized on glass surface should be high enough to permit its repeated use. Although there are many examples where cross-linked β -CD was applied repeatedly for cholesterol extraction from dairy products (Kwak et al. 2001, 2005; Kim et al. 2004; Han et al. 2005) but isolation of β -CD from reaction mixture after cholesterol extraction is a laborious and time consuming process. It can be simplified by using β -CD covalently attached on solid support as reported in this study. Moreover, this methodology can be used to develop continuous process at industrial scale.

In this study, we developed a method using β -CD covalently attached on glass surface which can be removed from ghee after cholesterol extraction and can be applied in repeated cycles after soaking in acetic acid and butanol mixture as described by some workers (Kim et al. 2004; Han et al. 2007). β -CD immobilized on glass beads surface was applied for cholesterol reduction from ghee repeatedly 10 times and HPLC results show that there was almost no change in cholesterol reduction efficiency. In 10 cycles, over all 78 $\pm$ 1 % cholesterol was extracted from the ghee using the same glass beads functionalized with β -CD. During preliminary experiments, we observed that there was almost no change in cholesterol reduction after 2 h. Therefore, ghee was treated

with modified glass surfaces for 2 h at 25 °C and 170 rpm during repeated use and cholesterol reduction was measured as described in “Experimental” section.

To follow the changes occurred on glass surface during repeated use for cholesterol reduction experiments, glass surface was characterized by XPS after 10 cycles. There was almost no change in elemental composition or relative proportion of (C–X)/(C–C) bond linkages (compare Figs. 2d and 3, Table 1). Number of C–X (C–O+C–N) bonds calculated from narrow-scan C_{1s} region of XPS spectrum recorded after 10 cycles of repeated use were decreased from 41.16 to 41.05 while relative number of C–C bonds were increased from 20.84 to 20.95 (Table 1). Because all bonds in β -CD molecules are C–O bonds, therefore, a very little decrease in C–X bonds and relatively little increase in C–C bonds indicate that most probably a few β -CD molecules were removed from the surface during repeated use but these changes are so minor that it can be ignored and overall it can be considered a stable functional surface. Results from repeated use of modified glass surfaces also indicate that these changes were so minor that cholesterol extraction efficiency was not affected.

Conclusions

β -CD can be immobilized successfully on glass beads and can be used as a solid support for cholesterol reduction from ghee. Functional glass surface was characterized by XPS and elemental analysis. 78 $\pm$ 1 % cholesterol reduction from ghee and advantage of easy separation, repeated use and possibility to use in continuous process at industrial scale show superiority of this method over other traditional methods. Modified glass surface remained stable and can be used for several cycles without losing cholesterol extraction efficiency.

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