Probing Solid−Liquid Interfaces Using Radiotracer Technology: Characterization of Functionalized Microspheres

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This study explores the use of nuclear probes (radiolabeling techniques) for the quantification of available, reactive groups on solid surfaces and their role in the engineering of materials for biosensor applications. Microspheres were synthesized with reactive carboxylate groups as conjugation sites for proteins. The carboxylate groups were reacted with three bifunctional ligands, sarar, aminobenzyl-cyclen, and aminobenzyl-dota. The reaction conditions used were similar to that employed for the conjugation of proteins and optimized for the concentration of reactants (bifunctional ligand and EDC), pH, and time at ~21 °C. Of the three bifunctional ligands conjugated to the microsphere, sarar proved to be the most efficient. Optimum reaction conditions employed molar ratios of 1:20:100 for microspheres (estimated carboxylate groups of 1.1×10^{-7} per milligram of microspheres):sarar:EDC at pH 5.0 for 1 h, followed by $^{57/nat}$Co radiolabeling at pH 7.0. The study demonstrates how nuclear probes with high specificity and sensitiviy can provide invaluable information on available reactive sites on solid surfaces at extremely low concentrations ($>10^{-10}$ M) in a range of media. More importantly the nuclear probes enable the characterization of the surfaces of new materials in a non-destructive manner under conditions relevant to the engineering of the materials and ultimately the desired biosensor.

Introduction

The demand for more rapid and accurate analytical techniques capable of providing reliable information about the presence of compounds such as drugs, metabolites, biomarkers, flavors, environmental, food contaminants, and toxic chemicals, without time-consuming isolation and sample preparation steps, continues to increase.[1] The biosensor area therefore strongly attracts the attention of the nanotechnology field. However, the ability to assemble and integrate various nanoscale components is essential for their effective application. One significant challenge of this area is the ability to characterize functionalized surfaces and to connect biomarkers to these functionalized solid surfaces with molecular precision.

Various strategies for the synthesis of polymeric microspheres using heterogeneous polymerization methods such as emulsion, suspension, dispersion, and precipitation polymerization continue to evolve.[2] We are now able to prepare microspheres, of varying size, potentially large surface areas, and high mobility that can be easily recovered from suspension. The interaction of these materials with their environment is largely controlled by their surface chemistry.[3] Hence the ability to effectively functionalize the surfaces of these systems as part of, or during, their engineering is critical in order to capitalizing on the significant unique features of these materials.[4]

A vital part of the design of sensing materials is the physical and chemical characterization of the functionalized surfaces with or without the sensor. Essential to optimizing both the application and the engineering of these materials is the ability to obtain the information about these surfaces in a non-destructive manner or with minimal impact. Information such as the number and type of *available functional groups* under the conditions that ultimately the sensor is to be applied is critical to optimizing the design and application of the biosensor. Hence for biosensors that may be used to determine the amount of protein or drug in an unknown solution, it is important that they are evaluated in relevant media systems.[5]

Conjugation of detection tags such as radiolabeled bifunctional chelators or fluorescence labels to complex tertiary structures such as proteins is well established.[6] Conditions for conjugation of multiple tags to a protein (which can contain multiple reactive sites) in order to sustain the activity of the protein are well understood. These methodologies are highly relevant where there is an interest to attach proteins to the functionalized surface of micro- or nanoparticles. These tags can be used to determine the number of active sites on

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(1) Coelho, M. A. N.; Gliozzi, A.; Möhwald, H.; Perez, E.; Sleytr, U.; Vogel, H.; Winterhalter, M. *IEEE Trans. Nanobioscience* **2004**, *3*, 3.
(2) Kawaguchi, H. *Prog. Polym. Sci.* **2000**, *25*, 1171.
(3) Dai, L.; He, P.; Li, S. *Nanotechnology* **2003**, *14*, 1081.
(4) Grubbs, R. B. *Nat. Mater.* **2007**, *6*, 553.
(5) Okubo, M.; Suzuki, T.; Tsuda, N. *Colloid Polym. Sci.* **2006**, *284*, 1319.
(6) Shim, H. W.; Lee, J. H.; Hwang, T. S.; Rhee, Y. W.; Bae, Y. M.; Choi, J. S.; Han, J.; Lee, C. S. *Biosens. Bioelectron.* **2007**, *22*, 3188.

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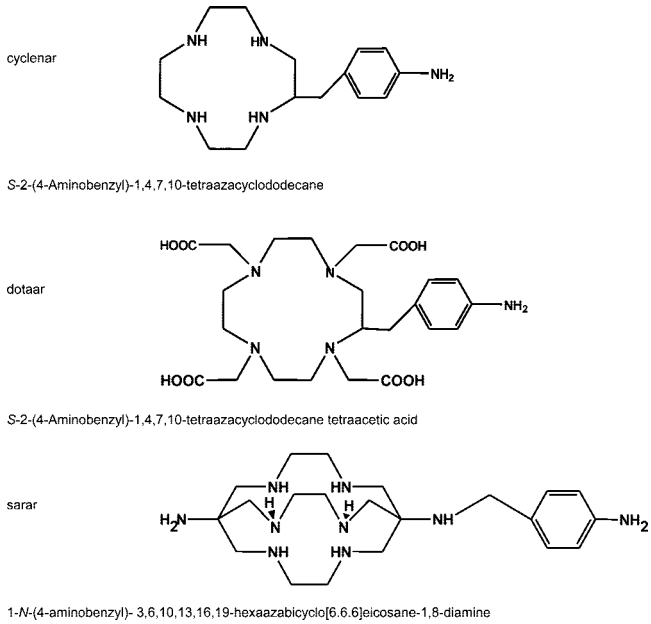


Figure 1. Bifunctional ligands used to radiolabel functionalized microspheres.

the microspheres and therefore predict the loading capacity of the desired protein. They can also be used to optimize the reaction conditions for further functionalization of the surface and provide valuable information for optimizing the engineering of the original microspheres.

Radiotracers are particularly attractive for such applications as they are highly sensitive, non-destructive tools that can be used to probe solid−liquid interfaces. Detection limits of $>10^{-3}$ ppb are readily achieved, and more importantly they are insensitive to matrix and media, that is, indiscriminate to whether the radioactive signals originate from solid surfaces or from media. Hence minimal sample sizes (<mg) and negligible preparation is required, giving the experimentalist added confidence in accuracy of the data.

Smith *et al.* have developed a novel bifunctional chelator, sarar (Figure 1), that is readily conjugated to carboxyl residues on peptides and antibody molecules via carbodiimide-mediated amide bonds.[7] This conjugation reaction can be carried out in neutral or slightly acidic pH conditions using standard aqueous buffers. The resulting sarar immunoconjugates are stable, allowing for advance preparation and storage for future labeling with the PET imaging radioisotope $^{64}Cu^{2+}$.[8] More recently they have demonstrated the feasibility of using this chelator to produce tumor-targeted immunoconjugates that can be used for *in vivo* imaging of neuroblastoma and melanoma.[9]

In this study the novel sarar technology is compared to two well-known bifunctional chelators, cyclenar and dotaar (Figure 1), for use in determining the number of available carboxylate (COO^-) groups on a polymeric microsphere. We investigate which bifunctional chelator is more efficient for reacting with the surface of the microsphere and determining the number of *available* carboxylates groups in the microspheres.

Experimental Section

Reagents. All reagents and solvents used were of analytical grade (used without further purification) and obtained from commercial sources. All water used for experimental purposes was Milli-Q grade. Silica impregnated glass fiber sheets for instant thin layer chromatography (ITLC-SG) were purchased from PALL Corp. Co-57 (carrier free) was obtained from GE Healthcare Bio. Pty. Ltd., Australia (specific activity typically 148 000 GBq g^{-1}). Analytical equipment used for this study was, for ^{1}H NMR, Bruker Avance DPX 400; gamma counter, Wallac Wizard 1480; and centrifuge, Spectrafuge 24D Digital Microcentrifuge. Sarar was synthesized in-house as previously described.[10] *p*-Nitro-benzyl-cyclen and *p*-nitro-benzyl-dota were purchased from Macrocyclics Inc. Buffers and mobile phases in this study were prepared as required and specifications are listed in Table 1.

Preparation of Microspheres. Core microspheres were synthesized via precipitation polymerization of divinyl benzene (DVB tech. 80%) in the presence of the RAFT acid. Subsequently, PEG-MEA was grafted from the microspheres via the mediation of the RAFT acid.

Preparation of *p*-Amino-benzyl-cyclen (4-(1,4,7,10-Tetraaza-cyclododecan-2-ylmethyl)-aniline) [Cyclenar]. Sodium borohydride (10 mg dissolved in 1 mL of D_2O) was added slowly to the catalyst [10% palladium on activated charcoal (10 mg) suspended in D_2O (2 mL)] and stirred under a nitrogen atmosphere. After the addition of sodium borohydride was complete, the reaction mixture was stirred for a further 5 min. *p*-Nitro-benzyl-cyclen (11.1 mg) dissolved in 1 mL of deoxygenated D_2O was added dropwise to the reaction mixture. The reduction was allowed to proceed for 20 min at 21 °C under nitrogen atmosphere. The Pd/C catalyst was filtered off (0.45 μm), and the filtrate collected in an ice-cooled glass vial. Deuterium chloride (10.5 M) was added dropwise (10 μL) to the cooled solution until gas evolution ceased (total volume 50 μL, HCl). The final pD was < 1. Completion of reduction of the aromatic nitro group was monitored by ^{1}H NMR. *p*-Nitro-benzyl-cyclen by ^{1}H NMR (400 MHz, D_2O): δ 8.20 (d, 2H, Ar-*H*), 7.51 (d, 2H, Ar-*H*), 3.07−2.49 (m, ArCH_2, 17H, CH_2N, CH_2CH_2N). *p*-Amino-benzyl-cyclen by ^{1}H NMR (400 MHz, D_2O): δ 7.40 (d, 2H, Ar-*H*), 7.34 (d, 2H, Ar-*H*), 3.51−2.81 (m, 17H, ArCH_2, CH_2N CH_2CH_2N).

Preparation of *p*-Amino-benzyl-dota 2-(4-Aminobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic Acid [Dotaar]. Sodium borohydride (11 mg dissolved in 1 mL of D_2O) was added slowly to the catalyst [10% palladium on activated charcoal (10 mg) suspended in D_2O (2 mL)] and stirred under a nitrogen atmosphere. After the addition of sodium borohydride was complete, the reaction mixture was stirred for a further 5 min. *p*-Nitro-benzyl-dota (10.59 mg) dissolved in 1 mL of deoxygenated D_2O was added dropwise to the reaction mixture. The reduction was allowed to proceed for 30 min at 21 °C under nitrogen atmosphere. The Pd/C catalyst was filtered off (0.45 μm) and the filtrate collected in an ice-cooled glass vial. Deuterium chloride (10.5 M) was added dropwise (5 μL) to the cooled solution until gas evolution ceased (total volume 20 μL, HCl). The final pD was < 1, and completion of reduction of the aromatic nitro group was monitored by ^{1}H NMR.

(7) Di Bartolo, N. M.; Sargeson, A. M.; Donlevy, T. M.; Smith, S. V. *J. Chem. Soc.* **2001**, *15*, 2303.
(8) Di Bartolo, N. M.; Sargeson, A. M.; Smith, S. V. *Org. Biomol. Chem.* **2006**, *4*, 3350.
(9) Voss, S. D.; Smith, S. V.; Di Bartolo, N. M.; McIntosh, L. J.; Cyr, E. M.; Bonab, A. A.; Dearling, J. L. J.; Carter, E. A.; Fischman, A. J.; Ted Treves, S.; Gillies, S. D.; Sargeson, A. M.; Huston, J. S.; Packard, A. B. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 17489.
(10) Di Bartolo, N. M.; Sargeson, A. M.; Donlevy, T. M.; Smith, S. V. *J. Chem. Soc.* **2001**, *15*, 2303.

Table 1. Buffers and Mobile Phases Used in This Study

buffer label	specification
buffer 1. (B1)	0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES); pH 5.0
buffer 2. (B2)	0.1 M sodium phosphate dibasic; pH 7.0
mobile phase 1 (MP 1)	0.15 M ammonium acetate pH 4.5; used to separate [^{57}Co-sarar]2+ from free $^{57}Co^{2+}$
mobile phase 2 (MP 2)	0.1 M sodium acetate pH 4.5 and ethanol (9:1, v/v); used to separate [^{57}Co-cyclenar]$^{2+}$ from free $^{57}Co^{2+}$
mobile phase 3 (MP 3)	0.1 M sodium acetate pH 4.5, H2O, methanol, and ammonium hydroxide (20:18:2:1 v/v). used to separate [^{57}Co-dotaar]$^{2-}$ from free $^{57}Co^{2+}$

p-Nitro-benzyl-dota: (400 MHz, D_2O): δ 8.26 (d, 2H, Ar-*H*), 7.55 (t, 2H, Ar-*H*), 4.28−2.81 (m, 21H ArCH_2, CH_2N, NCH_2COOH). *p*-Amino-benzyl-dota, ^{1}H NMR (400 MHz, D_2O): δ 7.46 (q, 2H, Ar−H), 7.40 (t, 2H, Ar−H), 4.33−2.70 (m, 21H ArCH_2, CH_2N, NCH_2COOH).

Complexation of ^{57}Co with Sarar, Cyclenar, and Dotaar. Typically equimolar amounts (10^{-3} M) of the ligand (cyclenar, dotaar, sarar) and a Co^{2+} solution doped with ^{57}Co ($^{57/nat}Co^{2+}$) in buffer (B1 or B2) were mixed at 21 °C for 1 h. The total volume of the final solutions was 40 μL. After 1 h the amount of $^{57/nat}Co^{2+}$ complexed by the conjugate was determined using ITLC-SG. The mobile phase established and the refractive index (R_f) for each species is given in Table 2. The processed ITLC-SG strips were then cut into 1 cm sections and associated radioactivity measured in a gamma counter. Percent $^{57/nat}Co^{2+}$ complexed by the ligand was calculated for each reaction.

Conjugation of Bi-Functional Ligands to Microspheres. Typically a stock solution of 5 mg/mL solution of microspheres (estimated to have 1.1×10^{-4} mol carboxylic acid groups per gram) in water was prepared. To varying aliquots (5, 10, 20, 40, 80 μL) of the stock solution of microspheres in an eppendorf tube was added varying molar ratios (0, 5, 10, 15, 20, 30, 60, and 80×) of each ligand (L1, L2, L3) and 100 -fold molar excess of 1-ethyl-3-(3-dimethyaminopropyl) carbodiimide hydrochloride (EDC) in buffer B1. The final volume of reactants in the eppendorf tubes was corrected to 1 mL with the addition of buffer B1. eppendorf tubes were then vortexed and incubated at ∼21 °C for 1 h and up to 48 h. The reaction mixtures were then centrifuged at 13 000 rpm for 10 min. The supernatant was removed, and the pellet resuspended in 900 μL of buffer B1. The microspheres were then centrifuged again and washed a further three times to remove any byproduct of the reaction.

Radiolabeling of microsphere-ligand conjugates. After the supernatant from the final wash had been removed, a solution of $^{57/nat}Co^{2+}$ in buffer B2 (10× and 1× molar excess) was added to each reaction tube. Microspheres were resuspended and allowed to incubate for a further hour at ∼21 °C. Tubes were centrifuged for 10 min at 13 000 rpm and washed with buffer B1 as previously described above. The supernatant for each wash was collected and transferred to kimble tubes for counting on the gamma counter. The washing step was repeated (∼5 times) until no further release of radioactivity could be detected. The radioactivity associated with each washing fraction and the pellets (microspheres) were counted on the gamma counter. The percentage of radioactivity associated with each fraction and the microspheres was determined and the moles of Co^{2+} calculated as outlined in eq 1.

$$\text{moles } Co^{2+}{}_B = Co^{2+}{}_I \times \%\text{ activity associated with pellet} \quad (1)$$

where B = bound to microspheres and I = initial moles of Co^{2+}.

Verifying Cyclenar and Dotaar Conjugation. Radiolabeled cyclenar and dotaar conjugated microspheres were further conjugated with sarar by incubating with 20 excess sarar and 100 excess EDC for 1 h. Pellets were then washed by centrifuging at 13 000 rpm and washed as described above. The microspheres were incubated again with an equimolar concentration of $^{57/nat}Co^{2+}$. The washing process described above was repeated and the remaining associated radioactivity monitored in a gamma counter. The percentage activity associated with the microspheres was determined and used to calculate the increase in moles of Co^{2+} bound to microspheres.

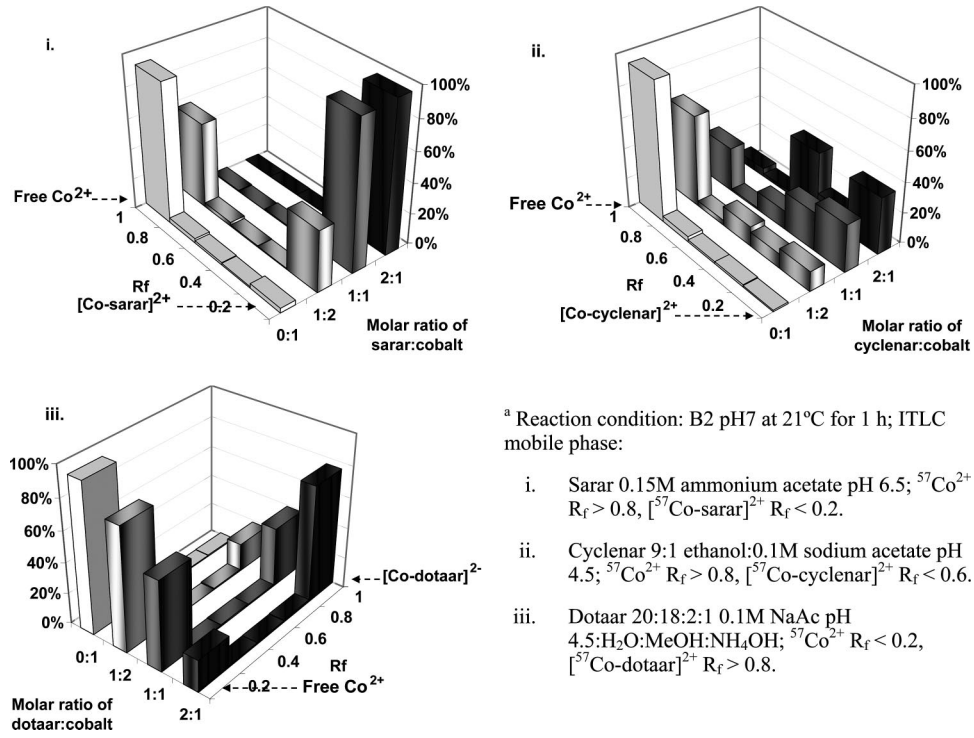
Table 2. Refractive Index of the Mobile Phases Used in This Study

mobile phase	ligands	Rf($^{57}Co^{2+}$)	Rf(^{57}Co-ligand)
0.15 M ammonium acetate pH 6.5 (MP 1)	sarar	>0.8	<0.2
9:1 0.1 M sodium acetate pH 4.5:ethanol (MP 2)	cyclenar	>0.8	<0.6
20:18:2:1 0.1 M sodium acetate pH 4.5:H_2O:MeOH:NH_4OH (MP 3)	dotaar	<0.2	>0.8

Results and Discussion

The (hydrophobic) core of the microspheres is synthesized via precipitation polymerization of divinyl benzene (DVB)[11] in the presence of a reversible addition fragmentation chain transfer (RAFT) agent. The microspheres contain RAFT end groups in the particle and on the surface of the particle that allow for subsequent grafting of polymers to form core−shell microspheres.[12] We have demonstrated that RAFT[13–17] polymerization can be applied to graft polymers with predetermined molecular weights and low polydispersity from these microspheres.[12,18] The RAFT technique utilizes dithio carbonyl compounds to mediate the polymerization. As a result of the RAFT mechanism (almost) all polymeric chains are capped with RAFT end groups which enable the polymerization to be reinitiated and to chain-extend the polymers. In addition, the RAFT agents (and therefore end groups) can contain additional functionalities (e.g., carboxyl groups). For this study, we have grafted a hydrophilic monomer, (polyethylene glycol) methyl ether acrylate (PEG-MEA), from DVB microspheres using the RAFT agent 3-benzylsulfanyl thiocarbonylsulfanyl propionic acid (RAFT acid) which contains one carboxyl group.[19]

(11) Li, K.; Stöver, H. D. H. *J. Polym. Sci., Part A: Polym. Chem.* **1993**, *31*, 3257.
(12) Barner, L.; Li, C.; Hao, X.-J.; Stenzel, M. H.; Barner-Kowollik, C.; Davis, T. P. *J. Polym. Sci., Part A: Polym. Chem.* **2004**, *42*, 5067.
(13) Perrier, S.; Takolpuckdee, P. *J. Polym. Sci., Part A: Polym Chem.* **2005**, *43*, 5347.
(14) Moad, G.; Rizzardo, E.; Tang, S. H. *Aust. J. Chem.* **2005**, *58*, 379.
(15) Moad, G.; Rizzardo, E.; Tang, S. H. *Aust. J. Chem.* **2006**, *59*, 669.
(16) Favier, A.; Charreyre, M.-T. *Macromol. Rapid Commun.* **2006**, *27*, 653.
(17) Barner, L.; Davis, T. P.; Stenzel, M. H.; Barner-Kowollik, C. *Macromol. Rapid Commun.* **2007**, *28*, 539.
(18) Joso, R.; Stenzel, M. H.; Davis, T. P.; Barner-Kowollik, C.; Barner, L. *Aust. J. Chem.* **2005**, *58*, 468.
(19) Stenzel, M. H.; Davis, T. P. *J. Polym. Sci., Part A: Polym. Chem.* **2002**, *40*, 4498.



[a] Reaction condition: B2 pH7 at 21ºC for 1 h; ITLC mobile phase:

i. Sarar 0.15M ammonium acetate pH 6.5; $^{57}Co^{2+}$ $R_f > 0.8$, $[^{57}Co\text{-sarar}]^{2+}$ $R_f < 0.2$.

ii. Cyclenar 9:1 ethanol:0.1M sodium acetate pH 4.5; $^{57}Co^{2+}$ $R_f > 0.8$, $[^{57}Co\text{-cyclenar}]^{2+}$ $R_f < 0.6$.

iii. Dotaar 20:18:2:1 0.1M NaAc pH 4.5:H_2O:MeOH:NH_4OH; $^{57}Co^{2+}$ $R_f < 0.2$, $[^{57}Co\text{-dotaar}]^{2+}$ $R_f > 0.8$.

Figure 2. Percent of $^{57}Co^{2+}$ complexed by sarar, cyclenar, and dotaar.[a]

All three parent species (cyclen, dota, and diamsar) of the bifunctional chelators (Figure 1) have been demonstrated to form thermodynamically stable complexes with Co^{2+} (log $K_{[Co\text{-}cyclen]2+} = 13.8$, log $K_{[Co\text{-}dota]2-} = 20.2$) and Cu^{2+} (log $K_{[Cu\text{-}cyclen]2+} = 23.3$, log $K_{[Cu\text{-}dota]2-} = 22.2$).[20]

Sarar is known to complex Cu^{2+} and Co^{2+} at micromolar concentrations within 30 min at pH 5 to 7.[7] However, for the present study it was important to establish if complexation of Co^{2+} by cyclenar and dotaar is complete at pH 5 and pH 7 at micromolar concentration of metal ions at room temperature. For this study the percent of complexation for each ligand was established by mixing various molar ratios of metal ion to ligand (0:1, 1:1, 2:1, and 1:2) and the amount of Co^{2+} complexed determined using instant thin layer chromatography. Figure 2 illustrates a typical profile for solutions of mixed molar ratios of metal to sarar, at pH 7 after 30 min of incubation at room temperature. Similar complexation experiments were conducted with cyclenar and dotaar. Both latter ligands complex Co^{2+} at pH 7. However, after 1 h only 70% of the $[Cocyclenar]^{2+}$ and 40% of the $[Codotaar]^{2-}$ was formed. The ITLC profiles are given in Figure 2.

Bifunctional ligands contain two parts, one for conjugating to the carboxylate groups on the microsphere and a second part used to chelate or capture the metal ion or radiometal ion. For this study the ligand is covalently attached to the microspheres via a covalent amide bond formed between the terminal reactive amino benzyl moiety and the available carboxyl groups on the functionalized microsphere in a similar manner to that employed for proteins.[21,22] This leaves the chelating moiety of the bifunctional ligand free to complex the radiometal ion, in this case the $^{57/nat}Co^{2+}$.

Carbodiimide,1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), perhaps one of the most popular reagents to conjugate biological substances, was used to mediate the conjugation or coupling of cyclenar, dotaar, and sarar to the microspheres. For the present study it was considered the most ideal activating agent as the authors would require similar conditions for the development of a biosensor, where a protein would need to be coupled to the carboxylic acid groups of the microspheres. EDC is water soluble and classified as a zero-length cross-linker, that effectively activates the carboxylate groups, for nucleophilic attack by the amino group of the linker arm of the bi-functional ligand. This water solubility allows it to be used with aqueous and proteinaceous systems without predissolution in organic solvents. Furthermore, excess reagent and the isourea formed as the byproduct of the cross-linking reactions are water-soluble and are easily removed by dialysis and/or centrifugation.

Nakajima and Ikada have demonstrated that EDC rapidly loses its activity in aqueous media of low pH, producing the corresponding urea derivative.[23] However, EDC was found to be very stable at neutral and higher pH. Previously, we have investigated the coupling of sarar to immunoglobulin protein (whole antibodies) using EDC as the coupling agent, and optimum yields were also obtained at pH 5.0 for this reaction.[8] The yield of reaction with EDC in aqueous media has also been reported to be approximately 50% efficient due to a number of competing reactions such as the

(20) Martell, A. E.; Smith, R. M. *Critical Stability Constants*; Plenum Press: New York, 1989; Vol *62*, Supplement.
(21) Hermanson, G. T. *Bioconjugation Techniques*; Academic Press: New York, 1996.
(22) Di Bartolo, N. M.; Sargeson, A. M.; Smith, S. V. *Org. Biomol. Chem.* **2006**, *4*, 3350.
(23) Nakajima, N.; Ikada, Y. *Bioconjugate Chem.* **1995**, *6*, 123.

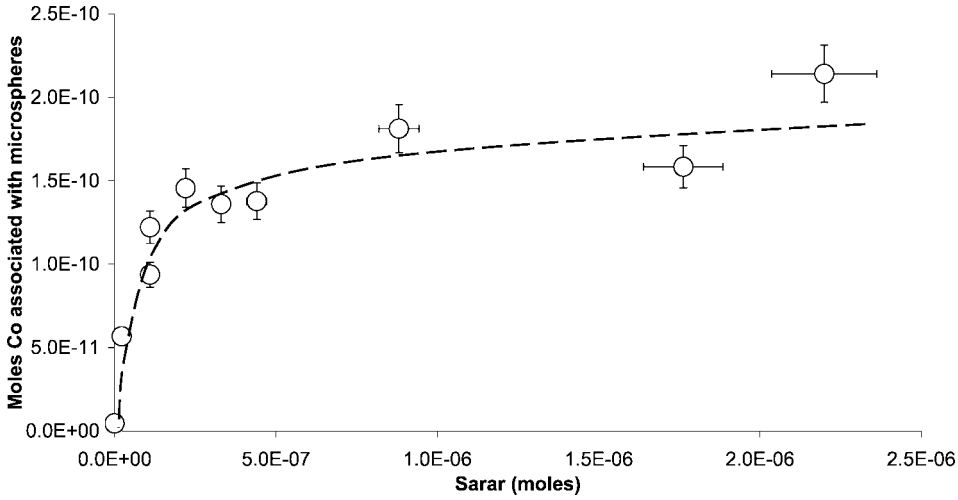


Figure 3. Resultant moles of [Co-sarar]$^{2+}$ attached to microspheres using various amounts of sarar. Reaction conditions: 0.2 mg of microspheres, 100:1 molar ratio of EDC to estimated carboxylate gropus, in B1 pH 5, 21 °C for 1 h.

hydrolysis of EDC to the corresponding urea derivative if the carboxylic acid group is not present. However, these competing reactions can be eliminated by a simple one-pot reaction where a molar excess of EDC is added to carboxylic acid groups in the presence of the benzylamino group at pH 5.

For this study various conditions for the conjugation of the ligands to the microsphere were investigated, including molar ratio of reactants (i.e., ligand and EDC, functionalized microsphere), pH (i.e., B1 = 5 and B2 = 7), and reaction time (i.e., up to 1 h and overnight). The reaction of the carboxylic acid groups on the microspheres was first optimized with respect to molar ratio of reactants such as sarar, EDC, and time. The reactions were conducted using a matrix of reaction conditions with molar ratios of carboxylic acid group to EDC to sarar equivalent of 1:100:(0, 5, 10, 15, 20, 30, 60, and 80). Reaction mixtures were incubated for 1−24 h. After each reaction, the conjugated microspheres were centrifuged, and the resultant pellet was washed several times to remove any excess reagents or byproducts. The isolated bifunctional conjugated microspheres were then incubated with 1- or 10-fold excess of $^{57/nat}Co^{2+}$ to carboxylate groups. The moles of $^{nat}Co^{2+}$ ion associated with the microspheres were calculated. Studies showed that the conjugation reaction was most effective using buffer B1 and that 1 h of incubation was sufficient to achieve the maximum amount of ligand conjugated to the microsphere.

The effect of concentration of bifunctional chelator to carboxylic acid groups on the microspheres was investigated by reacting microspheres (0.2 mg) with increasing excess of sarar (0, 5, 10, 15, 20, 40, and 80 molar). The microsphere conjugates were also exposed to various concentrations of $^{57/nat}Co^{2+}$ ions (1- to 10-fold excess to estimated carboxylic acid groups) to ensure all ligand binding sites attached were available to complex the $^{57/nat}Co^{2+}$ ions. The number of Co^{2+} ion associated to the microspheres for these reaction mixtures was equivalent, demonstrating that all conjugated ligands on were readily available to Co^{2+} ions complexation. Figure 3 illustrates a saturation curve for the reaction of varying concentrations of sarar to a fixed concentration of microspheres. It clearly shows that the number of moles of sarar required to conjugate all available carboxylate groups on 0.2 mg of microspheres is approximately 20-fold.

The assay was further tested by reacting increasing amounts of microspheres (0, 0.1, 0.2, 0.3, 0.4, and 0.5 mg) with sarar. The data, illustrated in Figure 4, clearly show that the moles of sarar attached to the microspheres increase linearly with the amount of microspheres. The linearity of the curve supports the reproducibility of the RAFT process in the production of the microspheres and the consistency of the resultant conjugated microspheres. The gradient of the curve gives the number of carboxylate groups per milligram of microspheres, which is of the order of 1.0×10^{-9} mol·mg^{-1} (error ± 10%).

The conjugation of sarar was compared with two other well know bifunctional ligands, cyclenar and dotaar. The percents $^{57/nat}Co^{2+}$ complexed with cyclenar and dotaar in B2 after 1 h at 21 °C were 70% ± 5% and 40% ± 5%, respectively. Hence an appropriate correction factor (cyclenar = 1.4; dotaar = 2.5) was used to determine the final number of carboxylate groups on the microspheres. Figure 5 summarizes the results of these comparative studies.

Figure 5 shows that the number of carboxylate groups determined using cyclenar and dotaar are considerably lower than those determined using sarar. This could either be the result of weaker complexation of the Co^{2+} by the cyclenar and dotaar compared to sarar or that the cyclenar and the dotaar do not conjugate as effectively under the same optimized conditions for sarar. Therefore a further investigation to assess the efficiency of the conjugation reaction with bifunctional ligands was performed. Cyclenar and dotaar were conjugated to the microspheres and then radiolabeled with $^{57/nat}Co^{2+}$. The dotaar microsphere and cyclenar microsphere conjugates were further reacted with sarar and the resultant conjugated microsphere radiolabeled with $^{57/nat}Co^{2+}$. The number of carboxylate groups was calculated, and the data is summarized in Figure 6. The data in Figure 6 clearly illustrate that the conjugation with sarar is more effective than with cyclenar and dotaar.

Furthermore, as the environment (i.e., pH) of each carboxylate groups may vary depending on where it is situated within and on the microsphere, the environment is likely to

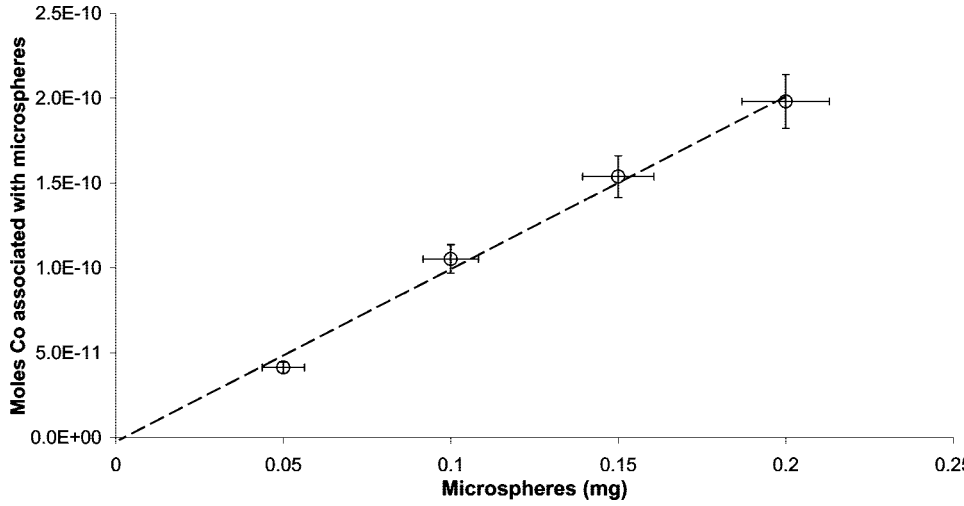


Figure 4. Available carboxylate groups correlated to weight of microspheres. Reaction conditions: 20:100:1 molar ratio of sarar to EDC to estimated carboxylate gropus, in B1 pH 5, 21 °C for 1 h.

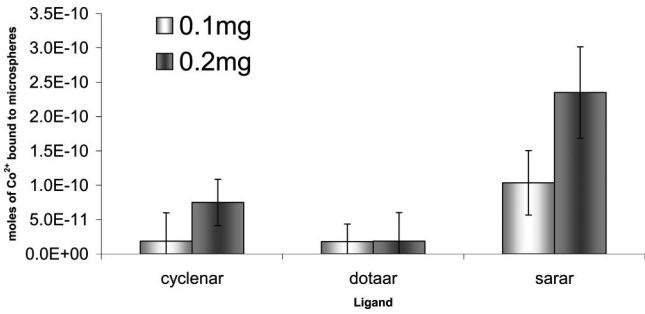


Figure 5. Number of available carboxylate groups on the microspheres using various ligand technologies. Reaction conditions: 20:100:1 molar ratio of ligand to EDC to estimated carboxylate gropus, in B1 pH 5, 21 °C for 1 h.

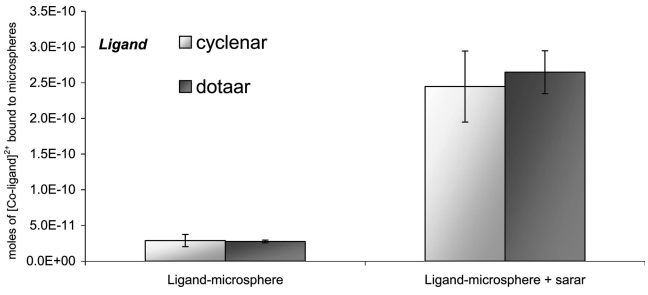


Figure 6. Available carboxylate groups on the microspheres after cyclenar/dotaar attachment. Reaction conditions: 20:100:1 molar ratio of ligand to EDC to estimated carboxylate gropus, in B1 pH 5, 21 °C for 1 h for both initial and final conjugation.

influence the efficiency of the conjugation reactions for each bifunctional ligand. Also, once attached, the chelating moiety of the conjugated microspheres may not be as effective as the free bifunctional ligands in complexing the metal ions. The unique ability of the sarar to complex transitions metal ions over a wider range of pH as the free ligand and attached to proteins explains the sarar's apparent greater efficiency in quantification of the available carboxylate groups on the microsphere.

This study demonstrates not only sarar's ability to complex radiotracers quantitatively at low concentrations and with high sensitivity but also the significant potential of nuclear probes to characterize the functional groups at solid−liquid interfaces. As in the development of ^{64}Cu-PET imaging agents, the sarar technology can be applied to a range of solid surfaces including functionalized nanoparticles and tracked *in vivo* using Positron emission tomography (PET) imaging for the risk assessment of drug delivery systems.

The ability to employ nuclear probes in various media and to design them specific for application will make them powerful tools for the engineering of new materials. This approach has significant potential for both the optimization of materials engineering as well as establishing conditions for conjugating the biosensor (e.g., protein, drugs, and so forth) on the surface of materials. The wider application of nuclear probes to the materials research and development pathway promises to have significant potential to assist in the fast through-put screening, optimizing the engineering of integrated systems, and for the risk assessment for final commercial application.

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