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Synthesis of polysaccharide-*b*-PEG block copolymers by oxime click†

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The oxime click reaction is shown to be a straightforward methodology for the synthesis of poly(ethylene glycol) (PEG)-polysaccharide diblock copolymers. The method is applicable to unmodified polysaccharides with a reductive end as demonstrated for dextran, hyaluronic acid and chitosan. Notably the oxime click reaction is applied for the first time to the end modification of polysaccharides.

Polysaccharides are abundant, renewable, ultra-lightweight, inexpensive, generally biocompatible and biodegradable, and therefore key compounds in the field of sustainable chemistry.[1] Additionally, polysaccharides can be used as active targeting moieties in drug delivery, as recently demonstrated by hyaluronic acid block copolymers.[2] In comparison with other families of block copolymers a small number of polysaccharide containing diblock copolymers have been described.[3] A short look into the literature shows that the number of publications on polysaccharide graft copolymers is one order of magnitude higher than on polysaccharide block copolymers. The difficulty in the preparation of these copolymers is mainly caused by the limited availability of a polysaccharide reducing end (0.0024% of aldehydo-D-glucose in D-glucose)[4] and the complexity of finding a common solvent for both blocks in the case of amphiphilic copolymers.

This underdevelopment is dramatic if one considers that, as compared to graft copolymers, block copolymers preserve the structure of the polysaccharide (none of their lateral groups are modified) and therefore will better preserve its chemical and biological properties. Moreover, it is well known that block copolymers allow a much greater control over nanostructure assembly than graft copolymers do.[5,6] In this way the combination of the nanoscale self-assembling properties of block copolymers with the above-mentioned properties of polysaccharides will permit interesting applications that range from drug delivery to materials science.[1,7]

Here we describe a new method for the preparation of PEG diblock polysaccharide copolymers. The method is applicable to different polysaccharides such as dextran, hyaluronic acid and chitosan. The use of charged polysaccharides will open the opportunity of preparing stoichiometric water soluble interpolyelectrolyte complexes (IPEC) with potential applications as protein or gene carriers, in a similar way to the strongly successful polypeptide-*b*-PEG copolymers.[4]

Three synthetic approaches have been described so far for the preparation of polysaccharide containing block copolymers: the extension of the polysaccharide block by radical polymerization, the enzymatic extension of the synthetic block, and the end-to-end coupling of both blocks.[3] The first method was discarded because PEG cannot be polymerized under conditions where the polysaccharide is soluble and the use of enzymatic growth was not taken into consideration because it should be optimized for each polysaccharide (lack of generality). Therefore, the end-to-end approach is more attractive for the synthesis of polysaccharide-*b*-PEG.

In fact, the synthesis of dextran-*b*-PEG (dex-*b*-PEG) has been described by end-to-end couplings.[8] The authors used reductive amination after the transformation of the reductive end of the dextran into a lactone. In spite of the lactonization step an excess of PEG is needed as a consequence of a relatively low efficiency of the reductive amination. In fact, a very important excess of the synthetic block is necessary if no previous lactonization is performed. For example, in the coupling of polystyrene and dextran by reductive amination, an excess of 50 to 100 equivalents has been described.[9]

Attempts to prepare chitosan-*b*-PEG (CS-*b*-PEG) have been described.[10,11] So far, the proposed procedures lead to CS radical depolymerisation or graft copolymer formation.[12] The preparation of hyaluronic acid-*b*-PEG (HA-*b*-PEG) remains unexplored to the best of our knowledge.

The recent use of copper-catalysed azide–alkyne cycloaddition, CuAAC, the most common of the click reactions, for the preparation of polypeptide-*b*-polysaccharide in DMSO as solvent inspired us to develop a method based on click chemistry. However, the CuAAC chemistry approach does not avoid the use of reductive amination to introduce the click active functionality. In addition, the reaction should be performed in DMSO because the use of Cu^{2+} ions in the presence of water leads to polysaccharide chain cleavage.[13] The use of DMSO as solvent restricts its application to polysaccharides with low molecular weight for solubility reasons. Therefore, alternatives to CuAAC are desirable.

As an alternative we propose the formation of an oxime by the reaction of an aldehyde and an aminooxy group, considered as a "click" reaction.[14] The oxime approach presents 3 major advantages: (i) it is active to the aldehyde in equilibrium at the reductive end of many natural polysaccharides (dextran, chitosan, hyaluronic acid, amylose, pullulan, among others)

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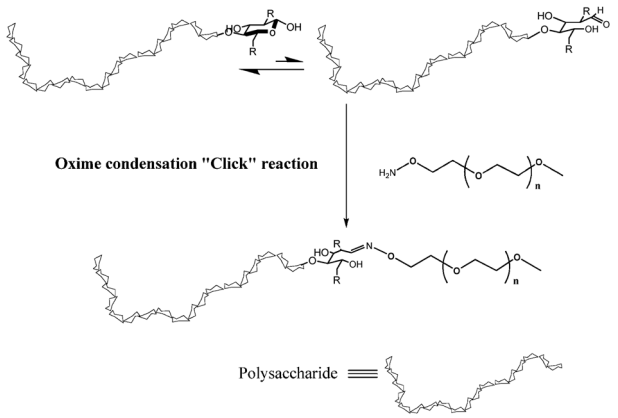

Fig. 1 Approach proposed for the polysaccharide-*b*-PEG synthesis.

avoiding a previous modification; (ii) it is performed without any metal catalyst and (iii) it can be performed under conditions where high molecular weight polysaccharides are soluble [typically water/acetonitrile (AcN) or dimethyl sulfoxide mixtures (DMSO)]. This reaction has been applied in the preparation of polymer–protein conjugates,[15] viral surface modification,[16] or the coupling of large peptide blocks.[17] Moreover, the formation of an oxime by the reaction of the oligosaccharide reductive end has been described for the preparation of glycopeptides.[18]

The proposed synthetic approach is shown in Fig. 1. The MeO–PEG–ONH_2 can be easily prepared from commercial MeO–PEG–OH using the Mitsunobu reaction with *N*-hydroxyphthalimide and deprotection with hydrazine (ESI†, Section 5).[16]

The conditions of the oxime formation were first investigated using glucosamine as a model and MeO–PEG–ONH_2 (2 kDa). The reaction was followed by the integration of the N–CH protons (*E* and *Z*) at 7.60 and 6.95 ppm in the ^{1}H NMR spectra. A faster oxime formation is observed if a non-aqueous solvent (AcN) is added to the buffer solution and when heating to 45 °C is applied (Table S1, ESI†). These observations are in agreement with oxime couplings reported previously.[15–18]

In a second set of experiments, low molecular weight dextran (3 kDa) was coupled to MeO–PEG–ONH_2 (2 kDa). The use of water/AcN or water/DMSO and two different pH values (3 and 5) were compared. The comparison is appropriate because different solvent mixtures and pH values have been reported for oxime formation.[15–18] The results show a faster and more efficient reaction using DMSO/pH 3 buffer solution at 45 °C. An amount of 5 equivalents with a reaction time of 24 h is sufficient for a complete dextran end modification (Table S2, ESI†).

Once optimal reaction conditions were determined the reaction was performed with dextran and PEG of higher molecular weights (up to 49 kDa for dextran and 5 kDa for PEG, Table 1). Dextrans with low polydispersity were obtained upon fractionation by ultrafiltration (ESI†, Section 2). Complete conversion was observed for dextran 6 kDa and MeO–PEG–ONH_2 2 and 5 kDa. For dextran 49 kDa the integration of the oxime signal is difficult and therefore the excess of PEG must be eliminated to confirm the success of the coupling.

Separation of the excess PEG by precipitation in different solvents (ethanol, dioxane and AcN) led to insufficient separation or very low mass recoveries due to partial solubility of the

Table 1 Polysaccharide-*b*-PEG prepared by oxime click reaction

Polysaccharide	10^{-3} M_n[a], g mol^{-1} (PDI) polysaccharide	10^{-3} M_n[b], g mol^{-1} MeO–PEG–ONH_2	10^{-3} M_n[c], g mol^{-1} (PDI)[d], PEG-*b*-polysaccharide
Dextran	6.2 (1.25)	2.0	8.2 (1.24)
		5.2	11.4 (1.10)
	48.8 (1.35)	5.2	54.0 (1.30)
Hyaluronic acid	6.0 (1.23)	2.0	8.0 (1.18)
		5.2	11.2 (1.20)
	9.3 (1.28)	2.0	11.3 (1.15)
		5.2	14.5 (1.24)
	54.3 (1.22)	2.0	56.3 (1.20)
		5.2	59.5 (1.40)
Chitosan	3.7 (1.32)	2.0	5.7 (1.55)
		5.2	8.9 (1.33)
	10.7 (1.26)	2.0	12.7 (1.60)
		5.2	15.9 (1.45
	53.6 (1.40)	2.0	55.6 (1.55)
		5.2	58.8 (1.76)

[a] For dextran determined by GPC with dextran standards, for HA and CS determined by GPC-MALS. [b] Determined by MALDI-TOF MS. [c] Determined by NMR. [d] Determined by GPC with dextran standards.

block copolymer. Similar difficulties were described in the synthesis of dextran-*b*-PEG by reductive amination.[8] Excess PEG could be successfully removed by dialysis of the milky or opalescent solution obtained by addition of ethanol to a water solution of the mixture. A membrane of a high molecular weight cut-off (50 and 100 kDa for PEG 2 and 5 kDa respectively) was used. Since ethanol is not a good solvent for PEG at room temperature, the dialysis bath was kept at 35 °C. The requirement of a high molecular weight cut-off membrane is explained not only by the shrinkage of the membrane pores in ethanol but also by calibration of the membranes for proteins with a much more compact conformation than PEG. The absence of free PEG was observed by gel permeation chromatography (GPC). GPC shows a low polydispersity of the block copolymers (Table 1 and Fig. S4–S6, ESI†).

Once the purity of the copolymer was demonstrated the composition was confirmed by the integration of the NMR spectra (Fig. 2 and ESI†, Section 8). The results demonstrate that the use of 5 equivalents is enough even for the coupling of dextran 49 kDa and PEG 5 kDa.

To generalize the synthetic approach to other polysaccharides, block copolymers with chitosan (CS) and the hyaluronic acid (HA) were prepared (Fig. 3).

Hyaluronic acid with different molecular weights was obtained from a commercial source while the CSs were obtained by deacetylation under basic conditions (ESI†, Section 3), controlled depolymerization and fractionation by ultrafiltration (ESI†, Section 4).

The procedure was successfully performed for HA with exact reaction and purification conditions as in the case of dextran for the three molecular weights. The block copolymer composition was also confirmed by NMR and GPC. A detailed synthetic procedure is included in the ESI† (Section 7). The characteristics of the HA-*b*-PEG are included in Table 1 (^{1}H NMR and GPC in the ESI†, Sections 8 and 9).

For CS the procedure was slightly modified. The citric acid based buffer solution at pH 3 was substituted by a solution of acetic acid. This change is necessary because the

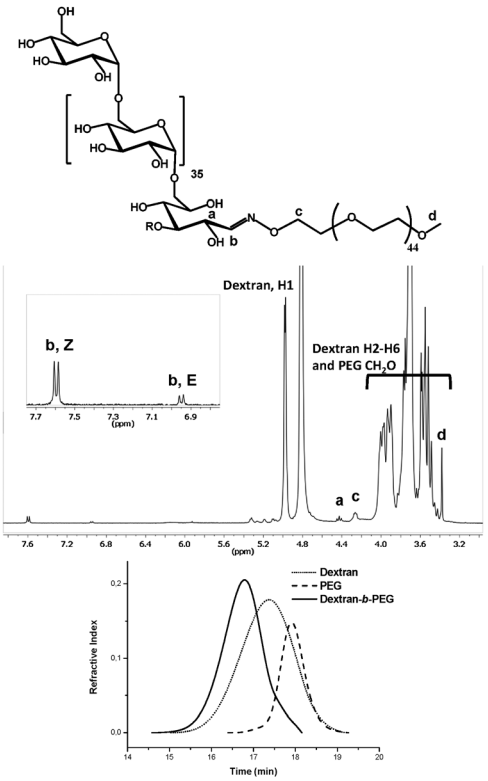

Fig. 2 Dex (6 kDa)-*b*-PEG (2 kDa): chemical structure, ^{1}H-NMR spectra in D_2O and GPC eluogram (0.1 M NaN_3, 0.01 M, NaH_2PO_4/20% MeOH).

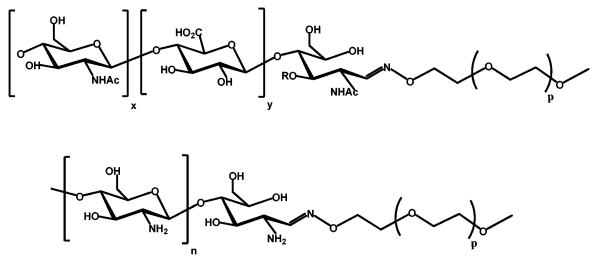

Fig. 3 Chemical structure of HA-*b*-PEG (top) and CS-*b*-PEG (bottom).

carboxylic acid groups of citric acid lead to solubility problems due to electrostatic interaction with the charged amino groups of CS. It should also be mentioned that the procedure selected for the depolymerisation of CS (nitric acid deamination) leads to the formation of some chitosan chains with 2,5-anhydro-D-mannose at the reducing end. This is an advantage since this form has a more favoured equilibrium with the aldehyde end group than native CS.[19] In fact, the aldehyde function could be detected in the NMR spectra of the depolymerized CSs. The reaction was also successful in the case of 54 kDa (not depolymerised). Therefore, we also demonstrated that the method can be applied to native chitosan. All the polysaccharide-*b*-PEGs synthesized are summarized in Table 1. The higher polydispersity of the CS-*b*-PEG is presumably caused by the use of dextran in the GPC calibration (CS 53.6 kDa shows a PDI of 1.80 using the same dextran calibration).

Since oximes are sensitive to acidic hydrolysis,[20] the stability of the formed oxime was studied for dex-*b*-PEG by NMR spectroscopy at different pH values (Table S3, ESI†). The oxime was completely stable for at least 55 h at pH 3 while it is hydrolysed to *ca.* 95% at pH 2 within 24 h. The stability of the oxime was also tested by GPC in the case of CS-*b*-PEG. The GPC conditions selected for these polymers (ESI†) include the use of a water based eluent at pH 2.5. In this case the stability was demonstrated by comparison of the eluograms of freshly dissolved CS-*b*-PEG and of the same solution after 24 h. We conclude that the stability of the oxime bond is sufficient for applications in pharmacological and materials science.

In conclusion, an efficient and general method for the synthesis of polysaccharide-*b*-PEG copolymers by means of the oxime click chemistry has been described. To the best of our knowledge, this is the first application of the oxime click chemistry to the end modification of a polysaccharide. The success of this approach opens the opportunity to use this chemistry as a general tool for the end-modification of polysaccharides instead of the commonly used reductive amination. Moreover, the prepared block copolymers bear an enormous potential in materials science and drug delivery.

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