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Synthesis and characterization of chelating agents responsible for tumor imaging

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ABSTRACT

*Present study shows the Rationale behind the synthesis of chelating agents and their thermodynamic stability along with kinetic inertness of metal complex in vivo is the prerequisites for the successful application of bifunctional chelating agent- conjugate. Macrocyclic bifunctional chelating agents (BFCs) with their established stabilizing properties are in the forefront for radio-immuno applications. In this study macrocyclic BFCs with increase in rigidity for complex stability besides requisite flexibility for complexation along with variation in ring size and donor ligands group were aimed to study for radio-diagnostic applications. Aromatic chelating agents (**5a**, **5b** and **5c**) synthesized by following the synthetic route shown in scheme 1 and 2. Radio-complexation with ^{99m}Tc and conjugation with monoclonal antibody for tumor diagnosis was designed for final evaluation of efficiency of synthesized chelating agents.*

Key Words: Chelating Agents, Synthesis, Tumor imaging, anti-EGFr monoclonal antibody**.**

INTRODUCTION

Radiodiagnosis and radiotherapy are well established techniques for the diagnostic analysis and therapy of various malignancies. Basic concept behind these techniques is near-about same except that γ-radiation source is used for the diagnosis and high energy β and α radiation source is utilized for therapeutic purpose.

In cancer diagnosis, the over expressed or specific cell surface receptors expressed by tumor cells are targeted by using ligands (bioligands or biomolecules). The bioligands that have been studied and attracted wide spread attention are monoclonal antibodies, antibody fragments,

peptides, steroids, folic acid and some heterocyclic molecules. Reagents used for the attachment of radio metal to biomolecules are usually called bifunctional chelating agents (BFCs) because they have a strong metal binding unit at one side and chemically reactive group at another side.

The choice of complexing agent is determined by the nature of metal ion to be bound. Initially open chain acyclic bifunctional chelating agent such as EDTA, DTPA were developed for use with many metal ions but the promiscuity of these acyclic chelating that bind several ions in solution was compromised by complex instability *in vivo* [1].

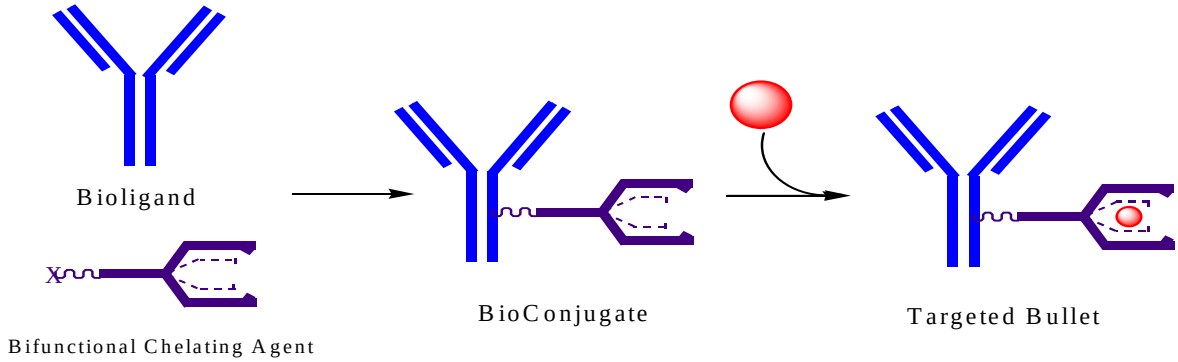

Figure 1 Concept of targeted bullet.

With copper, indium and yttrium, for ex., are either readily protonated or attract other competing metal ions (Ca^{2+} is 1.26 mmol dm^{-3}, Mg^{2+} is 0.8 mmol dm^{-3} and Zn^{2+} is 10^{-5} mol dm^{-3} in human serum) to form mixed complexes of reduced kinetic stability. The ensuing dissociation of metal ion *in vivo* is essentially irreversible as the metal ion will be rapidly sequestered by serum proteins (e.g. transferin [10^{-5} mol dm^{-3}] or albumin [10^{-3} mol dm^{-3}] and the complexing agent will be simultaneously occupied by one of the abundant serum cations. Dissociation of radioactive metal from bioconjugate is most undesirable as this increases background radiation while imaging and thus reduces signal to noise (S/N) ratio. Beside that radioactive metal used for diagnosis and therapy is not a part of biological system and thus causes chemical toxicity to the animal. Because of the insufficient stability of anionic metal complexes of EDTA and DTPA at low pH or in presence of serum cations (Ca^{2+}, Mg^{2+}) hindered their successful use *in vivo*[2,3].

Basic Requirements of a good Bi-functional Chelating Agent (BFC)
A BFC usually contain three parts:
1. Binding unit
2. Ligand framework
3. A conjugation group

There are several requirements for an ideal BFC. The BFC must form a metal chelate with high thermodynamic stability and kinetic inertness at neutral pH in order to keep the metal chelate intact under physiological conditions. Decomposition of metal chelate produce free lanthanide metal ion, which may deposit on the bone and cause bone marrow toxicity. The BFC must form a minimum number of isomer. A BFC must have high hydrophilicity, which helps to improve the

blood clearance and renal excretion of both the labeled and unlabeled BFC-BM conjugate. Fast renal clearance of the unlabeled BFC-BM will minimize its competition with radio-labeled BFC-BM forthe receptor [4,5].

The most common way to increase the thermodynamic stability and kinetic inertness of a metal complex is to use a polydentate chelator. The **denticity** requirement of a BFC is largely dependent on the size and coordination geometry preference of metal ion. e.g. Gadolinium, some other lanthanide (Y) ion are large and need eight to nine donor atoms to complete the coordination sphere and form stable complexes with macrocyclic chelate, such as DOTA and its derivatives [5].

The ligand framework is the spatial arrangement of the bonding unit. Polydentate ligands with three dimensional cavities are of particular interest because of their capability to adopt a preorganized conformation in the uncomplexed form. The higher the degree of preorganization of an uncoordinated ligand, the more stable will be the metal complex. Preorganization of the ligand framework also improves the kinetic inertness. Also minimizes the freedom of motion of the donor atoms and the ligand framework during the complexation process in such a way that the free ligand has a conformation more similar to that in the complex [6,7]. The highly preorganised macrocyclic framework of DOTA forces the four acetate chelating arms to adopt a conformation that wraps the metal ion. Therefore, the preorganisation should be an important factor in the design of new BFCs for the metal labeling of biomolecules [8,9].

Polyaminocarboxylate ligands based on cyclene are known to be well preorganised and form highly stable lanthanide complexes due to endocyclic orientation of the Nitrogen donors. Polyazamacrocycles have been widely used as chelator for a variety of transition metal ion. Macrocyclic BFCs such as DOTA, are of particular interest for several reasons [10,11].The macrocyclic ligand framework is well organized so that they form metal complexes with high thermodynamic stability and kinetic inertness. The pKa values for the carboxylic group are in the range 2-5. Lower pKa values results in less competition from proton, high stability of metal complex and minimum acid assisted demetalation, even under acid condition [12].

MATERIALS AND METHODS

The starting materials and reagents for the synthesis of BFC agents were purchased from S.D. Fine, CDH, Spectrochem, Fluka (Germany), Aldrich and Lancaster. All the solvents were either of analytical grade or were purified by distillation. For reactions performed under dry conditions, solvents were dried by usual reported laboratory procedures. TLC was run on the silica gel coated aluminum sheets (Silica gel 60 F_{254}, E merck, Germany) and visualized in UV light 254nm. Melting point was determined at Buchi B540 instrument. The corresponding 3, 5-bis-bromoethylnitrobenzene was synthesized by following the reported procedure. 2-aminothiophenol was procured from Acros Organics (USA) and distilled prior to use. 5-nitroisophthalic acid was synthesized by nitration of isophthalic acid essentially following the reported procedure. Infrared Spectra were recorded on the Perkin Elmer (spectrum BX) or Simadzu (FT-IR –8300) on Potassium Bromide pallets. ^{1}H-Nuclear NMR spectral

characterization was carried out on Bruker 400 MHz NMR instrument operating near 400 (^{1}H) and 100 (^{13}C) MHz and Bruker 300 MHz NMR instrument operating near 300 (^{1}H) and 75 (^{13}C) MHz. The FAB-MS spectra were recorded on JEOL SX 102/DA-6000 mass spectrometer using *m*-nitrobenzyl alcohol as matrix. EI-MS spectra were recorded on a JEOL SX102 / DA (KV 10 mA) instrument.

Synthesis of Intermediates-

1, 2-Bis(2-aminophenylthio)ethane (1a): The 1,2-dibromoethane (3.7 g, 20 mmol) was added drop wise to a refluxing solution of 2-aminothiophenol (5 g, 40 mmol) and sodium ethoxide (3.7 g, 50 mmol) in dry ethanol (20 mL). The refluxing reaction mixture was stirred for 6-7 h and it was monitored by TLC. On completion of reaction the solvent was removed *in vaccuo* and the reaction mixture was cooled to 0^{o}C. Water (50 mL) was added and reaction mixture was extracted with dichloromethane (20 mL × 4). The organic layer was dried over anhyd. Na_2SO_4 and evaporated to dryness *in vaccuo* to get a solid product, which on recrystallization from ethanol gave white colored solid (4.3 g, 78%), mp 72-73 ^{o}C. IR (KBr pallets, cm^{-1}) 3385, 3356, 3290, 3018, 2988, 2925, 1617, 1582, 1479, 1446, 749. ^{1}H NMR (60 MHz, $CDCl_3$) δ ppm 6.70-7.50 (m, 8H, ArH), 4.30 (br s, 4H, 2 x NH_2, exchanged with D_2O), 2.80 (s, 4H, 2 x S-CH_2). Anal. Calcd for $C_{14}H_{16}N_2S_2$: C 60.83; H 5.83; N 10.13; S 23.20. Found C 60.63; H 5.76; N 10.60; S 23.01 FAB-MS: Found m/z 276 $[M]^+$; calcd for $C_{14}H_{16}N_2S_2$: 276. EI-MS: 276, 138, 124 (base peak), 94.

1, 3-Bis(2-aminophenylthio)propane (1b): The 1,3-dibromopropane (4.02g, 20 mmol) was added dropwise to a refluxing solution of 2-aminothiophenol (5 g, 40 mmol) and sodium methoxide (3.2 g, 50 mmol) in dry methanol (20 mL). The refluxing reaction mixture was stirred for 6-7 h and it was monitored by TLC. On completion of reaction the solvent was removed *in vaccuo* and the reaction mixture was cooled to 0^{o}C. Water (50 mL) was added and reaction mixture was extracted with dichloromethane (20 mL × 4). The organic layer was dried over Na_2SO_4 and evaporated to dryness *in vaccuo* to get a crude product, which was further purified by column chromatography [column of SiO2 (100 g); pre-adsorption of the residue at SiO2 (*ca*. 8 g) with ethyl acetate; elution with petroleum ether/ethyl acetate=60:40 (v/v)] to obtain transparent oily liquid (4.5 g, 82%). IR (NaCl plates, cm^{-1}) 3434, 3355, 3018, 2988, 2925, 1606, 1478, 1448, 1301, 748. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.20 (dd, J_{ab}=7.5 Hz, J_{ac}=1.5 Hz, 2H, ArH), 7.00 (dt, J_{ab}=7.6 Hz, J_{ac}=1.4 Hz, 2H, ArH), 6.68 (dd, J_{ab}=8.0 Hz, Jac=1.2 Hz, 2H, ArH), 6.64 (dt, J_{ab}=7.6 Hz, J_{ac}=1.2 Hz, 2H, ArH), 4.10 (br s, 4H, 2 x NH_2, exchanged with D_2O), 2.80 (t, J=7.1 Hz, 4H, 2 x S-CH_2-), 1.75 (quintet, J=7.1 Hz, 2H, S-C-CH_2-). Anal. Calcd for $C_{15}H_{18}N_2S_2$: C 62.03; H 6.25; N 9.64; S 22.08. Found C 62.14; H 6.26; N 9.82; S, 21.88. FAB-MS: Found m/z 290 $[M]^+$; calcd for $C_{15}H_{18}N_2S_2$: 290. EI-MS:

1, 5-Bis(2-aminophenylthio)-3-oxapentane (1c): The diethyleneglycolditosylate (8.2 g, 20 mmol) was added dropwise to a refluxing solution of 2-aminothiophenol (5 g, 40 mmol) and sodium methoxide (3.2 g, 50 mmol) in dry methanol (20 mL). The refluxing reaction mixture was stirred for 6-7 h and it was monitored by TLC. On completion of reaction the solvent was removed *in vaccuo* and the reaction mixture was cooled to 0^{o}C. Water (50 mL) was added and reaction mixture was extracted with dichloromethane (20 mL × 4). The organic layer was dried

over Na_2SO_4 and evaporated to dryness *in vaccuo* to get a crude product, which was further purified by column chromatography [column of SiO2 (100 g); pre-adsorption of the residue at SiO2 (*ca*. 8 g) with ethyl acetate; elution with petroleum ether/ethyl acetate=60:40 (v/v)] to obtain viscous liquid (4.56 g, 72%). IR (KBr pallets, cm^{-1}) 3432, 3349, 3058, 2921, 2855, 1607, 1479, 1105, 748. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 7.30 (d, J=7.5 Hz, 2H, ArH), 7.10 (t, J=7.5 Hz, 2H, ArH), 7.00 (d, J=7.5 Hz, 2H, ArH), 6.60 (t, J=7.8 Hz, 2H, ArH), 4.00 (br s, 4H, 2 xNH_2, exchanges with D_2O), 3.30 (t, J=7.1 Hz, 4H, 2 x O-CH_2-), 2.73 (t, J=7.0 Hz, 4H, 2 x S-CH_2). ^{13}C NMR ($CDCl_3$) δ ppm 32.15, 67.32, 112.71, 114.06, 114.97, 127.81, 133.97, 148.19. Anal. Calcd for $C_{16}H_{20}N_2OS_2$: C 59.96; H 6.29; N 8.74; O 4.99; S 20.01. Found C 59.66; H 6.08; N 8.44; S, 20.00. EI-MS: 320, 319, 227, 195, 136 (base peak), 166, 94, 57.

General procedure for the synthesis of Macrocycle 3a, 3b and 3c:
1,3-Bis(2-aminophenylthio)propane **1b** (2.9 g, 10 mmol) in 150 ml dry dichloromethane and 5-nitroisophthaloyl dichloride **2** (2.5 g, 10 mmol) in 150 dichloromethane were added simultaneously drop wise from two separate dropping funnels to the fast stirring reaction mixture of K_2CO_3 (2.8 gm, 20 mmol) in 200 ml dry dichloromethane at room temperature. After complete addition, reaction mixture was further stirred for 1 hr. Solvent was removed under vacuum and solid left was washed with water, dried under vacuum as puff colored solid product.

Macrocycle 3a: (1.9 g, 42 %). IR (KBr pallets, cm^{-1}) 3276m (N-H str.), 1680s (C=O str), 1653s, 1579s, 1513s, 1436s, 1348s, 757m, 722w, 681w. ^{1}H NMR (300 MHz, DMSO) δ ppm: 10.1 (s, 1H, ArH), 8.9 (d, J=1.24 Hz, 2H, ArH), 8.6 (d, J=8.32 Hz, 2H, ArH), 7.6 (dd, J=7.7 Hz, J=1.48, 2H, ArH), 7.4 (dt, J=8.04, J=1.44 Hz, 2H, ArH), 7.1 (dt, J=7.4 Hz, J=1.44 Hz, 2H, ArH), 2.8 (s, 4H, 2 x S-CH_2-). FAB-MS: 452 (M^++1).

Macrocycle 3b: (2.4 g, 52 %). IR (KBr pallets, cm^{-1}) 3311m, 1681s, 1580s, 1517s, 1434, 1347m, 756m, 733m. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 9.7 (s, 1H, ArH), 9.1 (d, J=1.24 Hz, 2H, ArH), 8.6 (d, J=8.32 Hz, 2H, ArH), 7.6 (dd, J=7.7 Hz, J=1.48, 2H, ArH), 7.4 (dt, J=8.04, J=1.44 Hz, 2H, ArH), 7.1 (dt, J=7.4 Hz, J=1.44 Hz, 2H, ArH), 2.7 (t, J=7.9 Hz, 4H, 2 x S-CH_2-), 1.8 (quintet, J= (merged), 2H, 2 x S-C-CH_2-). FAB-MS: 466 (M^++1).

Macrocycle 3c: (2.9 g, 59 %). IR (KBr pallet, cm^{-1}) 3431w, 3257w, 3084, 2922m, 2864m, 1719, 1646s, 1578, 1528vs, 1440, 1351m, 731m, 664m. ^{1}HNMR (400 MHz, $CDCl_3$) δ ppm: 9.7 (s, 1H, ArH), 9.1 (d, J=1.24 Hz, 2H, ArH), 8.6 (d, J=8.32 Hz, 2H, ArH), 7.6 (dd, J=7.7 Hz, J=1.48, 2H, ArH), 7.4 (dt, J=8.04, J=1.44 Hz, 2H, ArH), 7.1 (dt, J=7.4 Hz, J=1.44 Hz, 2H, ArH), 3.6 (t, J=4.88 Hz, 4H, 2 x O-CH_2-), 3.0 (t, J=4.88, 4H, 2 x S-CH_2-). FAB-MS: 496 (M^++1).

General procedure for the synthesis of 4a, 4b and 4c from 3a, 3b, 3c respectively:
Synthesis of Macrocycle 4a
A solution of **3a** (1.15 g, 2.5 mmol) in 20 ml of dry THF was cooled to 0 ^{o}C, 88 ml of BH_3.THF solution (1M) was then added. The reaction mixture was stirred at 0 ^{o}C for 1 hr and then refluxed for 20 hr. The mixture was cooled to 0 ^{o}C and 6 ml of dry methanol was added to decompose excess BH_3. The reaction mixture was brought to room temperature and the solvent was evaporated under reduced pressure. The residue was dissolved in 8 ml of ethanol and solution

was saturated with HCl(g) and refluxed for 2 hr. On cooling, the precipitated product was collected, washed with water and then with methanol to give compound **4a** (522 mg, 49%).

Synthesis of Macrocycle 4b

1,3-Bis(2-aminophenylthio)propane **1b** (2.9 g, 10 mmol) in 100 ml DMF and Bis-3,5-bromomethylnitrobenzene **7b** (3.09 g, 10 mmol) in 100 ml DMF were added simultaneously drop-wise from separate dropping funnels under nitrogen atmosphere at 120 ^{o}C with constant stirring to the reaction flask containing 100 ml of dry DMF and K_2CO_3 (2.8 g, 20 mmol). Stirred the reaction mixture at same temperature for 7 hrs and on completion of reaction (TLC), removed the DMF under vacuum and triturated the solid left with water thoroughly and decanted away the water phase. Residual solid was again washed with methanol (20 ml) and dried under vacuum to afford yellow solid product (Obtained 2.8 g, calcd. 4.37 g, % yield 64). mp 206-207^{o}C IR (KBr pallets, cm^{-1}): 3389m (N-H str.), 1585s, 1520s (NO_2 asym str) , 1499s, 1352s (NO_2 sym str), 1321s, 745s. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 8.06 (s, 2H, ArH), 7.6 (s, 1H, ArH), 7.3 (d, J=6.9 Hz, 2H, ArH), 7.0 (t, J=7.3 Hz, 2H, ArH), 6.5 (t, J=7.2 Hz, 2H, ArH), 6.1 (d, J=7.9 Hz, 2H, ArH), 5.6, (s (broad), 2H, NH), 4.5 (d, J=5.5 Hz, 4H, 2 x Ar-CH_2-N), 2.7 (t, J=7.9 Hz, 4H, 2 x S-CH_2-), 1.7 (quintet (merged), 2H, 2 x S-C-CH_2-). ^{13}CNMR (75 MHz, $CDCl_3$) δ ppm: 142.2, 135.6, 129.9, 128.6, 120.2, 117.5, 109.9, 46.3, 34.7, 30.4. Anal Calcd for $C_{23}H_{23}N_3O_2S_2$ C 63.13, H 5.30, N 9.60, O 7.31, S 14.66. Found C 62.98, H 5.21, N 9.89, S 14.78. FAB-MS: Calcd for $C_{23}H_{23}N_3O_2S_2$ 437

4b (437 mg, 1mmol) was suspended in ethyl acetate: tetrahydrofuran (50:50) (25 ml) in a nitrogen flushed flask. Zinc dust (130 mg) and hydrazine hydrate 99% (5 ml) was added to the above reaction mixture and stirred at room temperature under nitrogen atmosphere for 8 hours. On completion of reaction (TLC: petroleum ether: ethyl acetate 60: 40, Rf. 3, UV (254 nm)), decanted the reaction mixture carefully leaving behind Zinc dust adhered to walls of rb flask. Solvent was removed under reduced pressure and kept the reaction mixture as such (still) for 1h. The solid separated out was filtered through filter paper by decantation so that most of the solid remains in flask. The solid was triturated in water, filtered and then washed with methanol (5 ml), filtered and dried under vacuum to obtain Pale whitish solid 5b. m. pt. 159-160^{o}C. Yield Calculated 407 mg, obtained: 350 mg, percentage yield: 85 %. Solubility of product: DMSO, DMF, THF, $CHCl_3$. IR (KBr pallets, cm^{-1}) 3385w, 3279m, 2923, 2850w, 1586s, 1499s, 1450m, 1319m, 1262m, 746s. ^{1}H NMR (DMSO-d_6, 300MHz) δ ppm: 7.2 (d, J=7.4 Hz, 2H, ArH), 6.9 (t, J=7.6 Hz, 2H, ArH), 6.3 (d, J=8.1 Hz, 2H, ArH), 6.4 (merged peaks, 3H, ArH), 5.8 (t, J=5.58 Hz, 2H, ArH), 5.05 (s), 4.1 (d, J=5.59 Hz, 4H, 2 x N-CH_2-), 2.7 (t, J=7.7 Hz, 4H, 2 x S-CH_2-), 1.6 (quintet (merged), 2H, S-C-CH_2-). ^{13}CNMR (DMSO-d_6) δ ppm: 148.6, 148.5, 140.6, 135.0, 129.4, 115.7, 115.4, 111.0, 110.2, 109.8, 46.1, 33.3, 29.9. Anal Calcd for $C_{23}H_{25}N_3S_2$ C 67.77, H 6.18, N 10.31, S 15.73. Found C 67.12, H 6.08, N 11.04, S 15.76. FAB-MS: cald for $C_{23}H_{25}N_3S_2$ 407 found 407 (M^+).

Synthesis of macrocycle 4a

1,2-Bis(2-aminophenylthio)ethane **1a** (2.7 g, 10 mmol) dissolved in 100 ml DMF and Bis-3,5-bromomethylnitrobenzene **7b** (3.09 g, 10 mmol) dissolved in 100 ml DMF were added simultaneously drop-wise from separate dropping funnels under nitrogen atmosphere at 120^{0}C

with constant stirring to the reaction flask containing 100 ml of dry DMF and K_2CO_3 (2.8 g, 20 mmol). Stirred the reaction mixture at same temperature for 7 hrs and on completion of reaction (TLC), removed the DMF under vacuum and triturated the solid left with water thoroughly and decanted away the water phase. Residual solid was again washed with methanol (20 ml) and dried under vacuum to afford yellow shining solid product (Obtained 2.2 g, calcd. 4.23 g, % yield 52). mp 222-224^{0}C IR (KBr pallets, cm^{-1}): 3384m (N-H str.), 1586m, 1524s (NO_2 asym str), 1498s, 1350s (NO_2 sym str), 1311s, 743s. H^1NMR (300 MHz, DMSO) δ ppm: 8.1 (s, 2H, ArH), 7.5 (s,1H, ArH), 7.2 (d, J=6.9 Hz, 2H, ArH), 6.9 (t, J=7.44 Hz, 2H, ArH), 6.4 (t(merged), J=7.4 Hz, 2H, ArH), 6.3 (d, J=8.1 Hz, 2H, ArH), 4.5 (d, J=6 Hz, 4H, Ar-CH_2-N), 2.8 (s, 4H, S-CH_2-). ^{13}CNMR (75 MHz, DMSO-d_6) δ ppm: 147.9, 142.7, 137.0, 130.3, 129.2, 120.3, 115.9, 114.8, 109.8, 44.1, 33.7. Anal Calcd for $C_{22}H_{21}N_3O_2S_2$ C 62.39, H 5.00, N 9.92, O 7.55, S 15.14. Found C 62.28, H 4.98, N 10.01, S 15.26. FAB-MS: Calcd for $C_{22}H_{21}N_3O_2S_2$ 423 found m/z 423 (M^+).

Synthesis of macrocycle 5a:

Synthesis of **5a** was performed by following the similar procedure as described for synthesis of **5b**. Pale whitish solid product. M. pt. 205-206 ^{o}C. Yield Calculated 393 mg, obtained: 348 mg, percentage yield: 89 %. Solubility of product: DMSO, THF. IR (KBr pallets, cm^{-1}) 3388w, 2922w, 1589s, 1501s, 1453m, 1319m, 737m. ^{1}H NMR (DMSO-d_6, 300MHz) ppm: 7.3 (d, J=7.4 Hz, 2H, ArH), 6.9 (t, J=7.6 Hz, 2H, ArH), 6.2 (d, J=8.1 Hz, 2H, ArH), 6.3 (merged peaks, 3H, ArH), 5.8 (t, J=5.58 Hz, 2H, ArH), 5.05 (s, NH), 4.1 (d, J=5.59 Hz, 4H, 2 x N-CH_2-), 2.8 (s, J=7.7 Hz, 4H, 2 x S-CH_2-). ^{13}C NMR (DMSO-d_6) δ ppm: 148.6, 140.6, 136.8, 134.9, 130.2, 116.4, 115.39, 114.49, 110.99, 109.92, 44.99, 33.77. Anal Calcd for $C_{22}H_{23}N_3S_2$ C 67.14, H 5.89, N 10.68, S 16.29. Found C 66.98, H 5.68, N 11.01, S 16.31. FAB-MS Calcd for $C_{22}H_{23}N_3S_2$ 393 found 393 (M^+).

Synthesis of macrocycle 4c

1,5-Bis(2-aminophenylthio)-3-oxapentane **1c** (3.19 g, 10 mmol) dissolved in 100 ml DMF and Bis-3,5-bromomethylnitrobenzene **7b** (3.09 g, 10 mmol) dissolved in 100 ml DMF were added simultaneously drop-wise from separate dropping funnels under nitrogen atmosphere at 120 ^{o}C with constant stirring to the reaction flask containing 100 ml of dry DMF and K_2CO_3 (2.8 g, 20 mmol). Stirred the reaction mixture at same temperature for 7 hrs and on completion of reaction (TLC), removed the DMF under vacuum and triturated the solid left with water thoroughly and decanted away the water phase. Residual solid was again washed with methanol (5 ml) and dried under vacuum to afford yellow solid product (Obtained 3.2 g, calcd. 4.67 g, % yield 68). mp 159.5-160^{o}C IR (KBr pallets, cm^{-1}): 3365m (N-H str.), 3055, 2919m, 2850w, 1588s, 1529s (NO_2 asym str) , 1497s, 1448m, 1350s (NO_2 sym str), 1317s, 1259m, 1085s, 751s. H^1NMR (300 MHz, $CDCl_3$) δ ppm: 8.1 (s, 2H, ArH), 7.8 (s, 1H, ArH), 7.4 (d, J=7.35 Hz, 2H, ArH), 7.1 (t, J=7.5 Hz, 2H, ArH), 6.6 (t, J=7.29 Hz, 2H, ArH), 6.3 (d, J=8.0 Hz, 2H, ArH), 5.6, (s (broad), 2H, NH), 4.4 (d, J=5.3 Hz, 4H, 2 x Ar-CH_2-N), 3.4 (t, J=6.9 Hz, 4H, 2 x O-CH_2-), 2.8 (t, J=6.9, 4H, 2 x S-CH_2-). ^{13}CNMR ($CDCl_3$) δ ppm: 149.2, 148.3, 142.5, 136.7, 130.4, 120.9, 117.8, 117.1, 110.5, 69.2, 47.9, 34.6. Anal Calcd for $C_{24}H_{25}N_3O_3S_2$ C 61.65, H 5.39, N 8.99, O 10.26, S 13.17. Found C 61.52, H 5.28, N 9.02, S 13.89. FAB-MS: Calcd for $C_{24}H_{25}N_3O_3S_2$ 467 found m/z 467 (M^+).

Conjugation with anti-EGFr monoclonal antibody (ior egf/r3)

1. Reaction of Glycolaldehyde with H2N-BFCs: The chelating agents H_2N-5b-c (1mmol) was incubated in THF at 80^0C for 4 hrs with 1mmol of glycolaldehyde. The aldoamine so obtained after Amadori rearrangement was directly used after removal of solvent to carry out the reaction with anti-EGFr antibody.

2. Reaction of Glycolaldehyde-NH-5a-c with anti-EGFr antibody: Ligand-glycolaldehyde adduct (25 µl of 20mM solution in THF/methanol) was incubated with ior egf/r3 (300 µl of a solution containing 3 mg of antibodies in 0.1 M sodium phosphate, pH 7) in presence of 20 µg (1 mg/ml of methanol solution) of sodium cyanoborohydride. The reaction mixture was incubated at 37^0C for 60min and then subjected to centrifuged column gel chromatography which removed the unreacted chelate and changed the buffer to 0.1M sodium acetate, pH 5.5. UV absorbance at 280nm for the centrifuged column effluent was used to determine the antibody concentration, and ^{57}Co assay to obtain the bound chelate concentration. In short, 1nmol of conjugated antibody, 2 nmol of ^{59}Co, and a tracer dose of ^{57}Co were mixed and incubated at pH 5.5 at room temperature for 2h. An aliquot was applied to thin layer chromatography with ITLC-SG (Gelman Sciences, Ann Arbor, MI) with ammonium acetate/CH_3OH (1:1v/v) solution as eluent. Free cobalt migrates to Rf=1 while conjugated cobalt stays at Rf=0.

Tumor imaging using immuno-conjugates

For imaging studies to compare the utility of the three chelates examined, three adult male mice were each injected i.v. with 100-200 µCi of 99mTc linked to anti-EGFr antibody by various ligands. Sequential images were obtained at different time intervals.

RESULTS AND DISCUSSION

Delivery of desired diagnostic and/or therapeutic agents (drugs/isotopes) to targeted tissues in a site specific non-toxic mode is the central theme of molecular imaging. Antigen-Antibody recognition processes have been widely used for targeting drugs to desired tissues. In principle, an antibody raised against a specific tumor antigen and labeled with a desired drug (radionuclide or fluorescent reagent) can be considered as a molecular truck that has specifically designed to deliver desired reagent to tumors, the targeted tissues. The application of antibodies for in vitro and in vivo diagnosis of tumor has potential with the recent advances in proteomic and genomic techniques [13,14].

The conjugation of chelating agent to an antibody as an anchoring site for a radionuclide is the first step in the successful preparation of a radiolabeled antibody for a diagnostic and therapeutic application. The high affinity of the protein bound chelator towards radionuclide ensures a higher selectivity in the delivery of the radionuclide to the targeted tissue [15,16].

Bifunctional chelating agent is key to successful application of receptor-ligands (biomolecules) based targeted radiopharmaceuticals and there is continued interest in designing and development of new bifunctional chelating agents for effective coordination of radioactive metals [17,18,19].

Bifunctionalized macrocyclic chelating agents has been successful for *in vivo* applications as complexes from macrocycles are more stable compared to acyclic chelating agents [20].Cyclam and cyclene framework is most studied and various modifications were done for required pharmacological applications. Recently aromatic chelating agents have been proposed as the introduction of reactive functional group, involved in conjugation with bioligands, is much easier in aromatic system compared to aliphatic chelating agents [21,22].In addition to above fact, the introduction of aromatic group in cyclic framework will increase the thermodynamic stability of complex by interactive involvement of π electrons of aromatic ring and will also enhance the kinetic inertness (stability) by increase in rigidity in framework [23,24].Based on above arguments, synthesis and evaluation of aromatic bifunctionalized macrocyclic chelating agents 5a-c (N_2S_2 and N_2S_2O system) derived from bis(2-aminophenylthio)alkane is described here. BFCs 5a-c were synthesized by following the two different routes (Scheme 1 and Scheme 2).

The bis(2-aminophenylthio)alkanes were prepared from 2-aminothiophenol by reaction with respective dibromoalkane and ditosylates. In one of the earlier attempt to cyclize the diamines with bromoacetyl bromide, 3-oxo-3,4-dihydro-2H-1,4-benzothiazine was obtained as sole product [22].Alternatively, 5-nitroisophtahlic acid was converted into diacidchloride (2) with PCl5 and used for cylization of 1a-c to obtain 3a-c. The slow addition of 5-nitroisophthaloyl dichloride (2) to a solution of diamine 1a in dichloromethane containing a suspension of K2CO3 (anhyd.) and TBA HSO4 (PTC) gives macrocycle 3a (42%) mp 245^0C. 3a-c were selectively reduced by diborane giving 4a-c macrocycles [25].Reduction of nitro group of 4a-c with Pd/C/H2 yielded 5a-c in very low yield and besides separation of product from reaction mixture was cumbersome. Gowda and Gowda method for reduction was the only successful method by which the product was easily obtained in high yield. In this scheme, the overall yield was low, so an alternate method was used to synthesize 5a-c (Scheme 2). In this scheme, prior to cyclization, the 5-nitroisophthaloyl chloride (2) was reduced to dialcohol and further converted into dihalide with phosphorus trihalide. In the last conversion, dicholoro was obtained in low yield while dibromo conversion was high yielding. The corresponding 3,5-bis-bromoethylnitrobenzene was synthesized by following the reported procedure. 5-nitroisophthalic acid was synthesized by nitration of isophthalic acid essentially following the reported procedure. The cyclization of 1b with bis-3,5-bromomethylnitrobenzene in dry DMF at 120oC in presence of K_2CO_3 and TBA HSO_4 gives 4b (62%). All the chelating agents (5a-c) are stable at room temperature and do not require any specialized condition for long term storage [26,27].

The final chelating agents (5a-c) are bifunctional bearing a free amine group that could be used for the conjugation with monoclonal antibodies, peptides and other biomolecules.

A variety of chemical procedures have been developed over the years for labeling antibodies with radionuclides. Preparation of radio-iodinated antibodies was one of the early approaches used to target the radionuclide to tissue of choice [28,29].Over the years, many limitations of this method have been recognized and alternative approaches have been advanced including the labeling of the antibodies with metal radionuclides. The latter approach involves the covalent attachment of a chelating agent to antibodies and then complexing the chelator antibody

conjugate with desired radionuclide. The chelating agent conjugated to antibodies, thus function as high affinity receptors of desired radionuclide [30,31].

The mAb ior egf/r3 recognizes EGFr and inhibits the binding to its ligand. This antigen has been used successfully as a target for imaging and therapy because of its over-expression in tumors of epithelial origin [32]. Although normal cells also express EGFr, the elevated number of receptors on tumor cells confers a degree of targeting specificity [33,34].Therefore, the tumor cells can proportionately bind more amounts of radiolabeled mAbs than can the normal cells. The anti-epidermal growth factor receptor (EGFr) mAb ior egf/r3 [35] was gift from CIMAB, Havana, Cuba. EGFr plays a crucial role in growth regulation through signal transduction mechanism. Reports of increased EGFr expression in epithelial cancer suggest its possible involvement in pathogenesis of certain epithelial neoplasms. The anti-EGFr antibody binds to external domain of the human EGFr [36].After binding to the receptor, the mAb–receptor complex is rapidly internalized and the fraction of the mAb can be found in the nucleus of the cell [37,38].

The conjugation of 5c to mAb was done at room temperature conditions. BFC 5c is pseudo-bifunctional reagent because it has a chelating functional domain at one end and an amino function at the other end, which could be modified to target this reagent to specific sites in protein. The amino function affords high flexibility to this reagent for purpose of conjugating this reagent to protein. Accordingly, for conjugating 5c to protein, a simple reported procedure was adopted [37,38].An alkylamine linkage is generated between the chelator and protein amino groups, using the latent cross linking potential of α-hydroxyaldehyde, glycoladehyde. To couple amino group of 5c to protein, its free amino group is derivatized into 2-oxoethylamino group. This intermediate, needed for alkylation of the protein with the chelator, was generated in situ and used directly. The amino BFC 5c is first incubated with nearly one equivalent of glycoldehyde at neutral pH and room temperature. The amino group of BFC 5c reacts with the carbonyl group of glycoldehyde to generate an aldimine adduct (a reversible adduct). The aldimine adduct then undergoes an intermolecular rearrangement (Amadori rearrangement) and enerates a stable aldo amine adduct, 2-oxoethylated analogue (Scheme 3).

In the second step of the reaction, the carbonyl group of the aldomine adduct is used to couple this reagent to the protein by reductive alkylation. The resultant alkylamine linkage between the chelator and protein is more stable than the thiocarbamyl linkage formed on the reaction of thiocyanatobenzyl derivative of BFCs with proteins. Modification of amino groups of proteins through reductive alkylation using aliphatic aldehydes is one of the mild and versatile procedures for derivatization of the amino groups. A major advantage of this procedure is that the alkyl chain is introduced mostly on the e-amino group of proteins without disturbing the surface charges of the proteins, since the secondary amino group modified amino group is expected to retain the positive charge just as the parent primary amino group. ^{57}Co binding assay indicated 2.1 ± 0.1 chelate molecules were conjugated per antibody molecules.

The conjugated antibodies were labeled with a specific activity 20–30mCi/mg of protein. Labeling efficiencies were measured by ascending paper chromatography on ITLC-SG strips. Results of radiolabeling of the immunoconjugates were found to be 98.5±0.30%. ITLC-SG

results in acetone showed that 1.5–2% or less free pertechnetate ran with the solvent front (Rf=0.7–1.0). This indicated that pertechnetate was reduced almost entirely. Using 10% NH_4Ac and methanol 1:1 as a solvent for migration showed all the activity on the base of ITLC-SG strips indicating the radiolabeled immunoconjugate, not the other species.

CONCLUSION

The aromatic macrocyclic chelating agents **5a-c** were successfully synthesized, characterized by using spectroscopic techniques and further evaluated for scintigraphic applications. The radio-complexation yields with ^{99m}Tc metal were more than 90 percent. The complexes formed ^{99m}Tc-**5b-c** are highly stable in dilute saline and in the serum under physiological conditions (*in vitro*). The BFCs **5b-c** can be utilized for the attachment of radioactive metals to the biomolecules; this was demonstrated using anti-EGFr monoclonal antibody. The bioactivity of the biomolecules is not altered by the presence of these macrocyclic chelating agents. Thus these bifunctional chelating agents **5b-c** show promising potential for radio diagnostic (and radiotherapy) applications with radioactive metals.

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